TITLE PAGE

Effects of Residues of Imidacloprid in Maize Pollen from Dressed Seeds on Honey Bees (*Apis mellifera*)

AUTHORS

[redacted]

TESTING FACILITY

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

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1.0 SUMMARY

Report: [redacted](2001): Effects of Residues of Imidacloprid in Maize Pollen from Dressed Seeds on Honey Bees (*Apis mellifera*)

Bayer AG, unpublished report No: [redacted]Am 012; 2001-04-17.
(Appendix XV contains data from study MR-111/01)

Guidelines: Internal Testing Method
Deviations: not applicable

GLP: yes (certified laboratory)

Material and methods: *test substance:* Gaucho WS 70, residues in maize pollen from dressed seeds, dressing rate: 49 g/unit a.i.. Residues of imidacloprid in the pollen were found to be below limit of quantitation (LOQ = 0.005 mg/kg). No olefine and hydroxy metabolites could be detected (limit of detection: 0.003 mg/kg and 0.0015 mg/kg, respectively).
Small bee colonies (appr. 700 honeybees) were confined in tent cages (ca. 20 m ²) on short grass meadows and exclusively fed with maize pollen which was harvested from plants, the seeds of which were dressed with Gaucho WS 70 or which were untreated (control). Sunflower honey was provided as carbohydrate source. The small bee colonies were examined for treatment-related impacts over a period of 38 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: 2000-08-21 to 2000-09-28.

Findings: Effects of Gaucho WS 70 residues in maize pollen on small honeybee colonies

Testing Endpoint	Control A	Control B	Treatment A	Treatment B
Mortality (no. of dead bees in front of bee hives)	32	27	20	30
Mortality (no of dead bees at the tent edges)	146	141	139	151
Foraging intensity (no. of bees at the pollen feeder)	1	15	29	2
Foraging intensity (no. of bees at the honey feeder)	267	253	274	255
Bee activity (no. of bees at the tent roof)	180	203	196	185
Pollen collected [g]	16	58	43	26
Honey collected [g]	736	853	819	877
Comb cell production [cm²]	606	618	660	664
Honey storage area at study termination [cm²]	434	254	417	399
Hive weight increase [% of the initial weight]	9.8	6.6	12.4	16.6
Egg laying activity[cm² comb area containing eggs at study termination)	19	63	15	18
Colony strength [cm² comb area covered with bees at study termination)	279	249	253	263

Observations: There were no treatment-related effects in the testing endpoints foraging activity, orientation, honey and pollen consumption, comb cell, production, honey storage, hive weight increase, population development, mortality, breeding activity, and breeding success. There are no hints that imidacloprid residues in pollen from maize seeds treated with Gaucho at the rate recommended might have any adverse effects to honey bee colonies.

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 residues of Gaucho WS 70 in pollen on the development of small bee colonies and on behavior and mortality of honey bees.

3.0 EXPERIMENTAL

3.1 Test Item

3.1.1. Test substance

Test substance:	Gaucho WS 70
Active ingredient(s):	NTN 33893 (Imidaclopride)
Chemical name of a.i. (CAS):	2-Imidazolidinimine, 1-[(6-chloro-3pyridinyl)methyl]-N-nitro-
CAS number of a.i.:	138261-41-3
Development number:	not applicable, commercially available product
Batch number:	not applicable, commercially available product
TOX-No.:	not applicable, commercially available product
a.i. content (acc. to analysis):	not applicable, commercially available product
Analytical method:	not applicable, commercially available product
Date of analysis:	not applicable, commercially available product
Expiry date:	not applicable, pollen fed immediately after analysis
Storage conditions:	Room temperature
Safety Precaution:	Routine hygienic precautions

3.1.1. Pollen from treated maize plants

Maize variety:	Mesnil
Seed dressing rate:	49 g a.i./unit (50.000 seeds)
A.i. content acc. to analysis:	below limit of quantitation
Date of analysis:	2000-08-17 to 21 (see appendix XV)
Expiry date:	not applicable, pollen fed immediately after analysis
Storage conditions:	ca. 12-15° C
Safety precautions:	Routine hygienic precautions

3.2 Reference Substance

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

3.3 Execution of the Test

Tent cages (20 m²) were placed on the study field on 2000-08-16 and confined the study plots. The hive colonies were placed inside the tent cages on 2000-08-21. The final evaluation on these hives were made on 2000-09-28.

Responsibilities:

Sponsor: BAYER AG
PF-E/PBA
D-40789 Monheim

Study director:
Responsible analyst:
Apicultural manager:
Study technician(s):
Quality assurance:
Laboratory study number:
[redacted]

3.4 Origin of Honeybees and Preparation of Hive Colonies

Honeybees were provided by a German beekeeper [redacted] Preparation of the hive colonies used for the test started on 2000-08-20. Honeybees of combs from a large commercially managed beehive were swept down into a drone sieving cage and moistened with water to suppress escape flights. These honeybees were divided into 170 g subsamples which were equivalent to a number of approximatey 700 honeybees. Each subsample was filled into a multiple-comb-fertilization-cage (= "Mehrwaben-Begattungskästchen") which contained 4 native comb strips (11 x 4 cm), i.e. only comb matrices. One queen in egg laying activity was added to each of these hive colonies. The hives were set up in the tents in 2000-08-21.

3.5 Origin of the Food Pollen

The maize pollen used in this study originated from France and was provided by [redacted] (Bayer France). The maize plants (variety "Mesnil") were cultivated in the enivrons of Louans (Dept. Indre et Loire, France). The maize seeds had been dressed with Gaucho WS 70 at a rate of 70 g Gaucho WS 70 (49 g a.i.) per unit (= 50.000 seeds) which is equivalent to 1 mg a.i. per seed. The seeds were sown on 2000-05-10 (treatment) and 2000-05-11 (control). Drilling rate was 93.000 seeds/ha (treatment) and 85.000 seeds/ha (control). Besides of the test substance treatment, the "Gaucho plot" was treated with the herbicides Duelor (2.2 l/ha), Atrazine (1 l/ha), and Eclat (0.5 l/ha), and with the insecticide Sherpa 2G (Cypermethrin) (50 g/ha); the insecticide was applied on 2000-07-16. The control plot received a herbicide treatment with 0.75 l/ha Mikado (a.i.: 300 g/l Sulcotrione), 0.75 l/ha Milagro (a.i.: 40 g/l Nicosulfurone), and 1 l/ha Atrazine, furthermore an insecticidal Curaterr treatment (12 kg: 600 g a.i./ha).

