TITLE PAGE

Effects of Imidacloprid Residues in Maize Pollen on the Development of Small Bee Colonies Under Field Exposure Conditions

AUTHOR

[redacted]

TESTING FACILITY

BAYER AG
Crop Protection-Development
Institute For Environmental Biology
D-51368 Leverkusen-Bayerwerk

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STATEMENT OF COMPLIANCE

This study was conducted in compliance with the Principles of Good Laboratory Practice (Chemicals Law (ChemG) of July 25, 1994, Annex 1 and OECD Principles of Good Laboratory Practice (GLP) of November 26, 1997 [C(97)186/Final]).

Signature:

[redacted]

21.9.99
Date

21.9.99
Date

CERTIFICATION OF AUTHENTICITY

[redacted]

Study Director

Title

21.9.99

Date

Responsible Analyst

Title

21.9.99

Date

APPROVAL

[redacted]

Head of Institute for
Environmental Biology

Title

28.9.99

Date

INQUIRIES

Inquiries should be directed to:

[redacted]

BAYER AG
Crop Protection - Development
Institute for Environmental Biology
D-51368 Leverkusen - Bayerwerk, FRG

Phone No. (02173) / 38 - 3888

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1.0 SUMMARY

Report: [redacted] 1999). Effects of Imidacloprid Residues in Maize Pollen on the Development of Small Beehives Under Field Exposure Conditions.
Bayer AG, unpublished report No: SXR/Am 005; 1999/09/15.
(Appendix XIII contains data from study MR-508/99)

Guidelines: Internal Testing Method
Deviations: not applicable

GLP: yes (certified laboratory)

Material and methods: *test substance:* imidacloprid techn., *purity*: 98.6%, *identity*: article no. 04145852, formulation/batch no. 230 824 088, no. of certificate TOX-No. 4941-00. Under field exposure conditions small bee colonies (appr. 500 honeybees) were confined on oat plots (50 m ², drilled on 1 April 1999) and exclusively fed with maize pollen which was fortified with either 0, 2, 5, 10 or 20 µg/kg imidacloprid. Sunflower honey was provided as carbohydrate source. The small bee colonies were examined for treatment-related impacts over a period of 39 days. In particular, the following endpoints were evaluated: mortality, comb cell production, food consumption, storage behavior, hive weight increase egg laying activity, breeding success, colony strength, foraging intensity and behavioral anomalies.

Dates of biological work: May 28 – July 6, 1998.

Findings: Effects of imidacloprid residues in maize pollen on small honeybee colonies

Testing Endpoint	Control	2 µg/kg	5 µg/kg	10 µg/kg	20 µg/kg
Mortality (no. of dead bees in front of bee hives)	10	5	6	8	7
Mortality (no of dead bees at the tent margin)	22	21	22	21	30
Foraging intensity (no. of bees at the pollen feeder)	22	19	23	37	24
Foraging intensity (no. of bees at the honey feeder)	104	124	123	130	128
Pollen consumption [g]	35	29	32	39	34
Honey consumption [g]	491	541	521	500	543
Comb cell production [cm²]	528	551	579	584	563
Honey storage area at study termination [cm²]	177	201	186	147	174
Hive weight increase	180	230	215	200	200
Egg laying activity[cm² comb area containing eggs at study termination)	144	153	181	205	153
Colony strength [cm² comb area covered with bees at study termination)	217	258	305	314	221

Observations: There were no differences between the control and the treatment groups nor a concentration-related trend among the treatment groups for any of the evaluated test parameters. In addition, no behavioral impacts (e.g. apathy, exaggerated motility, discoordinated movements) or suspicious mortality was observed on the honeybees of the treatment groups.

2.0 INTRODUCTION

According to EU directive 91/414/EEC the impacts of pesticides on honeybees have to be examined. If laboratory studies indicate a potential hazard to honeybees, higher Tier studies are required for a field-relevant risk assessment. The present study aims to examine the effect of imidacloprid residues in maize pollen on the development of small bee colonies.

3.0 EXPERIMENTAL

3.1 Test Substance

Test substance:	Imidacloprid techn.
Active ingredient(s):	Imidacloprid (NTN 33893)
Chemical name(s) of ai(s):	1-<(6-CHLORO-3-PYRIDINYL)=METHYL>-=N-NITRO-2-=IMIDAZOLIDINIMINE
CAS number of ai(s):	138261 - 41 - 3
Article number:	04145852
Formulation/batch number:	230 824 088
No. of certificate:	TOX-No. 4941-00
AI content (acc. to analysis):	98.6%
Analytical method:	HPLC, ext. Std.
Date of analysis:	March 12, 1998
Expiry date:	September 3, 1999
Physical appearance:	beige powder
Storage conditions:	Room temperature
Residue level(s) tested in the study:	0, 2, 5, 10, 20 µg/kg in maize pollen
Safety Precaution:	Routine hygienic precautions

3.2 Reference Substance

For this type of material and use pattern, a reference compound is not specified.

3.3 Execution of the Test

The study field site was drilled with oat on 1 April 1999. Tunnel cages (50 m²) were placed on this oat field on 19 and 20 May 1999 and confined the study plots. The hive colonies were placed inside the tunnel cages on 28 May 1999. The final evaluation on these hives were made on 6 July 1999.

Sponsor:	BAYER AG PF-E/PBA D-40789 Monheim
Study director:	[redacted]
Responsible analyst:	
Study technician(s):	
Quality assurance:	
Laboratory study number:	SXR/Am 005

3.4 Origin of Honeybees and Preparation of Hive Colonies

Honeybees were purchased from a German beekeeper ([redacted]. Preparation of the hive colonies used for the test started on 27 May 1999. Honeybees of 12 combs from a large commercially managed used beehive (beehive no. 5) were swept down into a drone sieving cage and moistened with water to suppress escape flights. These honeybees were divided into 70 g subsamples which is equivalent to a number of approximatey 500 honeybees. Each subsample was filled into one of 11 multiple-comb-fertilization-cages (= "Mehrwaben-begattungskästchen") which contained 4 native comb strips (13 x 2 cm), i.e. only comb matrices. One queen in egg laying activity was added to each of these hive colonies within a separate and closed cage. On the next day, the colonies were installed within the tunnel cages and the queen cage disclosed. Two days later (May 30, 1999), the queen cage was removed. At this time, all qeens had started to lay eggs in the small hives except in the control hive. The control queen was replaced on 31 May 1999 by a substitute queen which was kept in reserve. The substitute queen started immediately with egg-laying.

Due to low night temperatures, hives were protected with a styropor cover during 8 and 14 June 1999 (study days 11 to 17). A brood check was made on 5 June 1999.

3.5 Preparation of the Food Substrate

Maize pollen was purchased from Brasil. On behalf of Bayer do Brasil, [redacted] collected approximately 750 g fresh maize pollen from flowering maize and shipped it within 2 weeks after sampling to the study director. The pollen arrived at the study site on 5 May 1999. Before flowering, the sampled maize field had received the following pesticidal treatments:

Time of Treatment	Product Applied	Application Rate
Prior emergence	Primoleo (= H, atrazine), 400 SC	6 L/ha
8-Leaf stage	Karate (= I, lambda-Cyhalothrin), 050 EC	0.35 L/ha

The maize pollen was divided into 110-130 g subsamples which were fortified with technical imidacloprid (see 3.1). Fortification levels were 0, 2, 5, 10 and 20 µg/kg. Before fortification, the maize pollen was analysed for background contamination. The analytical results are reported in appendix I. According to these results, the maize pollen was free of imidacloprid and free of pyrethroids and organophosphates.

For fortification, a stock sample was prepared which contained 50 µg/kg imidacloprid. The stock sample was prepared as following:

50 mg imidacloprid techn. was solved in 500 ml drinking water (solution was stirred over night). This stock solution was diluted by 1:20 (1 ml added to 19 ml drinking water). This diluted sample was then again diluted by 1:20. Some 20 ml of the final dilution was sprayed onto 100 g maize pollen which was continuously mixed during spraying within a mixing drum. This stock sample (sample A) was then diluted with untreated maize pollen as reported in the table below.

Target Concentration [µg/kg]	Amount of Treated Pollen [g]	Amount of Untreated Pollen [g]
0	0	110
20 (= sample B)	90 (sample A)	135
10 (= sample C)	105 (sample B)	105
5	80 (sample C)	80
2	50 (sample C)	75

Five 1 g samples were taken from each preparation for an analytical verification of the target concentration. Sampling spots were on the left and right site of the top and bottom position and the centre of the pollen sample surface within the 1 L glass containers (filling height was about 1 cm). The analytical findings are summarized in Table 1 and reported in detail in appendix XIII. During the study, the prepared pollen samples were stored within a refrigerator between +6 and +9°C.

3.6 Location of the Trial Site and Description of the Study Plots

The trial site was located in the vicinity of Euskirchen-Billig, adjacent to the area „Billiger Wald“. Owner of the test field was [redacted]. In order to prevent honeybees from collecting honey and/or pollen from the study plot this part of the field which was confined with the tunnel cages was cropped with oat (variety „Jumbo“). The required oat strips were 100 x 5 m large and drilled on 1 April 1999 with a drilling rate of 150 kg/ha. The drilled oat was treated with the combined fungicide Sibutol mit Haftmittel (37.5% bitertanole and 2.3% fuberidazole) at 150 g/dt. There were no other pesticidal treatments till study termination.

On 19 and 20 May 1999, six 50 m² tunnel cages (10 x 5 m) were installed on one of two 100 x 5 m large field strips cropped with oat. The tunnel cages consisted of an aluminium frame covered by plastic gauze material (2 x 2 mm mesh size). For operational purposes, a walkway was created by removing all plants along a 50 cm wide transect from the tunnel entrance to the opposite end.

3.7 Treatment Design

After preparation of the bee colonies (see 3.4), they were allocated to one of the six tunnel cages by using a random list. Installment of the bee colonies was on 28 May 1999. The allocation of the colonies to treatment was as follows:

Colony no.	Tunnel no.	Treatment level
21	3	5 µg/kg
30	4	0 µg/kg
33	1	2 µg/kg
40	6	10 µg/kg
49	5	20 µg/kg

The colonies were fed with sunflower honey purchased from a commercial beekeeper. Before study initiation, the honey was analysed for background contamination. The analytical results are reported in appendix I. According to these results, the honey was

free of imidacloprid and free of the other contaminants for which samples were analysed (mainly pyrethroids and organophosphates). The sunflower honey was provided in an elevated and sheltered glass container which was positioned on the tunnel end opposite to the entrance. The honey was provided in small, weighed portions. Each third day, a fresh portion was offered and the remaining old portion removed and reweighed.

The fortified maize pollen was provided in 10-30 g subsamples at two different places. One portion was offered within a separate, sheltered container next to the honey feeder. A second portion was offered in an open glass bowl which was placed on the hive bottom. As the sunflower honey, the fortified pollen subsamples were stored during the study within a refrigerator between +6 and +9°C. Pollen subsamples of the feeders were replaced by fresh portions on the following study days:

Feeder	Fresh Pollen Subsample on Days
Hive feeder	5, 18, 25, 33
Field feeder	15, 25, 33

At each replacement event and finally on day 38, the amount of collected pollen was determined gravimetrically. The amount of pollen collected between days 0 and 5 could not be precisely determined since the feeder was robbed by mice. For this reason, the amount of collected pollen represents an underestimate of the total amount of collected pollen.

3.8 Climatic Conditions During the Study

During the study, temperature and precipitation events were continously recorded using thermohygrographs and precipitation measuring devices. The following records were made during the evaluation checks (always between 10:00 and 16:00):

Day after first exposure	Air temperature [°C]	Soil temperature [°C]	Precipitation [mm]	Cloudness (% sky coverage)	Wind speed (estimates)*
0 (13:45)	21	25	0	n.r.	n.r.
1 (14:30)	30	32	0	n.r.	n.r.
2 (15:10)	20	22	0	100	n.r.
3 (13:40)	17	19	8	100	n.r.
4 (15:00)	25	30	0	0	n.r.
5 (11:00)	27	29	0	0	+
7 (14:00)	18	16	8	100	++
10 (11:40)	17	20	12	100	+
11 (13:00)	15	17	3	100	++
12 (15:00)	15	17	1	100	++
13 (14:00)	15	17	1	100	-
14 (14:00)	15	16	0.5	100	+
17 (15:00)	24	25	38	60%	n.r.
18 (14:30)	23	28	0	70%	+
19 (13:00)	25	28	0	30%	+
20 (14:00)	25	28	0	40%	-
21 (14:00)	17	17	0	80%	++
24 (15:00)	15	16	9	95	++
25 (14:00)	14	14	2	70%	++
27 (14:00)	20	22	0	80%	++

Day after first exposure	Air temperature [°C]	Soil temperature [°C]	Precipitation [mm]	Cloudness (% sky coverage)	Wind speed (estimates)*
29 (13:00)	21	24	0	5%	+
32 (15:30)	20	20	7	90%	++
34 (15:30)	20	20	6	70%	++
35 (15:00)	22	25	0	n.r.	+

* - = calm, + = slight wind, ++ = moderate wind velocity, +++ = high wind velocities, stormy
n.r. = not reported

3.9 Observations on Honeybees Colonies

All anomalies in the development and behavior of the exposed honeybee colonies were recorded together with the date of observations. In particular, the following behavioural endpoints were evaluated:

Mortality: In front of the colony hives, cotton sheets of 60 x 50 cm were spread on the ground. Dead bees were collected from these sheets daily except during weekends. Any conspicous mortality within the oat strip or the tunnel margins was also recorded but no formal counts were made on these bees.

Comb cell production: The increase in the comb cell area was regularly assessed. For this estimation, the U-shaped form of each comb was mentally transformed to a virtual rectangular quadrat and the size of this virtual rectangle recorded (length x width). This endpoint allowed to evaluate potential impacts of the test compound on wax gland activity (starting about 13 days after ecdysis). A proper function of the wax glands indicates an appropriate supply of young worker bees with pollen.

Food consumption: The amount of pollen and honey consumption was determined by reweighing the respective feeders.

Honey storage behavior: The amount of sampled and processed sunflower honey was regularly assessed in two different ways. The weight increase of the small colonies was recorded which reflects mainly the amount of stored honey. In addition to these weight records, the percentage of comb cells which was filled with honey was also regularly estimated. The percentage values were then converted into an absolute area by taking into account the actual comb cell area at the time of evaluation (see above).

Egg laying activity: The egg laying activity of the queen was assessed by regular inspection of the brood combs. During each inspection, the percentage of comb cells which contained an egg was estimated. The percentage values were then converted into an absolute area by taking into account the actual comb cell area at the time of evaluation (see above).

Breeding success: During each inspection, the percentage of comb cells which contained a honeybee larva or pupa was estimated. The percentage values were then converted into an absolute area by taking into account the actual comb cell area at the time of evaluation (see above). This endpoint does not only evaluate potential influences of the test compound on the queen health (e.g. egg laying activity, egg fertilization) but also the devlopment of the hypopharyngeal glands of young workerbees. A proper functioning of the hypopharyngeal glands indicates an appropriate supply of young worker bees with pollen which is vital for their nursery activity (between day 4 and 12 after ecdysis).

Colony strength: During each inspection, the percentage of comb cell area covered by honeybees was estimated. The percentage values were then converted into an absolute area by taking into account the actual comb cell area at the time of evaluation (see above). This endpoint integrates potential impacts of the test compound on breeding success, longevity and mortality of honeybees.

Foraging intensity: Daily except weekends the number of bees foraging during a 5 minute observation period on the honey and pollen feeder were recorded. In addition, the number of honeybees encountered on the tunnel roof was counted. This figure may give an indication of possible disorientation or repellent/antifeedant phenomena.

Behavioral Anomalies: Whenever observed, behavioral anomalies were recorded with the date and daytime of observation. In particular, honeybees were observed for any of the following symptoms:
- exaggerated motility
- discoordinated movements (trembling, flight incapability)
- apathy, lethargic behavior.

4.0 FILING

All raw data, the study protocol and the original of the report are filed in the Central GLP archive of PF/F, Crop Protection Center 40789 Monheim, FRG. Reserve samples of the test substance are stored in the pertinent archive of that test facility which provided or certified the test substance.

5.0 RESULTS AND DISCUSSION

5.1 Climatic Conditions During the Study

Climatic conditions were recorded within the control study tent with a thermohygrograph. Records are listed in appendix I. Air temperatures fluctuated between 13 and 30°C. Precipitation was recorded on 13 of the 39 study days with a total rainfall of 103.5 mm. The sky was most of the time cloudy. Wind relations were slight to modest during the study period.

5.2 Activity Pattern of Foraging Honeybees and Food Storage Rates

As shown in Fig. 1, activity patterns of foraging honeybees did not differ in relation to the treatment. On average, the same number of foraging honeybees were encountered on either the honey or the pollen feeder. There was also no higher number of honeybees on the tent roof after exposure to imidacloprid residues. The latter endpoint was recorded as an indicator of an antifeedant or disorientated response.

Figure 2 illustrates the quantity of honey and pollen which was collected by the foraging honeybees. All test hives collected lots of pollen and honey and no treatment-related differences were apparent in the substrate consumption rate.

All hives started immediately with the production of new comb cells. Again, no treatment-related difference is found for this testing endpoint (Fig. 3). This evidences that residue levels of up to 20 µg/kg imidacloprid in the pollen do not influence the wax production of young worker bees.

The amount of the honey stores fluctuated considerably in time and with treatment. These fluctuations are most presumably associated with the varying breeding activity of the hive nuclei (Fig. 4). However, no dose-response relationship can be established for this endpoint either and it is, therefore, concluded that imidacloprid residue concentrations up to 20 µg/kg does not adveresely affect the food storage rate of *Apis mellifera carnica*.

Pollen was not stored within the combs over longer periods but directly invested in offspring production. From the breeding performance of all groups it is evident that honeybees of all treatment groups collected and fed sufficient pollen to allow a strong population increase.

A more precise figure for honey storage and comb cell production is derived from the hive weight development. As shown in Fig. 5 there was no treatment-related difference in this endpoint.

5.3 Population Strength Development and Breeding Performance

Fig. 6 reveals the changes in population strength over time. Population strength development shows the same trend for all treatment groups with an increase towards study termination (Fig. 6). Mortality was not related to treatment either (Fig. 7) which demonstrates that the tested imidacloprid residue level had no impact on honeybee longevity.

The egg laying cycle of the queen was different between the treatments but the overall laying activity was rather comparable (Fig. 8). Thus, it can be concluded that the treatment had no influence on the reproductive capacity of the hive nuclei.

The different egg laying cycle is also evident from the abundance of larval and pupal stages in the nuclei combs (Fig. 9 and 10). However, the amount of pre-imaginal stages produced by the nuclei was very comparable between the treatment groups.

FIGURES

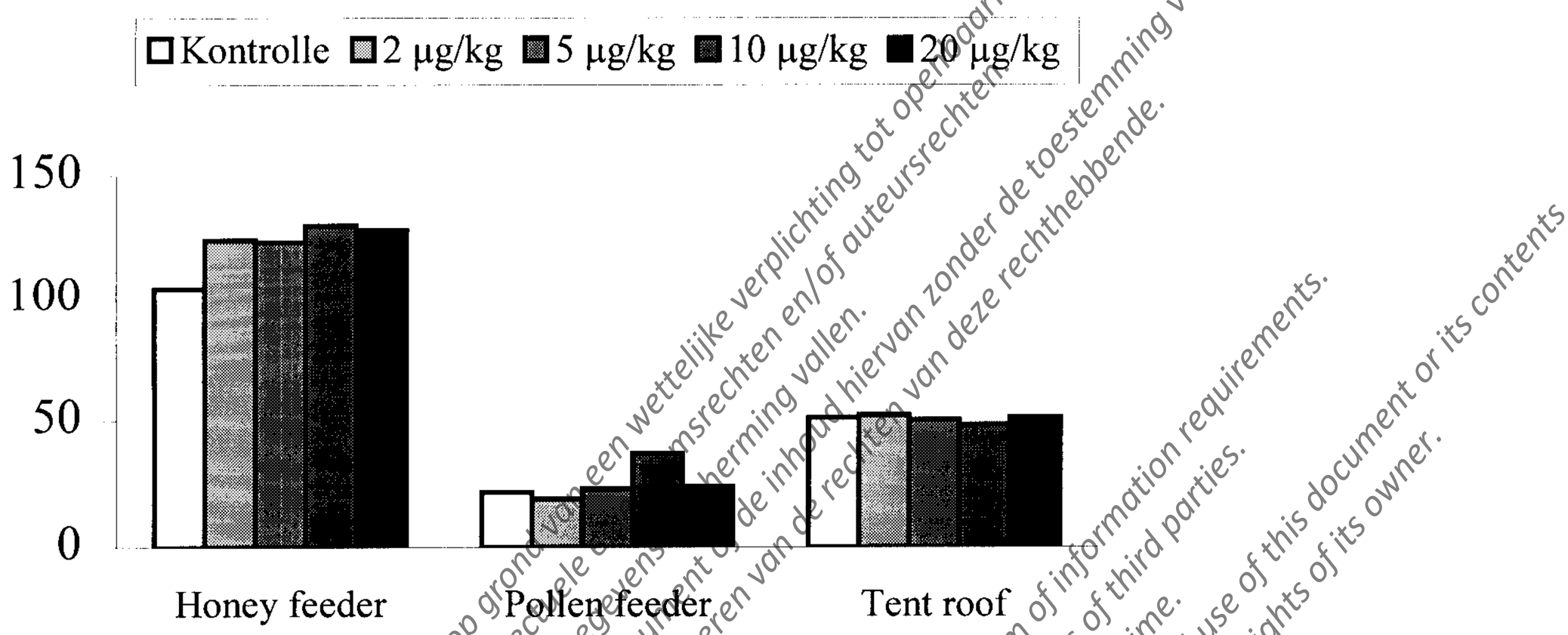
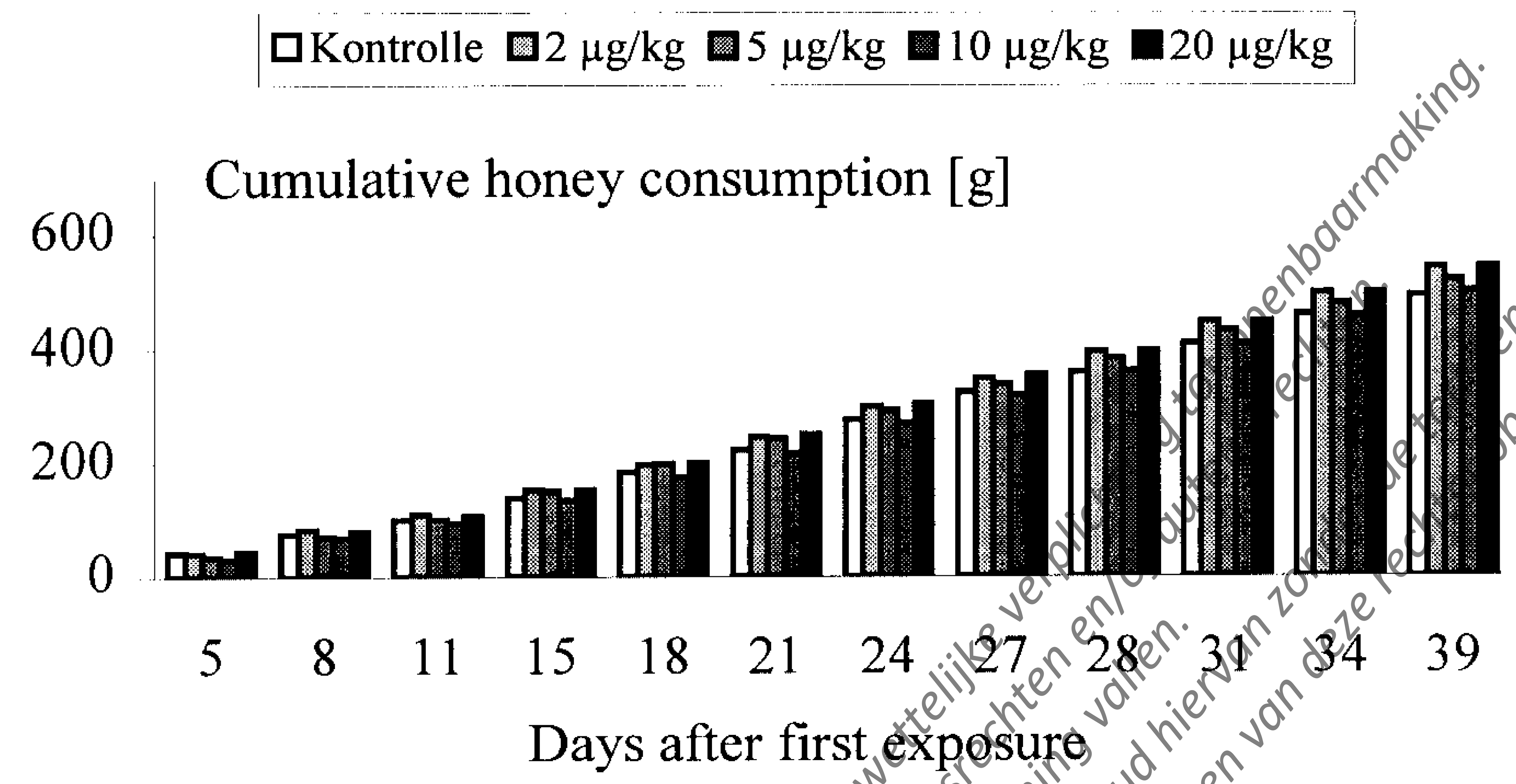


Figure 1: Activity pattern of foraging honeybees in relation to treatment.
Small bee hives were fed with sunflower honey and maize pollen. Maize pollen was fortified with different concentrations of technical imidacloprid. Bars give the average number of foraging honeybees which were recorded per day either on the pollen feeder, the honey feeder or at the tent roof.