The culture which has grown before the maize was wheat on the treatment plot, and ray grass on the control plot. The latter plot has not received an imidaclopride treatment since at least 4 years. The soil on both control and treatment plots was characterized as "Bournais Battant". The treatment plot was one time irrigated before flowering of the maize (30 mm), but not so the control plot.

Flowering of the maize began on 2000-07-24; the pollen was sampled between 2000-07-24 and 2000-08-02.

After arrival at the testing facility, the pollen was ground in a grinding machine (Fa. Braun) to obtain a fine powder; subsequently, samples were taken from the pollen for analysis to determine the residue level of the test substance. The analytical results are summarized in appendix I and reported in appendix XV. The Imidacloprid residue level in the samples was below the limit of quantitation; metabolites of the test substance were not detected.

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

The trial site was located in the vicinity of Euskirchen-Billig (Germany, Nordrhein-Westfalen). The test field was a reaped meadow, covered with short grass. There have no pesticide treatments or other agricultural measures been carried out during or shortly before the study. In order to prevent honeybees from leaving the test plots and from collecting nectar or pollen from other sources than the ones offered in the test, four plots on the test field were confined with tent cages of ca. 20 m^2 floor-space 10 x 2 m, height: 2 m). On 2000-08-16, the four tents were set up on the field. The tent cages consisted of an aluminium frame covered by plastic gauze material (ca. 2 x 2 mm mesh size).

3.7 Treatment Design

After preparation of the bee colonies (see 3.4), each of them was allocated to one of the four tent cages by using a random list. The bee colonies were transferred into the tents on 2000-08-21. The allocation of the colonies to control and treatment was as follows:

Colony no.	Tent no.	Treatment level
7	1	control A
16	4	treatment B
18	3	treatment A
20	2	control B

Additionally to the pollen, sunflower honey (as commercially available) was offered to the bees as food ressource in a feeder placed outside the bee hive. The sunflower honey was provided in elevated and sheltered glass containers which were positioned in a distance of some meters from the hives. The honey was provided in small, weighed portions. Each to each 7th day, a fresh portion was offered and the remaining old portion removed and reweighed.

The pollen was provided in ca. 2-10 g portions at two different places (in- and outside the hive). One portion was offered in 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 pollen was stored within a refrigerator between ca. +12 and +15°C during the study. Pollen and honey in the feeders were replaced by fresh portions on the study days depicted in appendix IV.

At each replacement event and finally after termination of the biological part of the study, the amount of collected pollen was determined gravimetrically.

3.8 Climatic Conditions During the Study

During the study, temperature and precipitation events were continously recorded using thermohygrographs and precipitation measuring devices (see nFig. 1, appendix II). The following records were made during the evaluation checks (always between 10:50 and 17:30):

Day after first exposure	Air temperature [°C]	Soil temperature [°C]	Precipitation [mm]	Cloudness (% sky coverage)	Wind speed (estimates)*
1 (12:00)	21	28	0	50	+
2 (12:30)	22	27	0	0	++
3 (12:30)	25	31	0	0	+
4 (11:45)	21	30	0	0	+
7 (13:10)	20	20	9	100	+
8 (14:20)	19	20	2	95	+
9 (16:30)	20	20	0	70	+
10 (16:45)	21	22	0	50	++
11 (13:50)	15	17	2	100	++
12 (14:40)	20	21	1	100	++
14 (11:40)	12	15	9	100	+
15 (11:50)	20	22	1	70	+
17 (17:30)	17	18	3	100	+
18 (12:00)	20	21	0	90	+
21 (11:40)	26	30	0	0	+
22 (12:10)	29	30	0	50	+
23 (16:10)	20	21	0	90	++
24 (15:40)	23	23	0	50	++
25 (10:50)	20	20	0	100	+
28 (11:50)	20	20	n.r.	90	+
29 (11:15)	20	25	0	40	++
32 (12:30)	22	22	20	40	++
35 (15:10)	21	21	1	50	+

- = 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 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 confined plots or at the tent edges 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 into a virtual rectangle 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: The amount of collected and processed sunflower honey was regularly assessed in two different ways. he weight increase of the 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 honeybee larvae or pupae 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 deylopment of the hypopharyngeal glands of young workerbees. A proper function 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 feeders were recorded. In addition, the number of honeybees observed the tent roof was counted. This endpoint may give an indication of possible disorientation or repellent/antifeedant effects.

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 maize pollen could not be retained, since the limited quantity of pollen at hand did not allow this, and all of the pollen was consumed in the course of the trial.

5.0 RESULTS AND DISCUSSION

5.1 Climatic Conditions During the Study

Climatic conditions were recorded in the control tent with a thermohygrograph. Records are listed in appendix II, further data in fig. 1. During the study, daily maximum temperatures ranged between 16 and 33°C, minimum temperatures between 7 and 15°C. Air humidity ranged from 45 to 100%. The sky was frequently cloudy during the assessments. Wind was mostly slight to modest during the study period.

5.2 Activity Pattern of Foraging Honeybees and Food Storage Rates

As shown in Fig. 2, there were no noticeable differences in the activity patterns of foraging honeybees between control and treatment. The numbers of bees recorded at the pollen feeders varied, but control and treatment lied within the same range; at the honey feeders, essntially similar numbers of bees were recorded in control and treatment. The same is true for the number of bees observed of the tent roof; thus, neither reduced flight activity nor signs of desorientation can be considered to be caused by the test item.
Figure 3 illustrates the quantity of honey and pollen which was collected by the foraging honeybees during the study. The amount of pollen collected was widely varying, however, possible treatment-related effects were not observed. The quantity of honey collected was on average higher in the treatment than in the control groups, although it lied essentially within the same range in all replicates.
All hives started immediately with the production of new comb cells. Again, no treatment-related difference is found for this testing endpoint (Fig. 4), the comb areas were always essentially equal in size in the control and treatment groups. This evidences that imidaclopride residues in the pollen at the residue level tested do not influence the wax production by young worker bees.
The amount of the honey stores fluctuated considerably over time and in all control and treatment groups (Fig. 5). These fluctuations are most probably caused by the food consumption associated with breeding activity of the hive nuclei. In the development of the honey stores over time, no treatment-associated patterns could be recorded. It is therefore concluded that imidaclopride residues at the level tested do not adveresely affect the food storage rate of *Apis mellifera*.
Pollen was not stored within the combs over longer periods but directly invested into offspring production. However, it is evident from the breeding performance of all colonies tested in this study that honeybees of all treatment groups collected and fed sufficient pollen to allow a strong population increase.
A more precise measure for honey storage and comb cell production is derived from the hive weight development. As shown in Fig. 6, hive weight increase was even greater in the treatment than in the control.