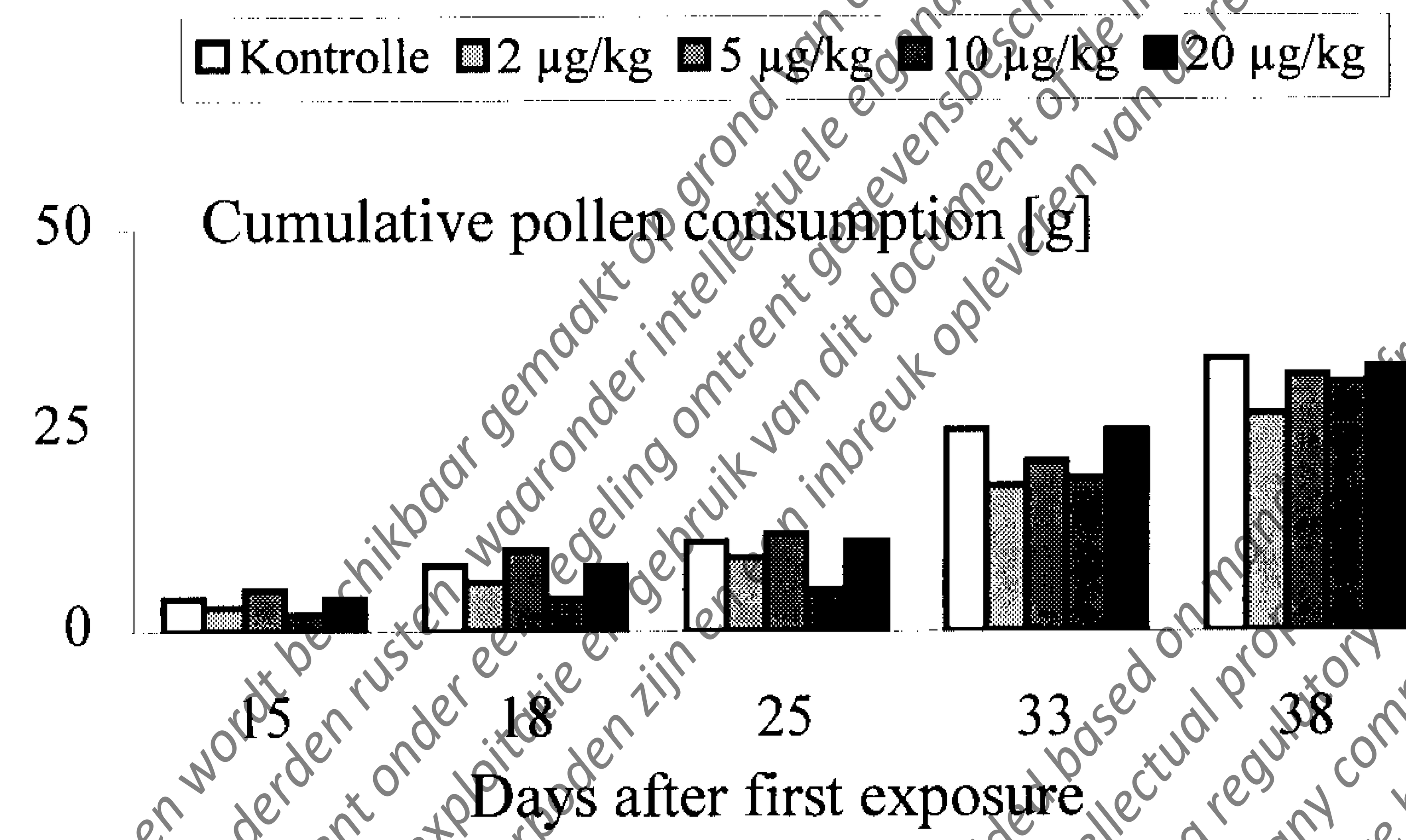


Figure 2: Honey (upper graph) and pollen (lower graph) foraging rate of honeybees in relation to treatment.

Small bee hives were fed with sunflower honey and maize pollen. Maize pollen was fortified with different concentrations of technical imidacloprid. Bars show the cumulative quantity of honey and pollen which was collected by the foraging honeybees over the study period.

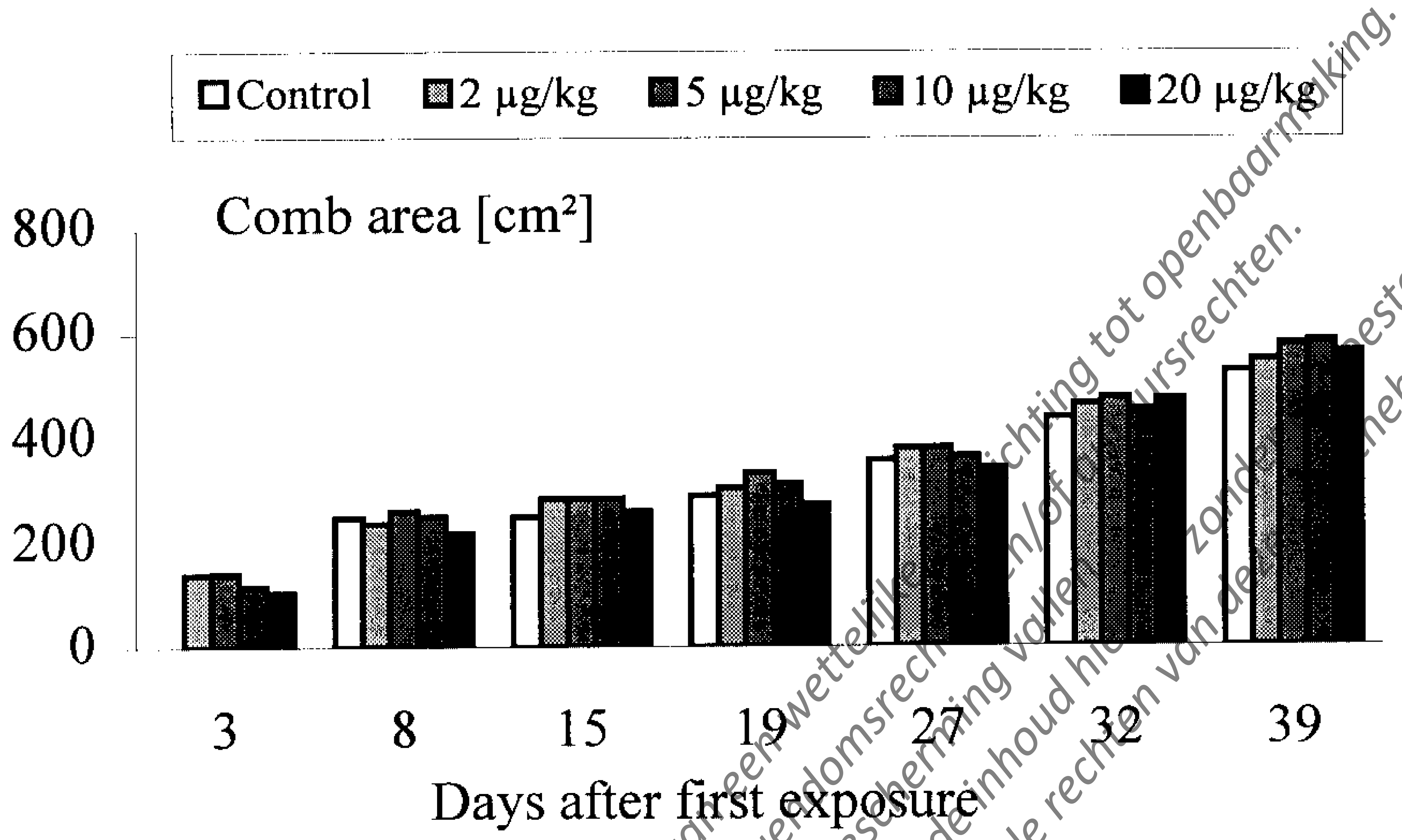


Figure 3: Development of the comb area over time in relation to treatment.
Small bee hives were fed with sunflower honey and maize pollen. Maize pollen was fortified with different concentrations of technical imidacloprid. Bars give the total comb cell area of 4 combs in cm².

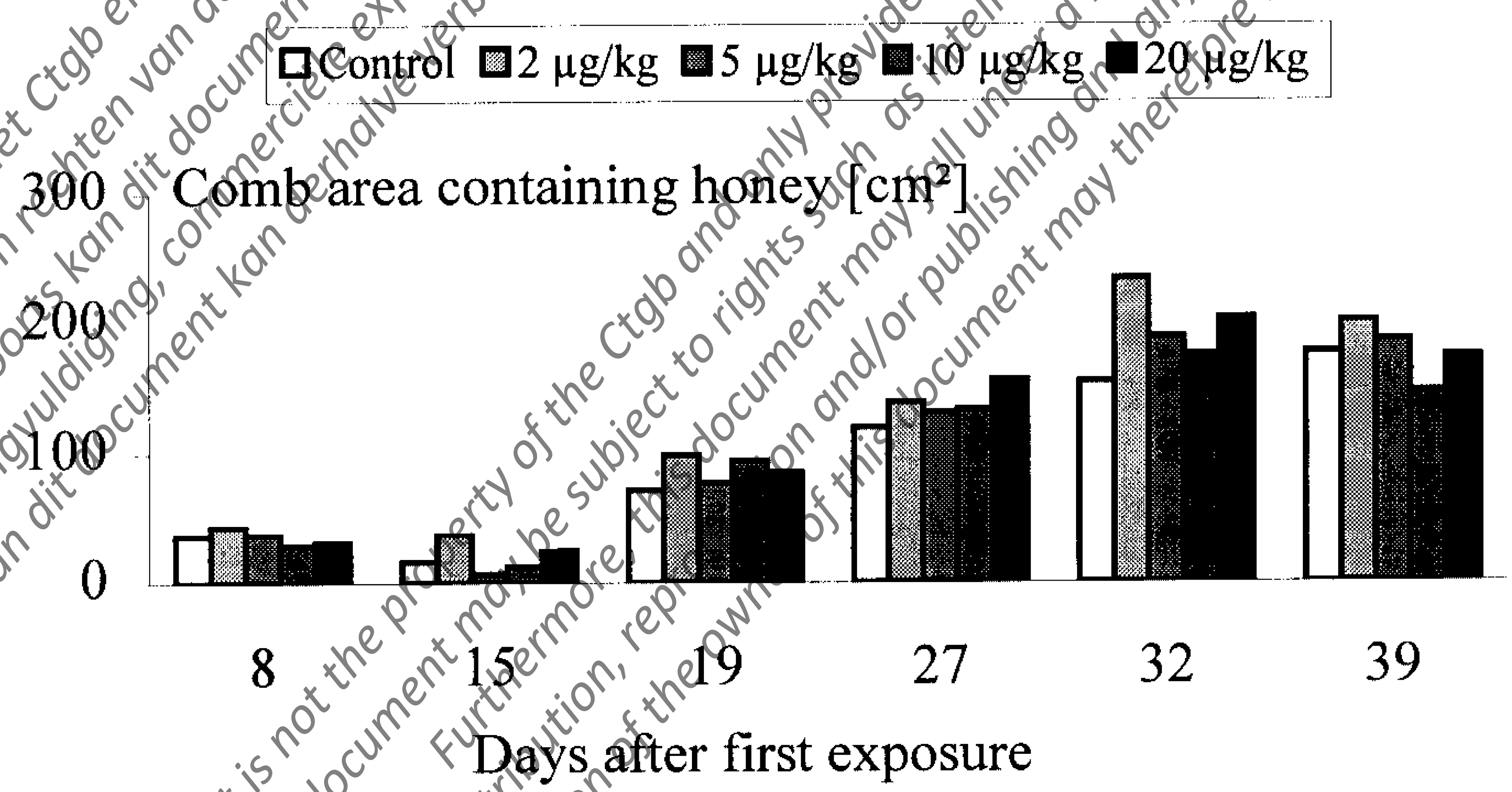


Figure 4: Amount of the honey stores over time in relation to treatment.
Small bee hives were fed with sunflower honey and maize pollen. Maize pollen was fortified with different concentrations of technical imidacloprid. Bars show the size of honey stores as cm² comb area which contained cells filled with honey.

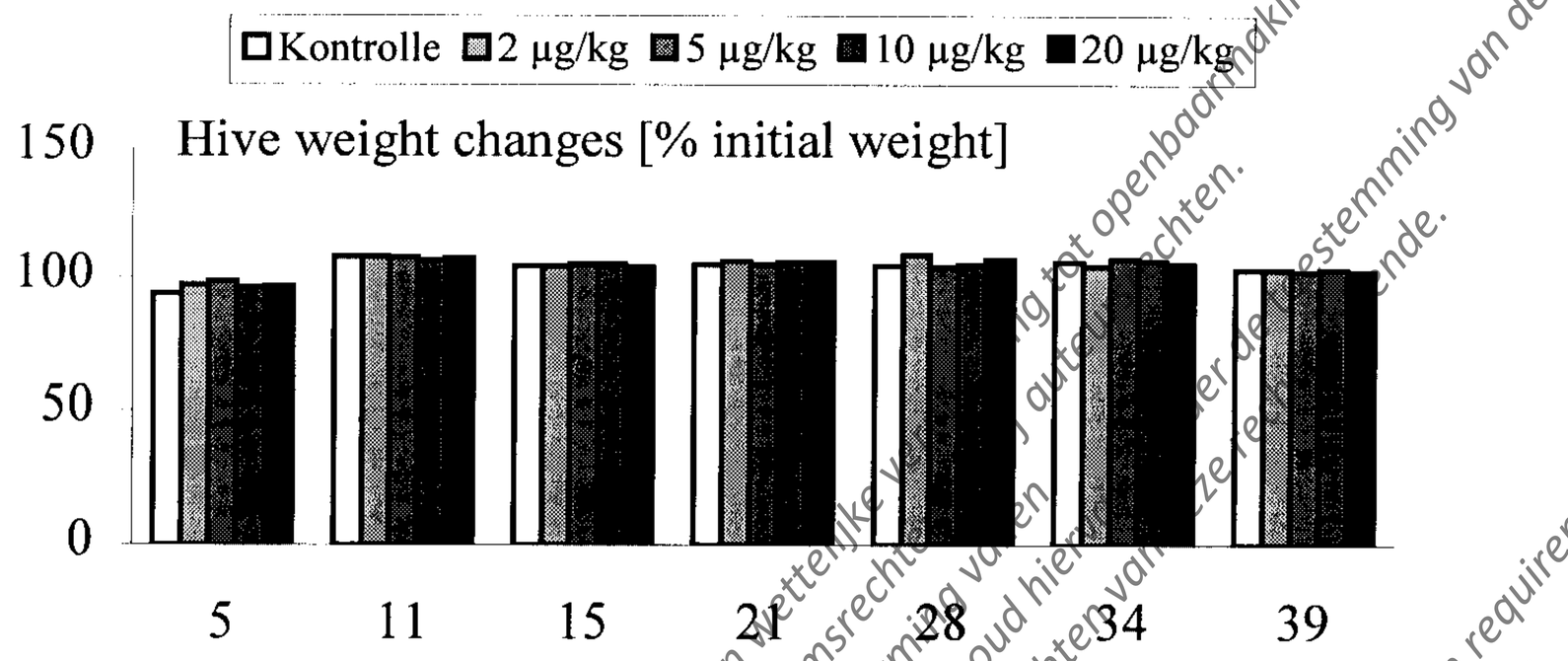


Figure 5: Weight increase of bee hives in relation to treatment.
Small bee hives were fed with sunflower honey and maize pollen. Maize pollen was fortified with different concentrations of technical imidacloprid. Bars show the weight increase relative to the initial hive weight.