5.3 Population Development and Breeding Performance

Fig. 7 shows the population development over time during the study. Comb areas covered by bees varied during the assessments and between the treatment groups, but there was no possibly treatment-related effect detectable. Values found lied essentially in the same range in the control and treatment groups.

Likewise, mortality was not related to treatment either (Fig. 8) which demonstrates that the tested imidaclopride residue level had no impact on honeybee longevity.

Egg-laying activity was very variable in control and treatment groups (Fig. 9). However, no treatment-related effects were recorded in any case. The same degrees of variation were observed in control and treatment, Thus the observed differences have to be attributed to natural variability, and not to the test item. The same is true for abundance of bee larvae and pupae as parameters of breeding activity and success (Figs. 10, 11). No correlation between treatments and differences in this endpoint between different treatment groups could be shown to exist.

FIGURES

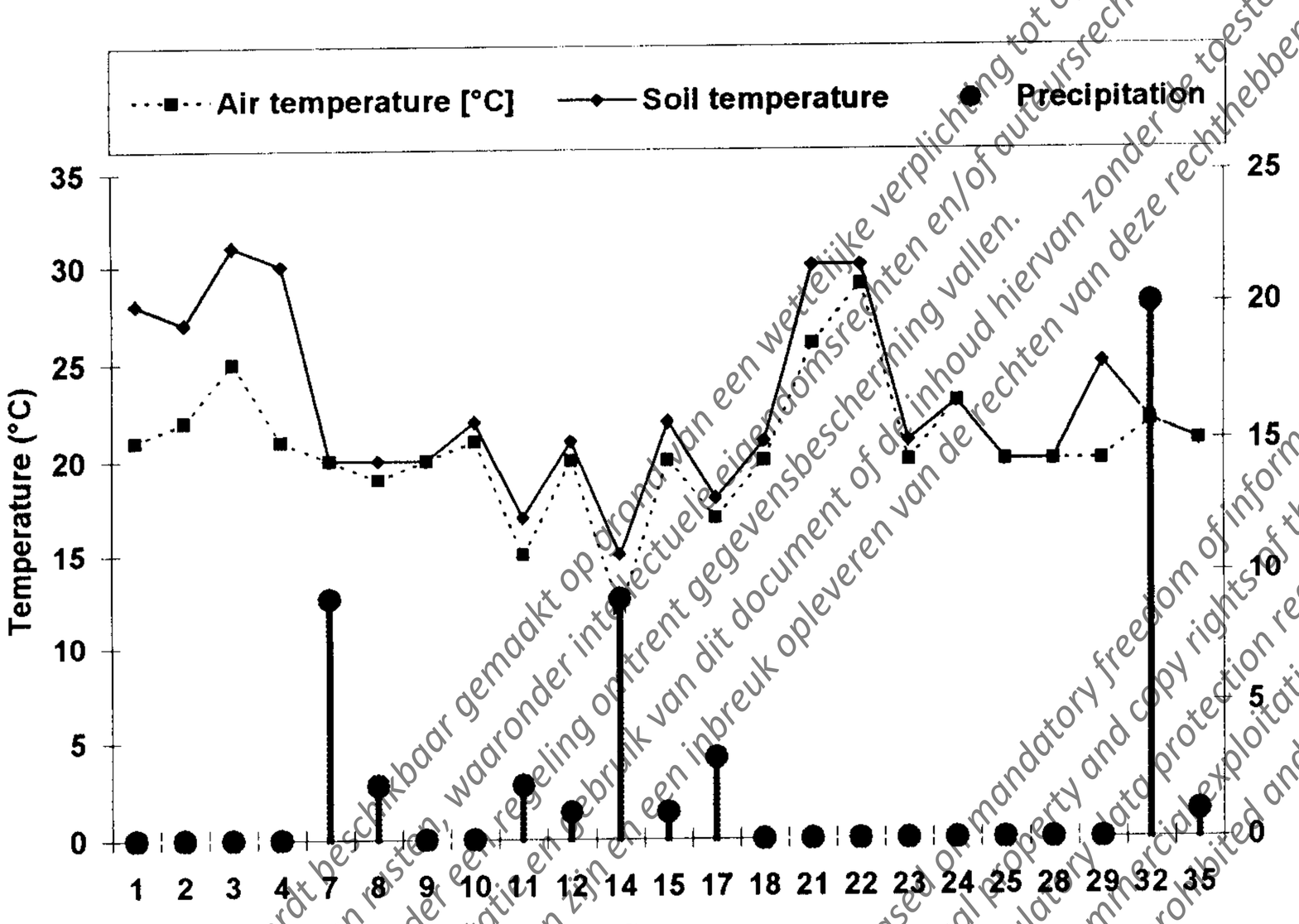


Figure 1: Temperature and precipitation during the study period.

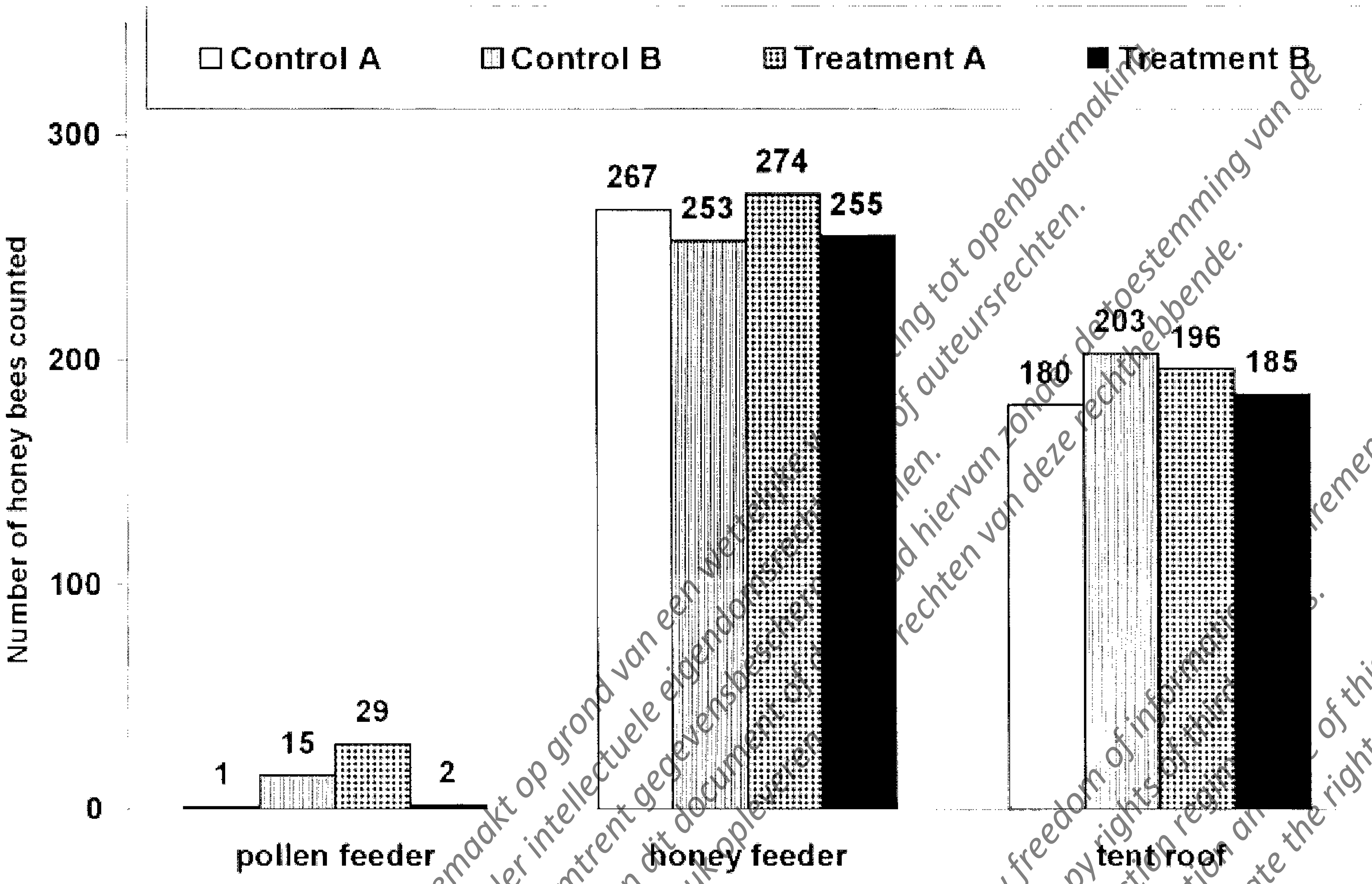


Figure 2: Activity pattern of foraging honeybees in control and treatment.

Small bee colonies were fed with sunflower honey and maize pollen. Maize pollen was taken from plants which had received imidaclopride seed-dressing. Colums give the average number of foraging honeybees recorded per assessment at the pollen feeder, the honey feeder or at the tent roof.

Number of foraging bees summed up for the whole sampling period (35 days) differed significantly between treatments on the pollen feeder ($\chi2=4.79$; $p<0.05$; d.f.=1) and did not differ significantly on the honey feeder ($\chi2=1.22$; n.s.; d.f.=3) and at the tent roof ($\chi2=1.71$; n.s.; d.f.=3).

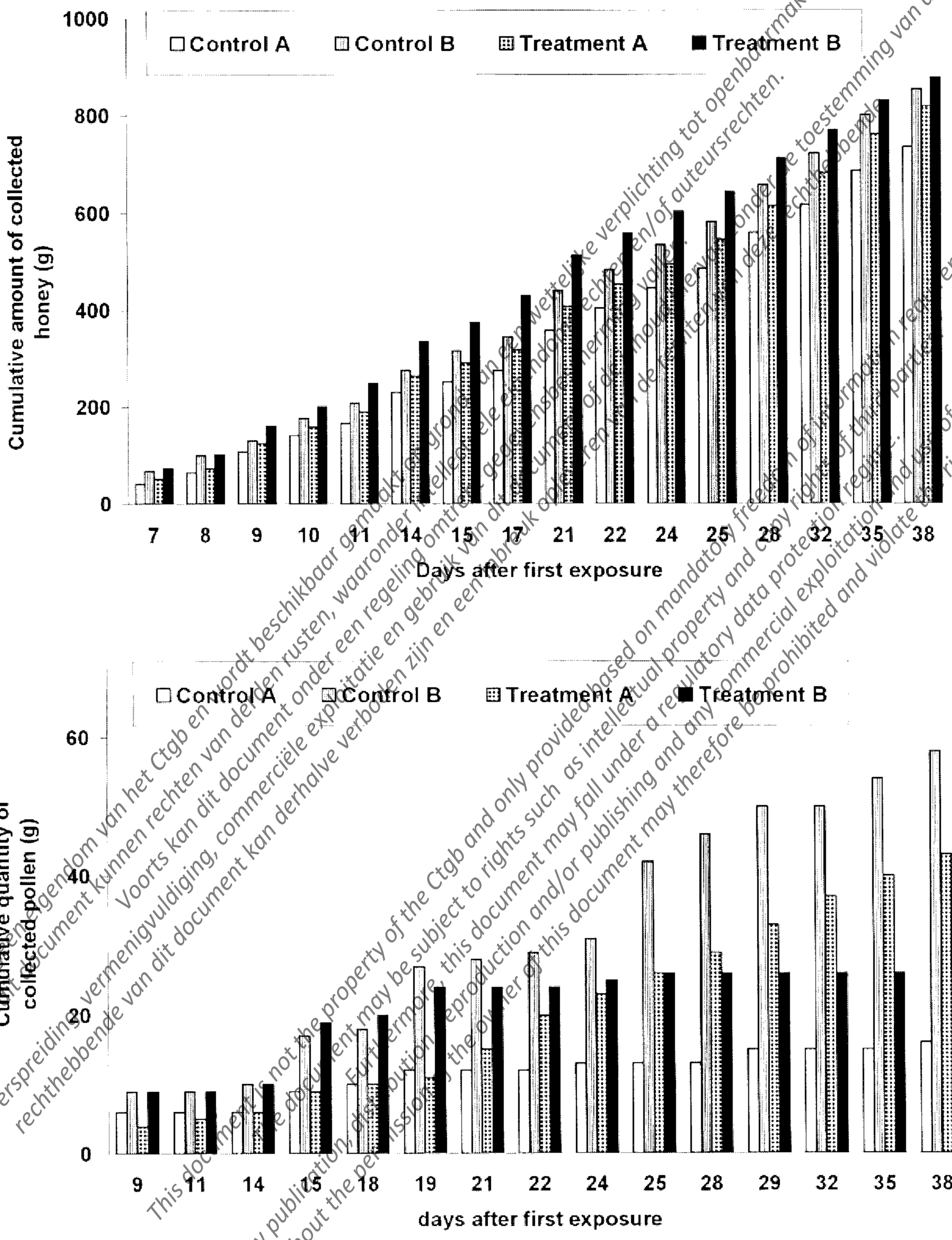


Figure 3: Honey (upper graph) and pollen (lower graph) collecting rate of honeybees in control and treatment.
Small bee colonies were fed with sunflower honey and maize pollen. Maize pollen was taken from plants which had received imidaclopride seed-dressing. Columns show the cumulative quantity of honey and pollen collected by the foraging honeybees during the study period.