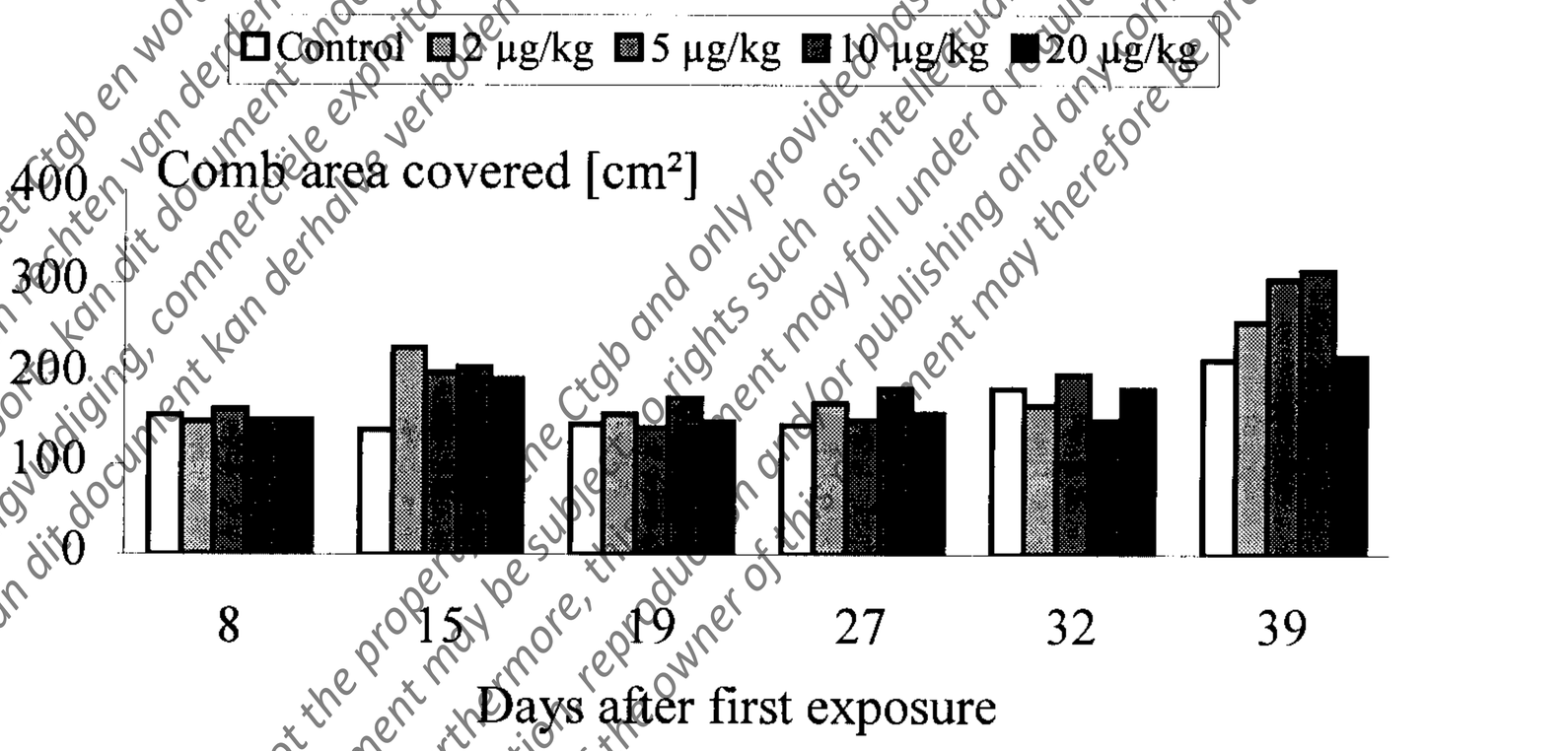


Figure 6: Population development in relation to treatment.
Small bee hives were fed with sunflower honey and maize pollen. Maize pollen was fortified with different concentrations of technical imidacloprid. Bars show the total comb area (four combs) covered by adult honeybees during evaluations taking into account the increase of the comb area over time (see appendix V).

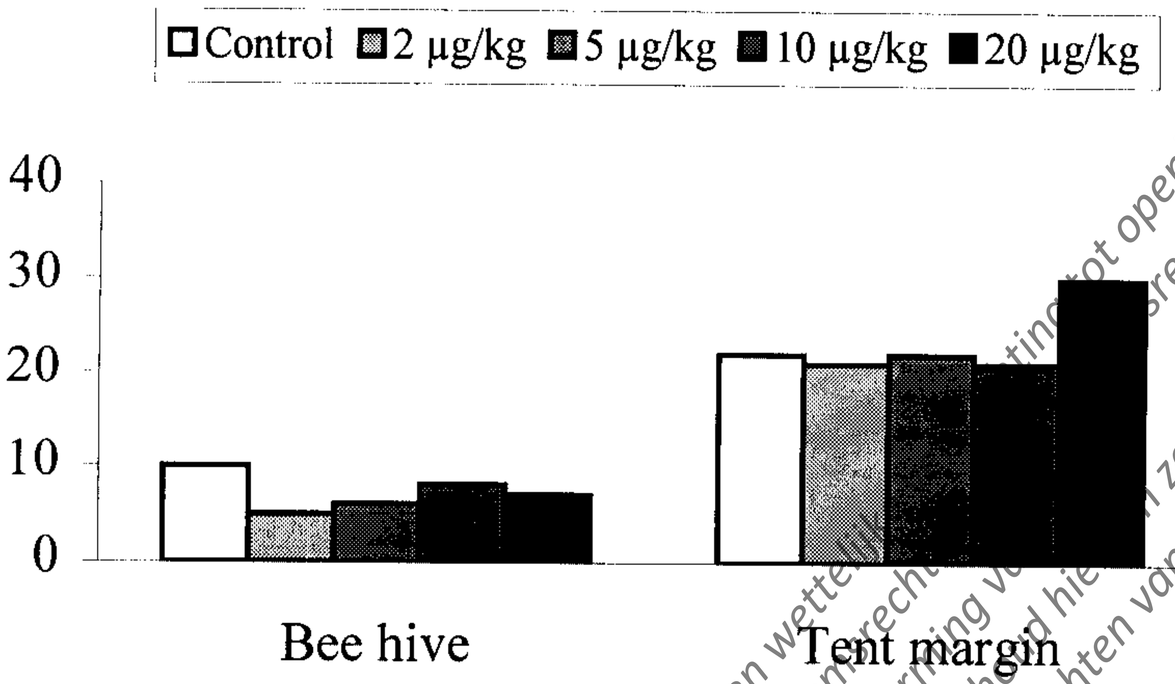


Figure 7: Mortality in relation to treatment.
Small bee hives were fed with sunflower honey and maize pollen. Maize pollen was fortified with different concentrations of technical imidacloprid. Bars give the total number of dead honeybees (workerbees & drones) which were found dead during the study either in front of the bee hives or at the tent margin.

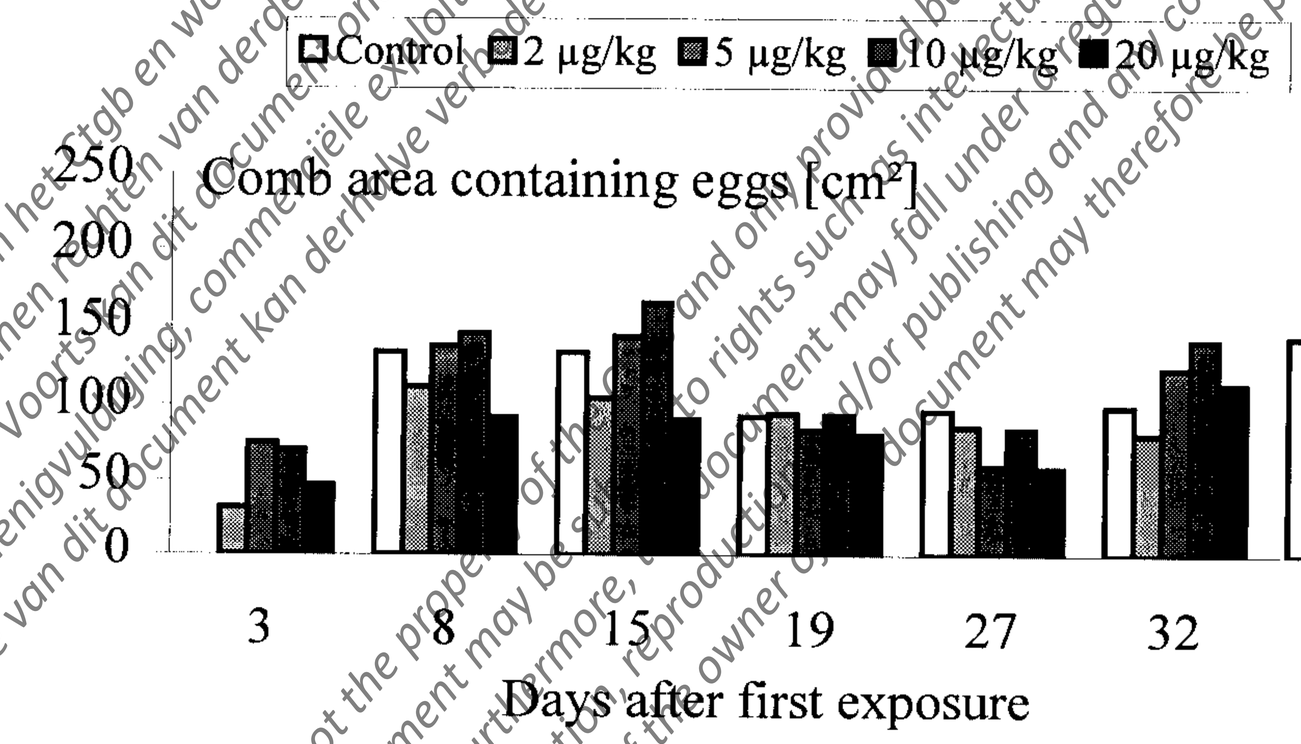


Figure 8: Egg laying activity of the queens in relation to treatment.
Small bee hives were fed with sunflower honey and maize pollen. Maize pollen was fortified with different concentrations of technical imidacloprid. Bars show the total comb area (four combs) where a egg was seen during evaluations taking into account the increase of the comb area over time (see appendix V).

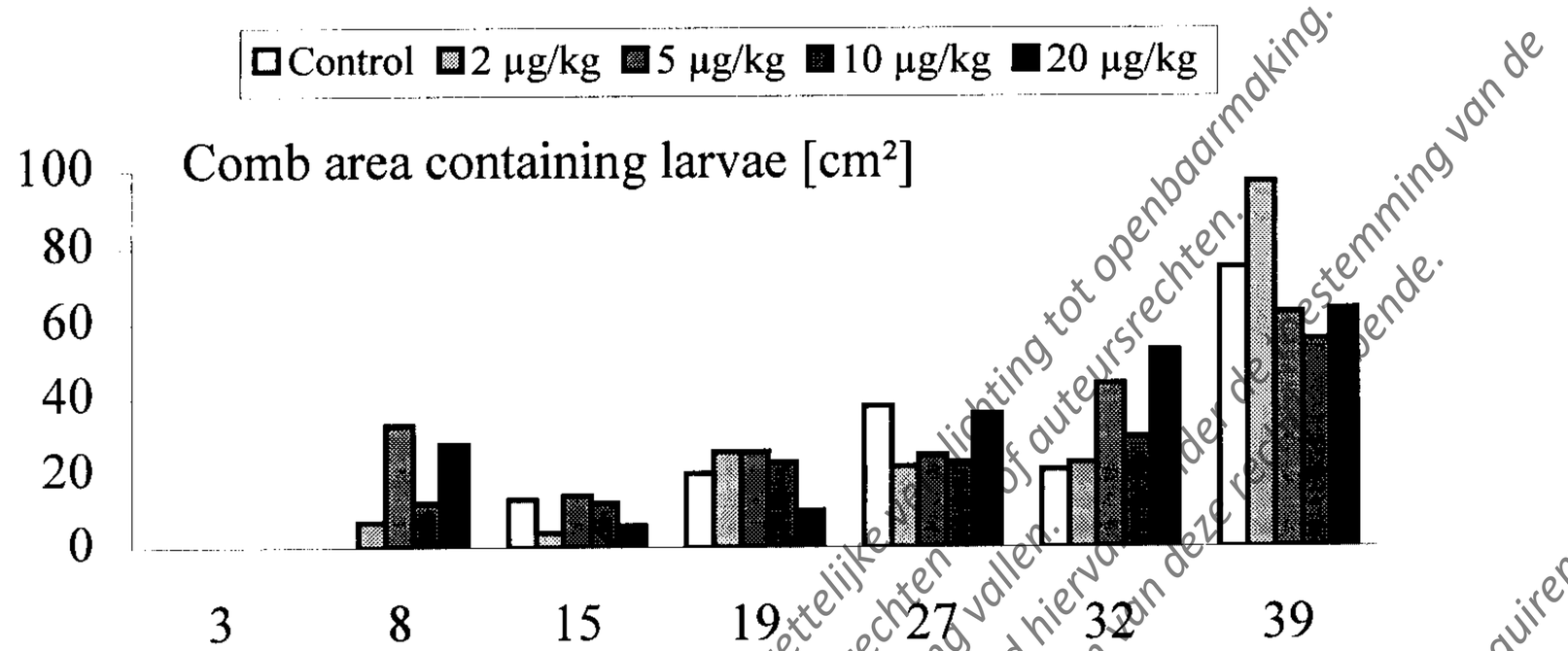


Figure 9: Abundance of Honeybee Larvae (non-capped brood) in Relation to Treatment.
Small bee hives were fed with sunflower honey and maize pollen. Maize pollen was fortified with different concentrations of technical imidacloprid. Bars show the total comb area (four combs) where a larva was seen during evaluations taking into account the increase of the comb area over time (see appendix V).

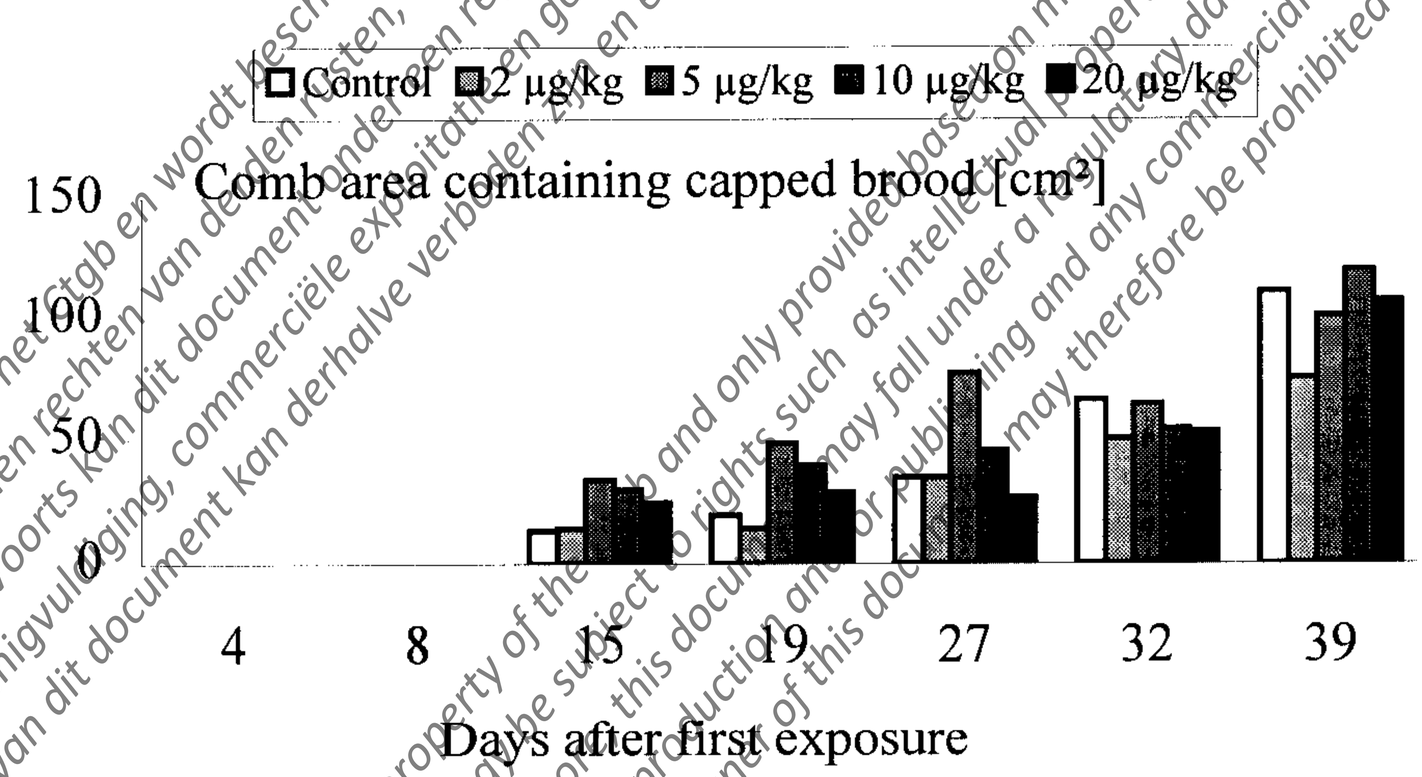


Figure 10: Abundance of Honeybee Pupae (= Capped Brood) in Relation to Treatment.
Small bee hives were fed with sunflower honey and maize pollen. Maize pollen was fortified with different concentrations of technical imidacloprid. Bars show the total comb area (four combs) where capped cells were seen during evaluations taking into account the increase of the comb area over time (see appendix V).

TABLES

Table 1: Summary of the Analytical Findings on Fortified Maize Pollen.

The summary sheet contains data which were generated and reported under study number MR-513/99. This study report is attached as appendix XIII to this report.

Sample Name	Fortification Level [µg/kg]	Residues of Imidacloprid [µg/kg]	% of Theoretical	Mean [%]	RSD* [%]
Control Pollen A	0	n.d.	0	0	0
Control Pollen B	0	n.d.	0		
Pollen 2 µg/kg A	2.0	< LOQ	-	-	-
Pollen 2 µg/kg B	2.0	< LOQ	-		
Pollen 2 µg/kg C	2.0	< LOQ	-		
Pollen 2 µg/kg D	2.0	< LOQ	-		
Pollen 2 µg/kg E	2.0	< LOQ	-		
Pollen 5 µg/kg A	5.0	4.2	84	90	13.3
Pollen 5 µg/kg B	5.0	4.7	94		
Pollen 5 µg/kg C	5.0	4.4	88		
Pollen 5 µg/kg D	5.0	3.8	76		
Pollen 5 µg/kg E	5.0	5.4	108		
Pollen 10 µg/kg A	10.0	9.4	94	88.6	12.4
Pollen 10 µg/kg B	10.0	9.4	94		
Pollen 10 µg/kg C	10.0	7.8	78		
Pollen 10 µg/kg D	10.0	7.6	76		
Pollen 10 µg/kg E	10.0	10.1	101		
Pollen 20 µg/kg A	20.0	18.8	94	99.5	5.0
Pollen 20 µg/kg B	20.0	21.3	106.5		
Pollen 20 µg/kg C	20.0	19.5	97.5		
Pollen 20 µg/kg D	20.0	19.4	97		
Pollen 20 µg/kg E	20.0	20.5	102.5		

Limit of quantitation: 0.005 mg/kg; limit of detection: 0.0015 mg/kg; 1.5 - 5 =residues below the limit of quantitation but above the limit of detection; n.d. = residues below the limit of detection.
** RSD = relative standard deviation in %*

Table 2: Summary of Findings.
Data are reported in detail in the pertinent appendices.

Testing Endpoint	Control	2 µg/kg	5 µg/kg	10 µg/kg	20 µg/kg
Mortality (no. of dead bees in front of bee hives)	10	5	6	8	7
Mortality (no of dead bees at the tent margin)	22	21	22	21	30
Foraging intensity (no. of bees at the pollen feeder)	22	19	23	37	24
Foraging intensity (no. of bees at the honey feeder)	104	124	123	130	128
Honey consumption [g]	35	29	32	39	34
Pollen consumption [g]	491	541	521	500	543
Comb cell production [cm²]	528	551	579	584	563
Honey storage area at study termination [cm²]	177	201	186	147	174
Hive weight increase	180	230	215	200	200
Egg laying activity[cm² comb area containing eggs at study termination)	144	153	181	205	153
Colony strength [cm² comb area covered with bees at study termination)	217	258	305	314	221

APPENDICES

APPENDIX I: Analytical Examination of the Food Fed During the Study (Sunflower Honey and Pollen) for Contaminants.

Contaminant analyses were performed by Bayer AG, PF-E/MR for imidacloprid and by Dr. Specht & Partner in D-20354 Hamburg for other contaminants. The latter analysis was not performed under GLP.

A. Sunflower Honey

Sample Description	Testing Facility	Contaminant	Analytical Findings
Sunflower honey	Bayer AG, PF-E/MR	Imidacloprid *	n.d.
Sunflower honey	Specht & Partner [1]	Pyrethroids **	n.d.
Sunflower honey	Specht & Partner [1]	Organophosphates ***	n.d.
Sunflower honey	Specht & Partner [1]	Tri-iso-butylphosphate **	n.d.
Sunflower honey	Specht & Partner [1]	Tris-2-butoxyethylphosphate	n.d.

[1] Analytical reference number: M5886/99

* *Limit of quantitation: 0.005 mg/kg; limit of detection: 0.0015 mg/kg; 1.5 - 5 =residues below the limit of quantitation but above the limit of detection; n.d. = residues below the limit of detection.*

** *Limit of detection: 0.01 mg/kg; n.d. = residues below the limit of detection.*

*** *Limit of detection: 0.02 mg/kg; n.d. = residues below the limit of detection.*

B. Maize Pollen from Brasil

Sample Description	Testing Facility	Contaminant	Analytical Findings
Maize pollen	Bayer AG, PF-E/MR	Imidacloprid *	n.d.
Maize pollen	Specht & Partner [1]	Pyrethroids **	n.d.
Maize pollen	Specht & Partner [1]	Organophosphates ***	n.d.
Maize pollen	Specht & Partner [1]	Tri-iso-butylphosphate **	0.13
Maize pollen	Specht & Partner [1]	Tris-2-butoxyethylphosphate	0.20

[1] Analytical reference number: M5886/99

* *Limit of quantitation: 0.005 mg/kg; limit of detection: 0.0015 mg/kg; 1.5 - 5 =residues below the limit of quantitation but above the limit of detection; n.d. = residues below the limit of detection.*

** *Limit of detection: 0.01 mg/kg; n.d. = residues below the limit of detection.*

*** *Limit of detection: 0.02 mg/kg; n.d. = residues below the limit of detection.*

APPENDIX II: Climatic Conditions as Recorded During Evaluation Dates.

Records were made within the study tents with thermohygrographs.

DAT	Minimum Temperature [° C]	Maximum Temperature [° C]	Air Humidity [%]
0	12	27	70-95
1	12	34	60-98
2	16	33	65-95
3	12	23	80-95
4	10	30	70-98
5	14	34	55-98
6	13	30	50-98
7	12	22	80-98
8	11	20	90-98
9	11	23	70-98
10	10	25	85-98
11	10	22	65-98
12	9	21	60-95
13	11	25	60-98
14	10	21	65-98
15	10	28	45-98
16	12	25	65-98
17	12	28	55-95
18	12	28	55-98
19	13	28	55-95
20	15	28	60-95
21	12	22	60-95
22	10	19	50-95
23	12	22	80-95
24	10	20	65-95
25	9	23	55-95
26	9	22	60-95
27	10	25	55-95
28	10	25	50-95
29	10	29	50-95
30	16	28	60-95
31	13	28	55-90
32	13	28	65-95
33	15	23	75-98
34	13	24	65-95
35	14	27	65-95
36	16	33	55-95
37	17	33	60-95

APPENDIX III: Activity Pattern of Foraging Honeybees in Relation to Treatment.

Figures give the average number of foraging honeybees which were recorded per day either on the pollen feeder, the honey feeder or at the tent roof.

Days after first exposure to treated substrate/comb area	Number of honeybees recorded on the pollen feeder				
	Control	2 µg/kg	5 µg/kg	10 µg/kg	20 µg/kg
0	0	0	0	0	0
1	0	0	0	0	0
2	0	0	0	0	0
3	0	0	0	0	0
4	0	0	0	1	0
5	1	0	0	1	0
7	1	0	0	0	1
10	1	0	0	2	1
11	0	1	1	1	0
12	1	2	1	1	1
13	2	1	2	1	2
14	1	0	1	2	1
17	1	2	1	3	2
18	1	0	0	0	0
19	1	2	0	3	1
20	2	1	2	2	2
21	0	1	1	1	1
24	1	1	2	2	1
25	1	1	1	4	1
27	1	2	2	2	2
28	2	2	3	4	3
31	1	0	3	1	2
33	1	1	1	2	1
34	3	2	2	4	2

APPENDIX III: cont´d.

Figures give the average number of foraging honeybees which were recorded per day either on the pollen feeder, the honey feeder or at the tent roof.