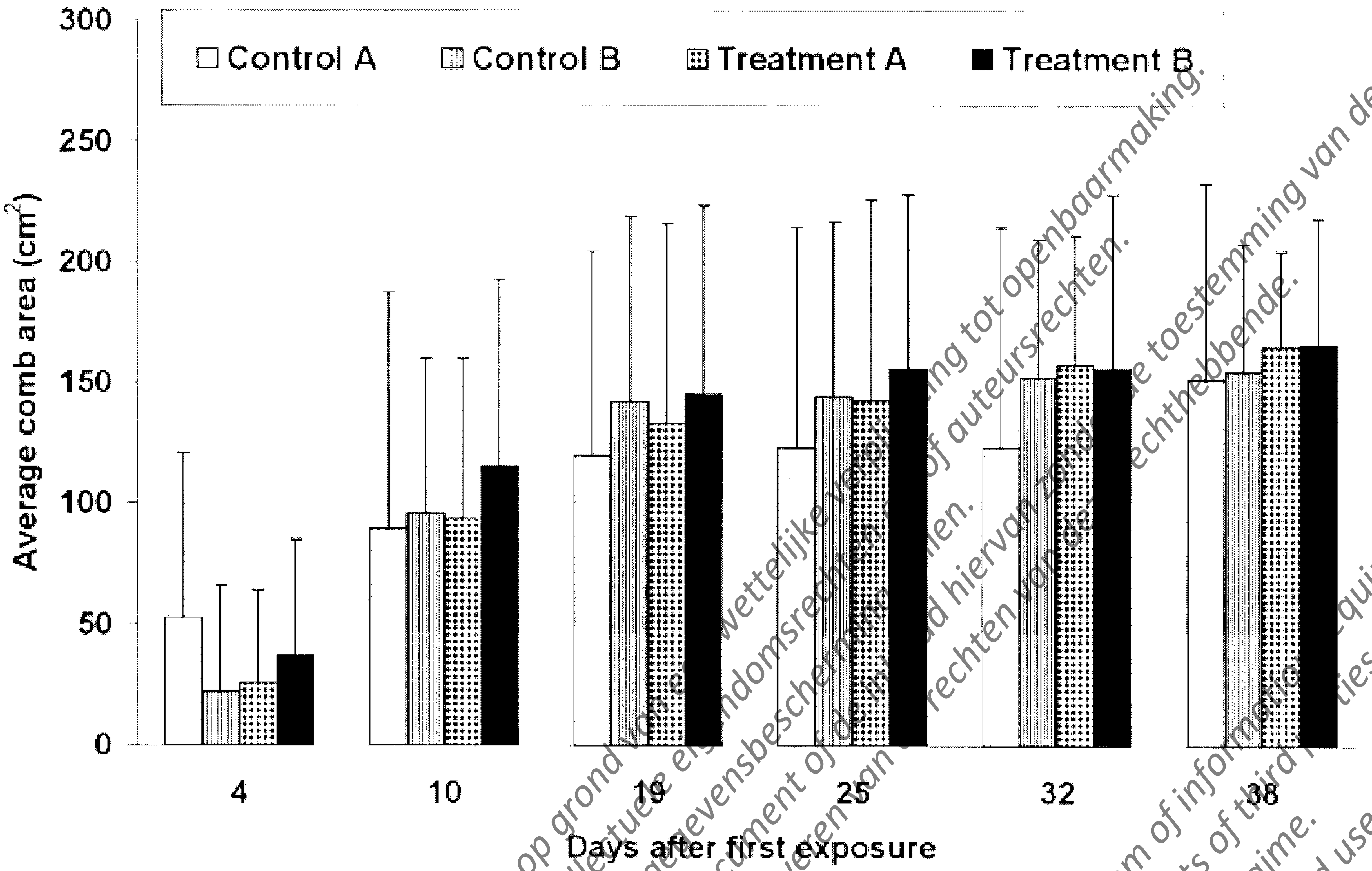


Figure 4: Development of the comb area over time in control and treatment.
Small bee colonies were fed with sunflower honey and maize pollen. Maize pollen was taken from plants which had received imidacloprid seed-dressing. Colums give the average comb cell area of 4 combs in cm² (mean ± s.d.).