Days after first exposure to treated substrate/comb area	Number of honeybees recorded on the honey feeder				
	Control	2 µg/kg	5 µg/kg	10 µg/kg	20 µg/kg
0	1	2	1	1	3
1	2	2	2	2	2
2	1	5	6	5	3
3	2	5	8	6	7
4	3	7	7	8	7
5	4	5	7	6	7
7	4	3	4	4	5
10	4	4	5	4	5
11	4	4	4	4	4
12	5	4	4	5	5
13	6	5	5	5	5
14	3	4	3	4	4
17	6	6	6	5	5
18	5	5	1	5	3
19	8	10	8	11	8
20	9	8	9	8	9
21	1	2	1	3	2
24	3	5	6	4	5
25	4	4	5	5	4
27	6	7	5	6	6
28	6	8	8	9	10
31	4	5	4	6	5
33	5	4	6	5	6
34	8	10	8	9	8

APPENDIX III: cont´d.

Figures give the average number of foraging honeybees which were recorded per day either on the pollen feeder, the honey feeder or at the tent roof.

Days after first exposure to treated substrate/comb area	Number of honeybees recorded on the tent roof				
	Control	2 µg/kg	5 µg/kg	10 µg/kg	20 µg/kg
1	1	1	2	2	2
2	1	1	2	3	1
3	0	1	1	1	1
4	3	1	3	3	3
5	1	2	2	2	2
7	0	0	0	0	0
10	0	0	0	0	0
11	0	0	0	0	0
12	0	0	1	0	0
13	0	2	2	2	0
14	0	0	0	0	0
17	6	5	2	4	3
18	15	15	11	10	13
19	4	6	5	3	4
20	3	4	4	4	5
21	0	0	0	0	0
24	1	1	1	0	1
25	2	3	2	2	3
27	4	3	3	4	2
28	5	5	3	4	5
31	1	1	0	1	0
33	2	0	3	1	2
34	2	1	3	2	4

APPENDIX IV: Quantity of Pollen and Honey Collected by the Foraging Honeybees in Relation to Treatment.

Days after first exposure to treated substrate/comb area	Quantity of Collected Honey [g]				
	Control	2 µg/kg	5 µg/kg	10 µg/kg	20 µg/kg
3	2	10	5	2	14
5	41	31	31	29	30
8	32	42	35	37	36
11	25	27	30	26	27
15	39	44	50	41	47
18	45	44	49	41	48
21	40	49	43	42	49
24	52	52	49	52	52
27	48	49	47	48	52
28	35	48	45	43	42
31	50	51	48	48	50
34	50	49	47	49	49
39	32	45	42	42	47

Days after first exposure to treated substrate/comb area	Quantity of Collected Pollen [g]				
	Control	2 µg/kg	5 µg/kg	10 µg/kg	20 µg/kg
15	4	3	5	2	4
18	4	3	5	2	4
25	3	3	2	1	3
33	14	9	9	14	14
38	9	9	11	12	8

APPENDIX V: Comb Production in Relation to Treatment.

Figures give the production of comb cells on four comb matrices over time, i.e. cumulative values. The newly produced comb area was not rectangular. Instead, it was added on to the rectangularly shaped comb matrix in the form of a half circle. The area of the irregularly shaped comb was estimated by extrapolation this geometric form into a rectangular form. The values in parenthesis give the one-sided mean vertical and the mean horizontal extension of this extrapolated quadrat for each of the four comb matrices. Since comb cells are produced simultaneously on both sides, the total area calculated had to be multiplied by factor 2.

Days after first exposure to treated substrate	Increase in comb area [cm²] Control	2 µg/kg	5 µg/kg	10 µg/kg	20 µg/kg
3	0 (0x 0)	0 (0x 0)	0 (0x 0)	0 (0x 0)	0 (0x 0)
	0 (0x 0)	0 (0x 0)	0 (0x 0)	0 (0x 0)	0 (0x 0)
	0 (0x 0)	70 (7x 5)	36 (6x 3)	48 (6x 4)	24 (4x 3)
	0 (0x 0)	72 (6x 6)	108 (9x 6)	72 (6x 6)	84 (6x 7)
	Total: 0	Total: 142	Total: 144	Total: 120	Total: 108
8	0 (0x 0)	0 (0x 0)	0 (0x 0)	0 (0x 0)	0 (0x 0)
	0 (0x 0)	0 (0x 0)	0 (0x 0)	0 (0x 0)	0 (0x 0)
	108 (12x 4^5)	108 (12x 4^5)	121 (11x 5^5)	110 (11x 5)	110 (11x 5)
	144 (12x 6)	132 (11x 6)	144 (12x 6)	144 (12x 6)	112 (8x 7)
	Total: 252	Total: 240	Total: 265	Total: 254	Total: 222
15	0 (0x 0)	0 (0x 0)	0 (0x 0)	0 (0x 0)	0 (0x 0)
	0 (0x 0)	0 (0x 0)	0 (0x 0)	0 (0x 0)	0 (0x 0)
	121 (11x 5^5)	144 (12x 6)	144 (12x 6)	144 (12x 6)	144 (12x 6)
	132 (12x 5^5)	144 (12x 6)	144 (12x 6)	144 (12x 6)	120 (8x 7^5)
	Total: 253	Total: 288	Total: 288	Total: 288	Total: 264
19	0 (0x 0)	0 (0x 0)	0 (0x 0)	0 (0x 0)	0 (0x 0)
	30 (5x 3)	20 (5x 2)	48 (8x 3)	16 (4x 2)	20 (5x 2)
	120 (12x 5)	144 (12x 6)	144 (12x 6)	144 (12x 6)	144 (12x 6)
	144 (12x 6)	144 (12x 6)	144 (12x 6)	156 (12x 6^5)	112 (8x 7)
	Total: 294	Total: 308	Total: 336	Total: 316	Total: 276
27	0 (0x 0)	0 (0x 0)	0 (0x 0)	0 (0x 0)	0 (0x 0)
	48 (8x 3)	48 (8x 3)	48 (8x 3)	20 (5x 2)	32 (8x 2)
	144 (12x 6)	168 (12x 7)	168 (12x 7)	168 (12x 7)	168 (12x 7)
	168 (12x 7)	168 (12x 7)	168 (12x 7)	180 (12x 7^5)	144 (9x 8)
	Total: 360	Total: 384	Total: 384	Total: 368	Total: 344
32	0 (0x 0)	0 (0x 0)	0 (0x 0)	0 (0x 0)	0 (0x 0)
	120 (10x 6)	120 (10x 6)	143 (11x 6^5)	120 (10x 6)	120 (10x 6)
	154 (11x 7)	168 (12x 7)	168 (12x 7)	156 (12x 6^5)	180 (12x 7^5)
	168 (12x 7)	180 (12x 7^5)	168 (12x 7)	180 (12x 7^5)	176 (11x 8)
	Total: 442	Total: 468	Total: 479	Total: 456	Total: 476
39	0 (0x 0)	0 (0x 0)	32 (8x 2)	20 (5x 2)	24 (6x 2)
	132 (11x 6)	143 (11x 6^5)	168 (12x 7)	156 (12x 6^5)	143 (11x 6^5)
	192 (12x 8)	192 (12x 8)	192 (12x 8)	192 (12x 8)	180 (12x 7^5)
	204 (12x 8^5)	216 (12x 9)	187 (11x 8^5)	216 (12x 9)	216 (12x 9)
	Total: 528	Total: 551	Total: 579	Total: 584	Total: 563

APPENDIX VI: Size of Honey Stores over Time in Relation to Treatment.

Figures give the proportion of comb areas (four combs) where stored honey was recorded during evaluation. The first values give the mean values of both comb sites. The values in parenthesis give the single values of the front and back site of each comb.
The total figure refers to the absolute area in cm² which contained honey taking into account the values of the newly produced comb area from appendix V.

Days after first exposure to treated substrate/comb area	Honey deposition area [%combs with honey/total = cm² combs with honey]				
	Control	2 µg/kg	5 µg/kg	10 µg/kg	20 µg/kg
3	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	40 (60; 20)	25 (30; 20)	3 (0; 5)	5 (5; 0)
	0 (0; 0)	25 (25; 25)	20 (20; 20)	25 (20; 30)	55 (30; 25)
	Total: 0	Total: 46	Total: 31	Total: 19	Total: 47
8	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	13 (20; 5)	13 (20; 5)	15 (20; 10)	15 (25; 5)	3 (0; 5)
	15 (20; 10)	23 (20; 25)	13 (10; 15)	8 (10; 5)	25 (25; 25)
	Total: 36	Total: 44	Total: 37	Total: 28	Total: 31
15	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	8 (15; 0)	18 (25; 10)	5 (10; 0)	3 (5; 0)	10 (20; 0)
	5 (0; 10)	8 (0; 15)	0 (0; 0)	5 (5; 5)	8 (0; 15)
	Total: 16	Total: 37	Total: 7	Total: 12	Total: 24
19	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	25 (0; 50)	0 (0; 0)	5 (10; 0)
	30 (35; 25)	40 (40; 40)	13 (0; 25)	35 (35; 35)	33 (35; 30)
	25 (25; 25)	28 (30; 25)	33 (30; 35)	28 (30; 25)	33 (35; 30)
	Total: 72	Total: 98	Total: 78	Total: 94	Total: 85
27	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	25 (0; 50)	0 (0; 0)	0 (0; 0)
	50 (60; 40)	50 (50; 50)	33 (35; 30)	50 (50; 50)	65 (65; 65)
	28 (30; 25)	33 (35; 30)	38 (35; 40)	28 (30; 25)	33 (30; 35)
	Total: 119	Total: 139	Total: 131	Total: 134	Total: 157
32	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	5 (5; 5)	23 (25; 20)	35 (30; 40)	23 (20; 25)	20 (20; 20)
	55 (50; 60)	80 (80; 80)	43 (45; 40)	60 (60; 60)	68 (65; 70)
	38 (40; 35)	40 (40; 40)	40 (45; 35)	30 (30; 30)	33 (30; 35)
	Total: 155	Total: 234	Total: 189	Total: 175	Total: 204
39	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	55 (50; 60)	68 (65; 70)	68 (75; 60)	45 (50; 40)	60 (70; 50)
	28 (25; 30)	28 (25; 30)	20 (20; 20)	20 (20; 20)	25 (30; 20)
	25 (30; 20)	23 (25; 20)	18 (15; 20)	18 (20; 15)	20 (15; 25)
	Total: 177	Total: 201	Total: 186	Total: 147	Total: 174

APPENDIX VII: Weight increase of bee hives in relation to treatment.

Days after first exposure to treated substrate/comb area	Total Hive Weight [g]				
	Control	2 µg/kg	5 µg/kg	10 µg/kg	20 µg/kg
0	655	635	640	625	620
5	615	615	630	600	600
11	665	665	680	640	645
15	695	695	715	675	670
21	730	740	750	715	710
28	765	805	780	750	760
34	810	840	835	800	800
39	835	865	855	825	820

APPENDIX VIII: Population Growth in Relation to Treatment.

Figures give the proportion of comb areas (four combs) which was occupied by adult honeybees during evaluation. The first values give the mean values of both comb sites. The values in parenthesis give the single values of the front and back site of each comb. The total figure refers to the absolute area in cm² covered by honeybees taking the values of the newly produced comb area from appendix V.

Days after first exposure to treated substrate/comb area	Population density [% occupied combs/total cm² occupied comb area]				
	Control	2 µg/kg	5 µg/kg	10 µg/kg	20 µg/kg
3	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	90 (100;80)	80 (80; 80)	75 (80; 70)	100(100;100)
	0 (0; 0)	70 (40;100)	75 (90; 60)	70 (50; 90)	95 (100; 90)
	Total: 0*	Total: 113	Total: 110	Total: 86	Total: 104
8	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	50 (50; 50)	45 (40; 50)	80 (90; 70)	45 (40; 50)	55 (60; 50)
	70 (70; 70)	75 (80; 70)	45 (30; 60)	70 (80; 60)	80 (80; 80)
	Total: 155	Total: 148	Total: 162	Total: 150	Total: 150
15	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	55 (80; 30)	80 (90; 70)	65 (50; 80)	70 (60; 80)	65 (70; 60)
	80 (80; 80)	80 (70; 90)	75 (70; 80)	75 (70; 80)	85 (90; 80)
	Total: 172	Total: 230	Total: 202	Total: 209	Total: 196
19	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	40 (40; 40)	25 (30; 20)	30 (40; 20)	60 (70; 50)	35 (20; 50)
	45 (50; 40)	50 (60; 40)	43 (50; 35)	60 (70; 50)	55 (35; 75)
	55 (70; 40)	55 (60; 50)	45 (40; 50)	50 (50; 50)	55 (50; 60)
	Total: 145	Total: 156	Total: 141	Total: 174	Total: 148
27	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	5 (5; 5)	18 (25; 10)	20 (30; 10)	5 (5; 5)	8 (5; 10)
	40 (20; 60)	50 (50; 50)	38 (40; 35)	45 (50; 40)	45 (20; 70)
	50 (30; 70)	45 (50; 40)	45 (20; 70)	60 (80; 40)	55 (50; 60)
	Total: 144	Total: 168	Total: 149	Total: 185	Total: 157
32	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	20 (20; 20)	33 (30; 35)	28 (30; 25)	18 (25; 10)	30 (40; 20)
	50 (20; 80)	43 (25; 60)	60 (70; 50)	30 (40; 20)	43 (35; 50)
	50 (50; 50)	30 (30; 30)	35 (50; 20)	45 (30; 60)	40 (50; 30)
	Total: 185	Total: 166	Total: 200	Total: 149	Total: 184
39	0 (0; 0)	0 (0; 0)	3 (5; 0)	3 (0; 5)	3 (5; 0)
	30 (20; 40)	30 (35; 25)	40 (40; 40)	35 (50; 20)	40 (50; 30)
	50 (40; 60)	50 (30; 70)	65 (50; 80)	45 (50; 40)	45 (50; 40)
	40 (40; 40)	55 (40; 70)	60 (70; 50)	80 (90; 70)	38 (35; 40)
	Total: 217	Total: 258	Total: 305	Total: 314	Total: 221

* Honeybees formed aggregates at the lower end of the comb matrix.