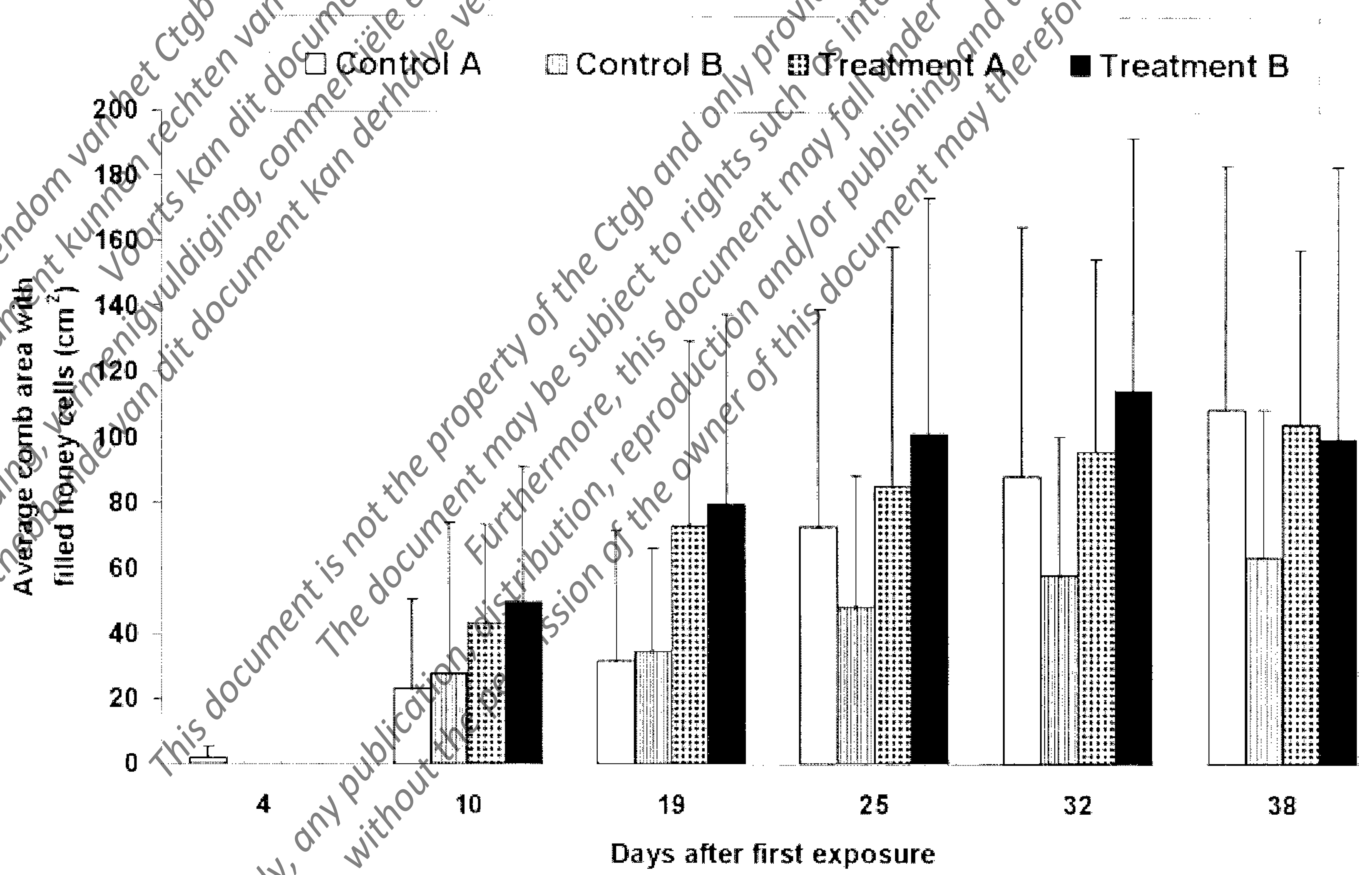


Figure 5: Amount of the honey stores over time in control and treatment.
Small bee colonies were fed with sunflower honey and maize pollen. Maize pollen was taken from plants which had received imidacloprid seed-dressing. Columns show the average size (mean ± s.d.) of honey stores as cm² comb area which contained cells filled with honey taking into account the increase of the comb area over time (see appendix V).

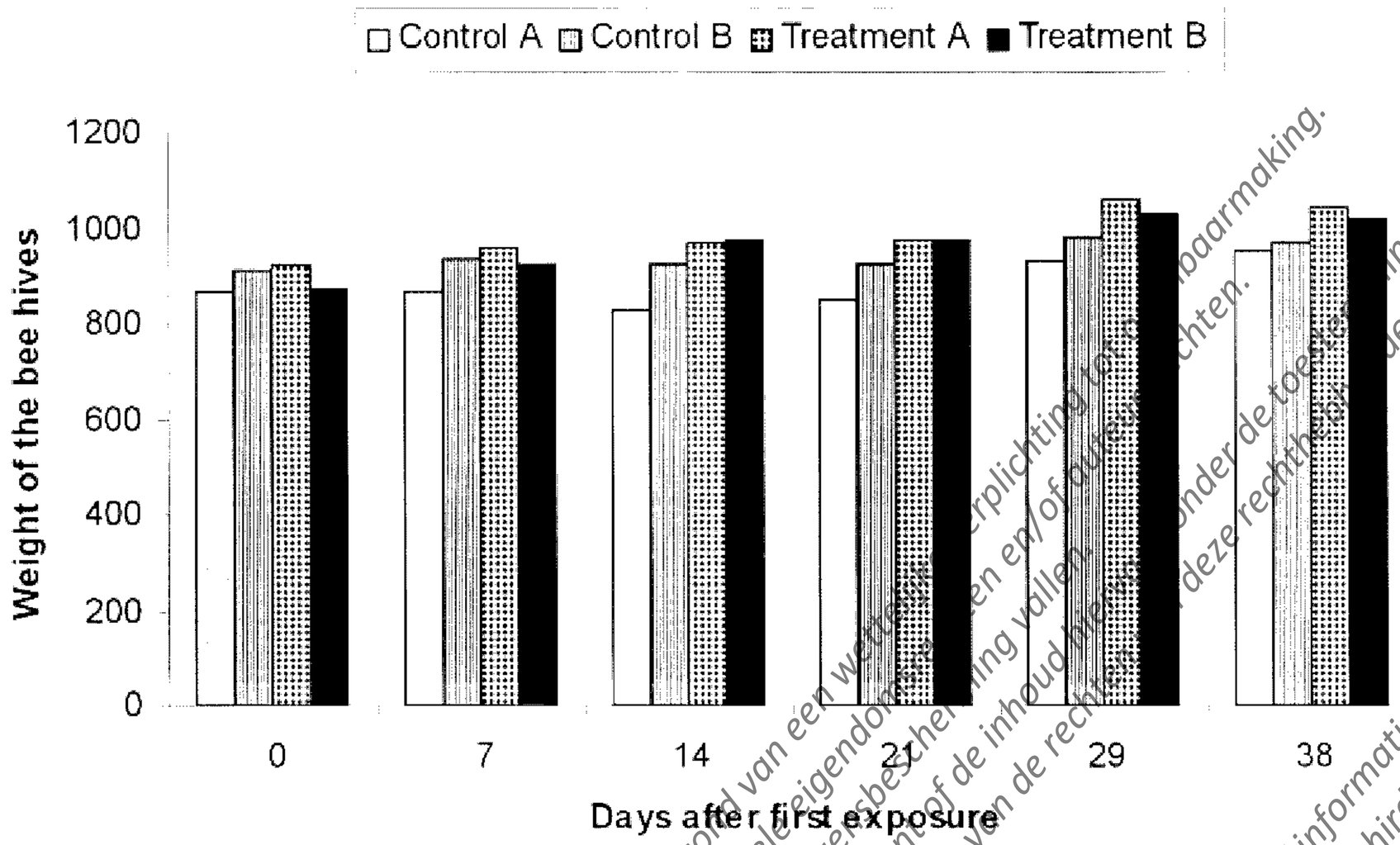


Figure 6: Weight increase of bee hives in control and treatment.
Small bee colonies were fed with sunflower honey and maize pollen. Maize pollen was taken from plants which had received imidacloprid seed-dressing. Columns show the weight of the bee hives at the days of assessment.

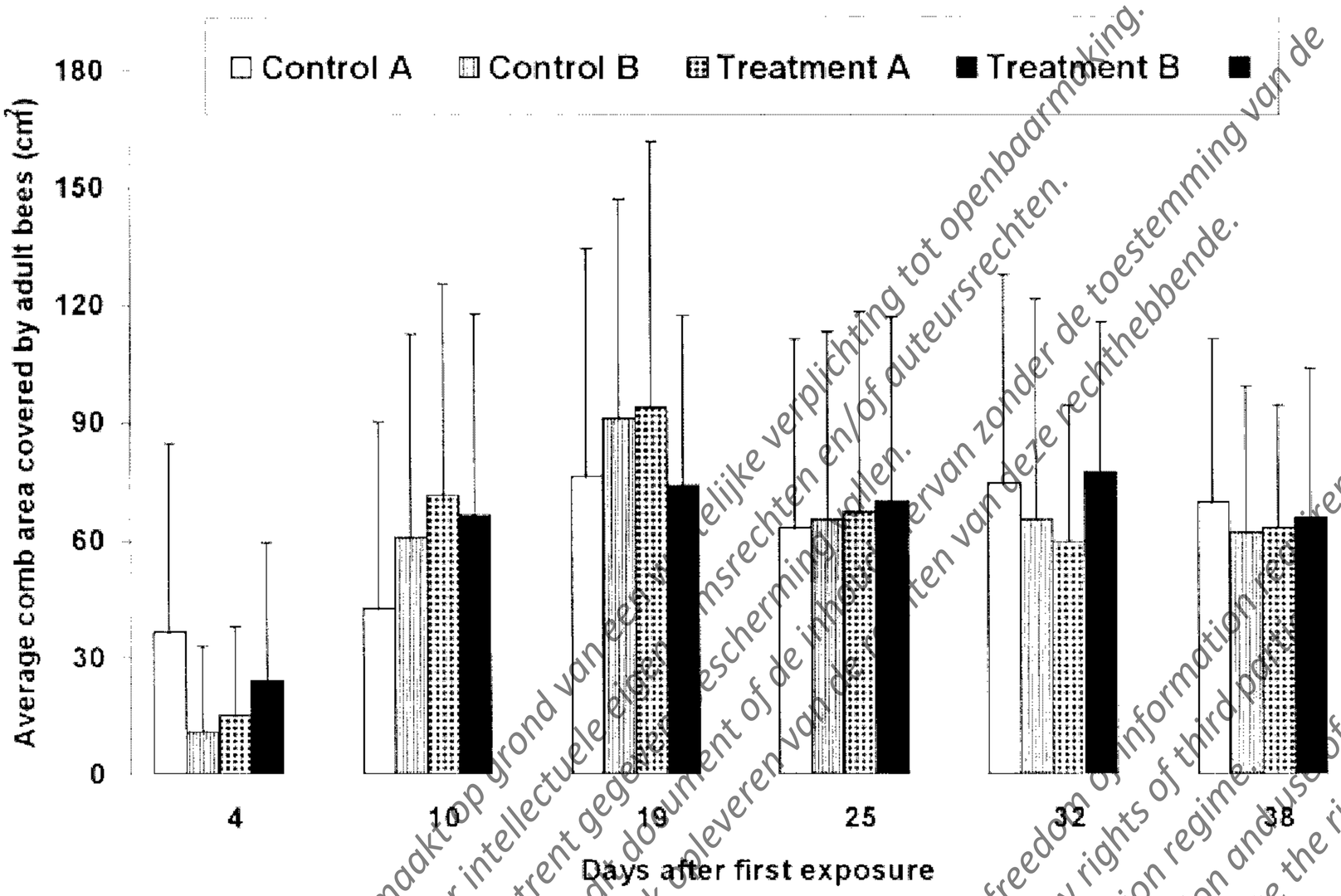


Figure 7: Population development in control and treatment.
Small bee colonies were fed with sunflower honey and maize pollen. Maize pollen was taken from plants which had received imidacloprid seed-dressing. Columns show the average comb area (mean ± s.d., n=4) covered by adult honeybees during evaluations taking into account the increase of the comb area over time (see appendix V).