APPENDIX IX: Mortality in Relation to Treatment.

Figures give the number of honeybees (worker bees and drones) which were found dead during the study either in front of the bee hive or at the tent margin.

Days after first exposure to treated substrate/comb area	Number of dead honeybees found in front of the bee hives				
	Control	2 µg/kg	5 µg/kg	10 µg/kg	20 µg/kg
1	1	0	0	0	1
2	0	0	0	0	0
3	1	0	0	0	0
4	0	0	0	0	0
5	0	0	0	0	0
7	1	0	0	0	0
10	0	0	0	0	0
11	0	0	0	0	0
12	1	0	1	0	0
13	1	1	0	0	0
14	0	0	0	1	1
17	0	0	0	0	1
18	0	0	0	0	0
19	0	0	0	0	1
20	0	0	1	0	0
21	0	1	0	1	0
24	1	0	0	1	1
25	1	0	1	1	0
27	2	1	1	1	1
28	0	0	0	0	0
31	1	1	0	2	0
33	0	1	1	0	1
34	0	0	1	1	0

APPENDIX IX: cont´d.

Figures give the number of dead honeybees (worker bees and drones) which were found dead during the study either in front of the bee hive or at the tent margin.

Days after first exposure to treated substrate/comb area	Number of dead honeybees found at the tunnel margin				
	Control	2 µg/kg	5 µg/kg	10 µg/kg	20 µg/kg
1	1	2	3	2	1
2	1	0	1	0	0
3	0	0	0	0	0
4	0	0	0	0	0
5	0	0	0	1	1
7	0	0	0	0	1
10	1	0	0	1	1
11	0	0	1	0	0
12	1	1	0	0	0
13	1	2	0	2	3
14	0	0	1	0	0
17	3	3	1	2	4
18	1	4	2	5	6
19	0	0	0	1	0
20	1	0	0	1	1
21	0	1	1	0	0
24	2	2	2	1	2
25	3	1	1	2	1
27	2	2	2	1	1
28	0	0	1	0	2
31	3	1	3	1	2
33	1	2	1	0	3
34	1	0	2	1	1

APPENDIX X: Queen Egg Laying Activity in Relation to Treatment.

Figures give the proportion of comb areas (four combs) where an egg was found during evaluation. The first values give the mean values of both comb sites. The values in parenthesis give the single values of the front and back site of each comb.
The total figure refers to the absolute area in cm² which contained eggs taking into account the values of the newly produced comb area from appendix V.

Days after first exposure to treated substrate/comb area	Egg deposition activity [% combs with eggs/total=cm² combs with eggs]				
	Control	2 µg/kg	5 µg/kg	10 µg/kg	20 µg/kg
3	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	5 (5; 5)	73 (70; 75)	5 (5; 5)
	0 (0; 0)	45 (40; 50)	68 (70; 65)	50 (50; 50)	55 (50; 60)
	Total: 0	Total: 32	Total: 75	Total: 71	Total: 47
8	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	40 (15; 65)	45 (50; 40)	63 (60; 65)	50 (40; 60)	50 (50; 50)
	63 (60; 65)	48 (60; 35)	43 (40; 45)	63 (60; 65)	33 (30; 35)
	Total: 134	Total: 112	Total: 138	Total: 146	Total: 92
15	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	58 (50; 65)	30 (30; 30)	55 (50; 60)	65 (65; 65)	38 (35; 40)
	48 (60; 35)	43 (50; 35)	45 (50; 40)	50 (50; 50)	30 (40; 20)
	Total: 134	Total: 105	Total: 144	Total: 166	Total: 91
19	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	38 (40; 35)	33 (30; 35)	38 (35; 40)	38 (35; 40)	38 (35; 40)
	33 (35; 30)	33 (35; 30)	20 (25; 15)	25 (25; 25)	23 (25; 20)
	Total: 93	Total: 95	Total: 84	Total: 94	Total: 81
27	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	38 (35; 40)	23 (20; 25)	23 (25; 20)	23 (20; 25)	18 (15; 20)
	25 (20; 30)	28 (25; 30)	13 (15; 10)	25 (25; 25)	20 (20; 20)
	Total: 97	Total: 86	Total: 60	Total: 84	Total: 59
32	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	43 (50; 35)	38 (35; 40)	40 (40; 40)	45 (40; 50)	58 (55; 60)
	20 (20; 20)	5 (5; 5)	20 (20; 20)	30 (30; 30)	10 (10; 10)
	10 (10; 10)	15 (20; 10)	20 (20; 20)	23 (25; 20)	15 (15; 15)
	Total: 99	Total: 81	Total: 124	Total: 142	Total: 114
39	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	25 (30; 20)	10 (10; 10)	20 (15; 25)	30 (30; 30)	10 (10; 10)
	28 (25; 30)	33 (35; 30)	33 (30; 35)	43 (45; 40)	35 (30; 40)
	28 (30; 25)	35 (40; 30)	45 (50; 40)	35 (30; 40)	35 (35; 35)
	Total: 144	Total: 153	Total: 181	Total: 205	Total: 153

APPENDIX XI: Abundance of Honeybee Larvae (non-capped brood) in Relation to Treatment.

Figures give the proportion of comb areas (four combs) where a larva was seen during evaluation. The first values give the mean values of both comb sites. The values in parenthesis give the single values of the front and back site of each comb.
The total figure refers to the absolute area in cm² which contained larvae taking into account the increase of the comb area over time (see appendix V).

Days after first exposure to treated substrate/comb area	Larval abundance [% combs with larvae/cm² combs with larvae]				
	Control	2 µg/kg	5 µg/kg	10 µg/kg	20 µg/kg
3	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	Total: 0	Total: 0	Total: 0	Total: 0	Total: 0
8	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	5 (0; 10)	23 (20; 25)	8 (10; 5)	25 (25; 25)
	Total: 0	Total: 7	Total: 33	Total: 12	Total: 28
15	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	5 (5; 5)	0 (0; 0)	0 (0; 0)
	10 (0; 20)	3 (0; 5)	5 (5; 5)	8 (10; 5)	5 (5; 5)
	Total: 13	Total: 4	Total: 14	Total: 12	Total: 6
19	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	5 (0; 10)	8 (5; 10)	8 (10; 5)	5 (5; 5)	3 (0; 5)
	10 (10; 10)	10 (5; 15)	10 (5; 15)	10 (10; 10)	5 (5; 5)
	Total: 20	Total: 26	Total: 26	Total: 23	Total: 10
27	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	3 (0; 5)	3 (5; 0)	5 (5; 5)	0 (0; 0)	0 (0; 0)
	20 (20; 20)	10 (5; 15)	10 (15; 5)	13 (10; 15)	25 (25; 25)
	Total: 38	Total: 22	Total: 25	Total: 23	Total: 36
32	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	3 (5; 0)
	8 (5; 10)	5 (5; 5)	13 (10; 15)	10 (10; 10)	13 (15; 10)
	5 (5; 5)	8 (5; 10)	13 (10; 15)	8 (10; 5)	15 (20; 10)
	Total: 21	Total: 23	Total: 44	Total: 30	Total: 53
39	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	23 (25; 20)	28 (25; 30)	20 (25; 15)	18 (15; 20)	20 (20; 20)
	15 (10; 20)	20 (10; 30)	13 (15; 10)	10 (10; 10)	13 (10; 15)
	Total: 75	Total: 97	Total: 63	Total: 56	Total: 64

APPENDIX XII: Abundance of Honeybee Pupae (capped brood) in Relation to Treatment.

Figures give the proportion of comb areas (four combs) where capped cells was seen during evaluation. The first values give the mean values of both comb sites. The values in parenthesis give the single values of the front and back site of each comb.
The total figure refers to the absolute area in cm² which contained larvae taking into account the increase of the comb area over time (see appendix V).

Days after first exposure to treated substrate/comb area	Pupal abundance [% combs with pupae/ total=cm² combs with pupae]				
	Control	2 µg/kg	5 µg/kg	10 µg/kg	20 µg/kg
3	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	Total: 0	Total: 0	Total: 0	Total: 0	Total: 0
8	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	Total: 0	Total: 0	Total: 0	Total: 0	Total: 0
15	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	5 (10; 0)	0 (0; 0)	0 (0; 0)
	10 (15; 5)	10 (10; 10)	18 (15; 20)	20 (20; 20)	20 (20; 20)
	Total: 13	Total: 14	Total: 33	Total: 29	Total: 24
19	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	10 (10; 10)	0 (0; 0)	0 (0; 0)
	13 (10; 15)	10 (15; 5)	23 (25; 20)	25 (25; 25)	25 (25; 25)
	Total: 19	Total: 14	Total: 48	Total: 39	Total: 28
27	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	23 (25; 20)	0 (0; 0)	0 (0; 0)
	20 (25; 15)	20 (25; 15)	20 (15; 25)	25 (25; 25)	18 (20; 15)
	Total: 34	Total: 34	Total: 77	Total: 45	Total: 26
32	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	10 (15; 5)	5 (5; 5)	20 (20; 20)	0 (0; 0)	0 (0; 0)
	30 (35; 25)	23 (25; 20)	18 (10; 25)	30 (30; 30)	30 (35; 25)
	Total: 66	Total: 50	Total: 64	Total: 54	Total: 53
39	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	23 (25; 20)	13 (15; 10)	28 (25; 30)	20 (20; 20)	20 (20; 20)
	33 (30; 35)	23 (25; 20)	25 (25; 25)	38 (40; 35)	33 (40; 25)
	Total: 111	Total: 75	Total: 101	Total: 120	Total: 107

APPENDIX XIII: Analytical Report on the Fortified Honey Samples

Bayer AG
Crop Protection Development
Institute for Metabolism Research
and Residue Analysis

D-51368 Leverkusen

STUDY TITLE

Effects of Imidacloprid Residues in Maize Pollen on the Development of Small Beehives under Field Exposure Conditions

Author

[redacted]

Testing Facility

Bayer AG
PF-E/MR, Building 6610
51368 Leverkusen, Germany

Study Completion Date

September 2, 1999

Study Number

E 370 1595-0

TABLE OF CONTENT

1 INTRODUCTION

Maize pollen was fortified with Imidacloprid to residue concentration levels of 2 µg/kg, 5 µg/kg, 10 µg/kg and 20 µg/kg. For quality control, samples of the fortified pollen diets were analysed for residues of imidacloprid. The results are tabulated in the table below. Extraction, sample clean up and determination of Imidacloprid by HPLC-MS/MS was performed according to method 00537/E001 (MR-551/99). The limit of quantitation was 5 µg/kg and the limit of detection was 1.5 µg/kg.

2 RESULTS OF HONEY DIET ANALYSIS:

Sample Name	Fortification Level [µg/kg]	Residues of Imidacloprid [µg/kg]	% of Theoretical	Mean [%]	RSD* [%]
Control Pollen A	0	n.d.	0	0	0
Control Pollen B	0	n.d.	0		
Pollen 2 µg/kg A	2.0	< LOQ	-	-	-
Pollen 2 µg/kg B	2.0	< LOQ	-		
Pollen 2 µg/kg C	2.0	< LOQ	-		
Pollen 2 µg/kg D	2.0	< LOQ	-		
Pollen 2 µg/kg E	2.0	< LOQ	-		
Pollen 5 µg/kg A	5.0	4.2	84	90	13.3
Pollen 5 µg/kg B	5.0	4.7	94		
Pollen 5 µg/kg C	5.0	4.4	88		
Pollen 5 µg/kg D	5.0	3.8	76		
Pollen 5 µg/kg E	5.0	5.4	108		
Pollen 10 µg/kg A	10.0	9.4	94	88.6	12.4
Pollen 10 µg/kg B	10.0	9.4	94		
Pollen 10 µg/kg C	10.0	7.8	78		
Pollen 10 µg/kg D	10.0	7.6	76		
Pollen 10 µg/kg E	10.0	10.1	101		
Pollen 20 µg/kg A	20.0	18.8	94	99.5	5.0
Pollen 20 µg/kg B	20.0	21.3	106.5		
Pollen 20 µg/kg C	20.0	19.5	97.5		
Pollen 20 µg/kg D	20.0	19.4	97		
Pollen 20 µg/kg E	20.0	20.5	102.5		

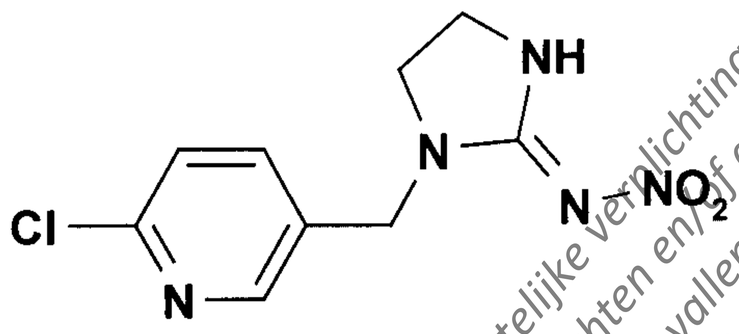
Limit of quantitation: 0.005 mg/kg; limit of detection: 0.0015 mg/kg; 1.5 - 5 =residues below the limit of quantitation but above the limit of detection; n.d. = residues below the limit of detection.
** RSD = relative standard deviation in %*

3 EXPERIMENTAL

3.1 Reference Substances

Imidacloprid

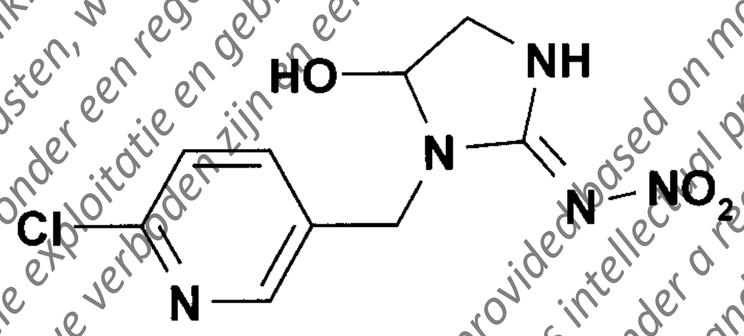
Structural formula:



Empirical formula:	$C_9H_{10}ClN_5O_2$
Molecular weight:	255.7 g/mole
Certificate of Analysis:	M00680, 03/13/98
Certified Assay:	99.4 %
Expiry Date:	March 2000

Hydroxy-Imidacloprid (WAK 4103)

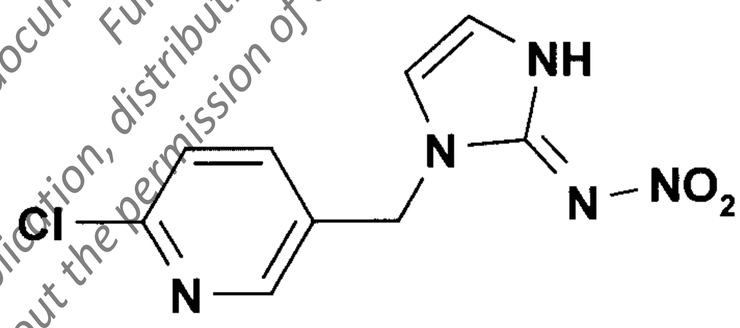
Structural formula:



Empirical formula:	$C_9H_{10}ClN_5O_4$
Molecular weight:	271.7 g/mole
Certificate of Analysis:	930323ELB03, 06/07/95
Certified Assay:	99.4 %
Expiry Date:	June 2000

Olefin-Imidacloprid (NTN 35884)

Structural formula:



Empirical formula:	$C_9H_8ClN_5O_2$
Molecular weight:	253.6 g/mole
Certificate of Analysis:	M00804, 07/22/98
Certified Assay:	98 %
Expiry Date:	June 2000

3.2 Residue Analytical Methodology

3.2.1 Extraction and Sample Clean-up

1. Place for e.g. 2.0 g of the sample material in a 150-ml beaker.
   Add 30 ml of methanol/water (3/1,v/v) and allow the sample to soak for 30 min.

2. Blend the sample using an ultra-turrax blender (or equivalent) for approximately 1 min.

3. Vacuum filter the suspension through 2.5 g of Celite filter aid using Schwarzband filter paper supported on a Büchner funnel into a 250-ml vacuum filter flask.

4. Wash the filtered solids with a total of 30 ml of methanol/water (3/1, v/v). Press residual solvent from the solids using rubber damming. Discard the filtered solids.

5. Transfer the filtrate to a 100-ml graduated cylinder. Determine the total volume of the extracts. Mix the solution well, and transfer the half (e.g. 1.0 g sample equivalent) to a 250-ml brown glass round-bottomed flask.

6. Concentrate the aliquot to an aqueous remainder of 5 to 10 ml using a rotary evaporator with a max. bath temperature of 50 °C.

3.2.2 ChemElut® Column Clean-up

1. Add 5 to 10 ml water to the aqueous solution from 3.2.1 step 6 to bring the total volume of the extracts to approx. 20 ml.

2. Place the aqueous solution on the top of the ChemElut® CE 1020 (20 ml volume) column fitted with a disposable stainless steel needle and wait for approx. 15 minutes to achieve an uniform distribution of the liquid on the column.

3. Elute the residues from the column with 140 ml of CH_2Cl_2. Collect the eluate in a 250-ml brown glass round-bottomed flask.

4. Evaporate the eluate from step 3 to dryness using a vacuum rotary evaporator and a max. bath temperature of 40 °C.

3.2.3 Silica Gel Column Clean-up

1. Dissolve the residues from 3.2.2 step 4 in 2 ml of toluene/ethyl acetate (85/15, v/v).

2. Apply the organic solution from step 1 onto a 0.5 g (3 ml) silica gel (SiOH) column (e.g. Varian).

3. Allow the solution to pass through the column at a flow rate of 1 ml/min.

4. Rinse the 250-ml brown glass round-bottomed flask with 10 ml of toluene/ethyl acetate (70/30, v/v) and apply the solution onto the column, too.

5. Elute the residues with 5 ml of acetonitrile at a flow rate of 1 ml/min. Collect the eluate in a 25-ml brown glass pear-shaped flask.

6. Evaporate the eluate from step 5 to dryness using a vacuum rotary evaporator and a max. bath temperature of 40 °C. Dissolve the residues in e.g. 1.00 ml of acetonitrile/water (2/8, v/v) and determine the residues with HPLC-MS/MS.

NOTE

1. The volumes to be used for flushing the column with toluene/ethyl acetate and for elution with acetonitrile must be newly determined for each batch of SiOH-column!

2. The flow rate should not be too high, since otherwise losses of the residues in may occur with recoveries below 70 % and the clean-up is less effective.

3. The Hydroxy-Metabolite may be converted to the Olefin-Metabolite (especially under acidic conditions).

4. The Olefin-Metabolite is degraded by light (ca. 50% in one day at natural daylight). Therefore, all solutions containing the Olefin-Metabolite must be protected from light and stored in a cool and dark place.

3.3 HPLC-MS/MS determination of Imidacloprid and Metabolites

3.3.1 Measuring equipment and HPLC conditions:

Instrument: HP 1100
Injector: Gilson 233 XL
Column: Phenomenex, Luna C18 (2), 5 µm, 15 cm, 0.46 cm i.d.
or equivalent
Injection Volume: 50 µl
Oven temperature: 40 °C
Mobile Phase: A: Water/ACN (90/10, v/v)+ 0.1 ml acetic acid per litre
B: Acetonitrile + 0.1 ml acetic acid per litre

Time Table	0 min	11.1 % B
	10 min	11.1 % B
	10.1 min	90 % B
	15 min	90 % B
	15.1 min	11.1 % B
	19 min	11.1 % B

Stoptime: 19 min
Flow (Column): 1.0 ml/min
Flow (into MS): 0.15 ml/min
Retention Time: Olefin-Metabolite: approx. 4.6 min
Hydroxy-Metabolite: approx. 5.5 min
Imidacloprid: approx. 9.1 min

NOTE: Conditiones may be adapted for other HPLC-MS/MS systems.

3.3.2 MS/MS-Detection

The experiments were performed on a triple-quadrupole mass spectrometer system, fitted with an electrospray interface operated in the positive ion mode under MRM conditions.
The mass spectrometer was tuned by infusing a standard solution of 0.5 mg/l Imidacloprid and ist metabolites (dissolved in water/acetonitrile 8/2 + 0.1 ml acetic acid per l) at a flow rate of 10-20 µl/min. Mass axis calibration was done by infusing a polypropylene glycol 3000 solution. Unit mass resolution was established and maintained in each mass resolving quadrupole by maintaining a full width at half-maximum of between 0.8 and 1.0 DA. After tuning and calibration, optimal collision-activated dissociation (CAD) conditions for fragmentation of Imidacloprid and its metabolites were determined. These experiments were performed with nitrogen as collision gas with a collision offset of -19 eV for Imidacloprid, -21 eV for the Hydroxy-Metabolite and -13 eV for the Olefin-Metabolite and at an approximate collision gas thickness of 1.46 x 10^{15} atoms/cm^2. Nebulizer gas is set at 1.48 l/min, curtain gas is set at 1.44 l/min collision gas is set at 0.87 l/min and turbo gas is set at 6.0 l/min.

Detector: Triple Quadrupol LC-MS/MS Mass Spectrometer, e.g.
Perkin-Elmer Sciex Instruments
API 300, Apple™ Macintosh System® 8.1

Interface: Electrospray, Turbo Ion Spray
Potential: + 4400 V
Temperature: 400 °C
Nebulizer Gas: Nitrogen 5.0 (99.999 % purity), 1.48 l/min
Curtain Gas: Nitrogen 5.0 (99.999 % purity), 1.44 l/min
Turbo Gas: Nitrogen 5.0 (99.999 % purity), 6.0 l/min

Scan Type: MRM (Multiple Reaction Monitoring Mode)

Polarity: Positive

Collision Gas: Nitrogen 5.0 (99.999 % purity), 0.87 l/min

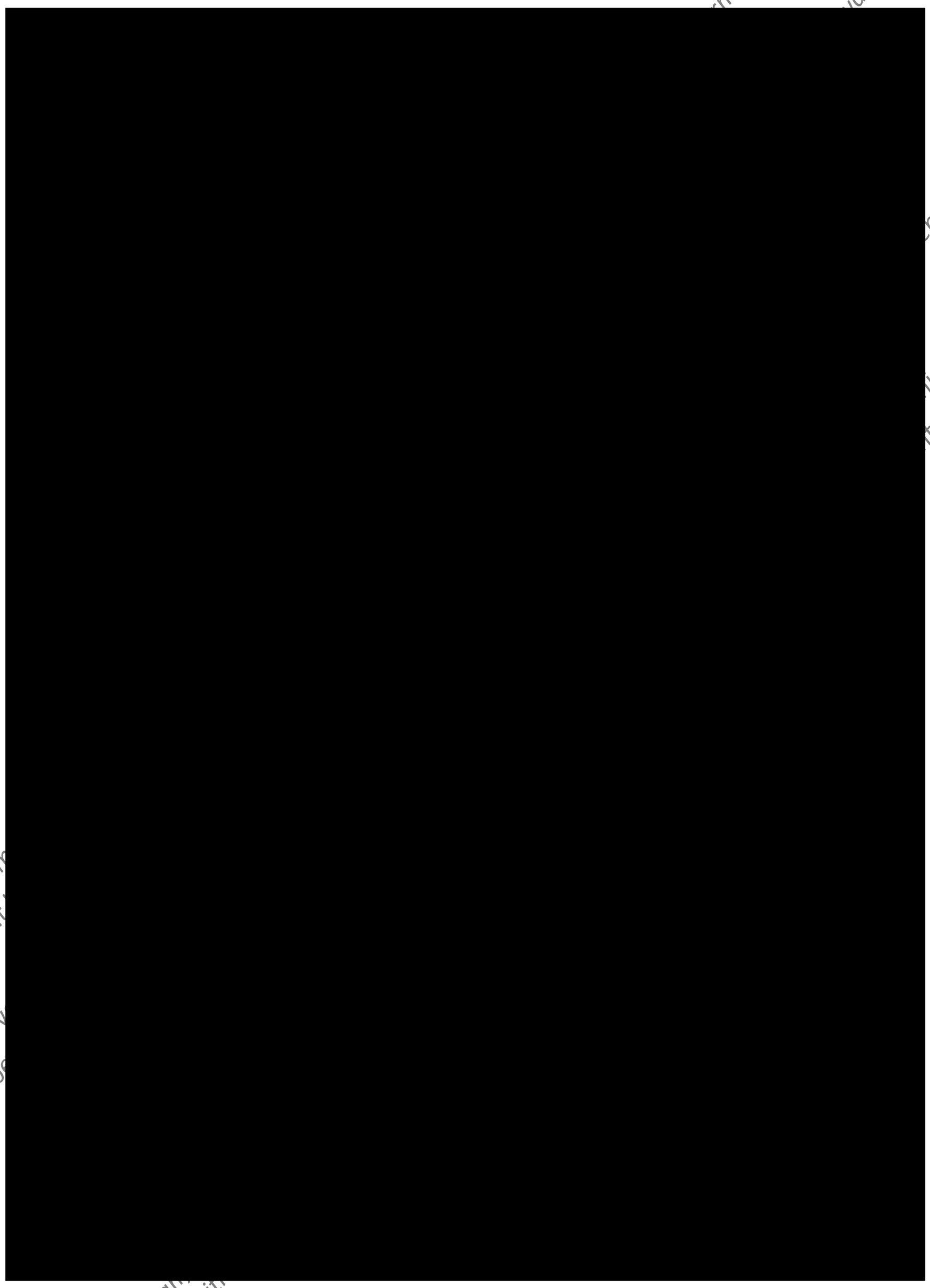
Mass spectrometer operating parameters:

Compound	Precursor Ion Q1 Mass (amu)	Product Ion Q3 Mass (amu)	Dwell Time (msec)	Collision Energy (eV)
Olefin-Metabolite (37)	256#	238	250	-13
Olefin-Metabolite (35)	254	236	250	-13
Hydroxy-Metabolite (37)	274#	191	250	-21
Hydroxy-Metabolite (35)	272	191	250	-21
Imidacloprid (37)	258#	211	500	-19
Imidacloprid (35)	256	209	500	-19

#: The Cl 37 isotope of all substances was detected to build the isotopes ratio

NOTE: Different MS/MS-instruments or instrument parameters may result in different ion transitions and different relative intensities.

Appendix XIV: Copy of the GLP Certificate



Appendix XIV: (continued):

[redacted]

Appendix XV: Quality Assurance Statement

Referat GLP

[redacted]