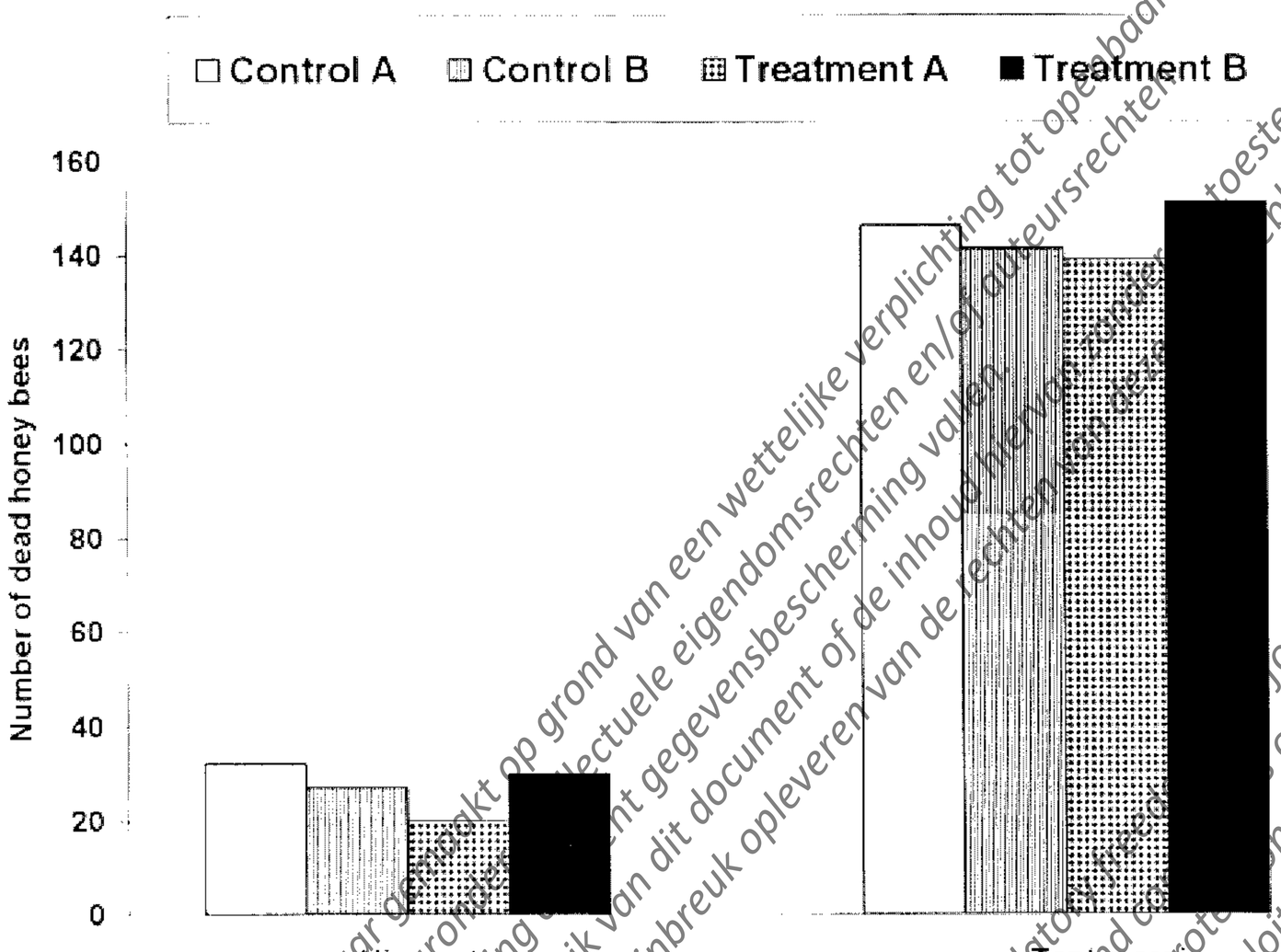


Figure 8: Mortality in control and treatment.
Small bee colonies were fed with sunflower honey and maize pollen. Maize pollen was taken from plants which had received imidaclopride seed-dressing. Columns give the total number of dead honeybees (workerbees & drones) which were found dead during the study either in front of the bee hives or at the tent edges. The number of dead bees at the hive entrance ($\chi 2=3.04$; d.f.=3; n.s.) and at the tent edge ($\chi 2=0.60$; d.f.=3; n.s.) did not differ significantly between treatments.

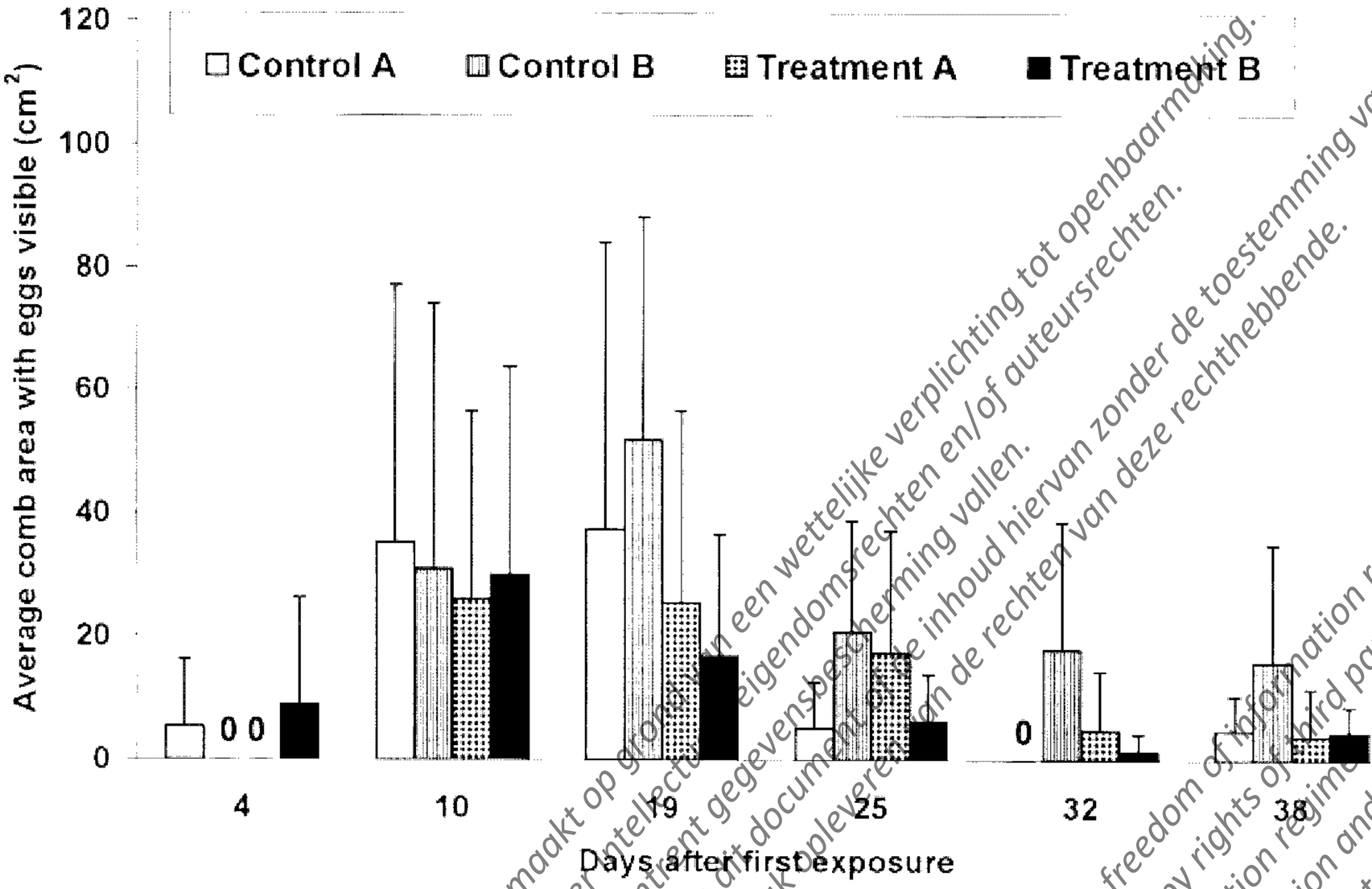


Figure 9: Egg laying activity of the queens in control and treatment
Small bee colonies were fed with sunflower honey and maize pollen. Maize pollen was taken from plants which had received imidacloprid seed-dressing. Columns show the average comb area (mean ± s.d., n=4) where eggs were seen in the cells during evaluations taking into account the increase of the comb area over time (see appendix V).

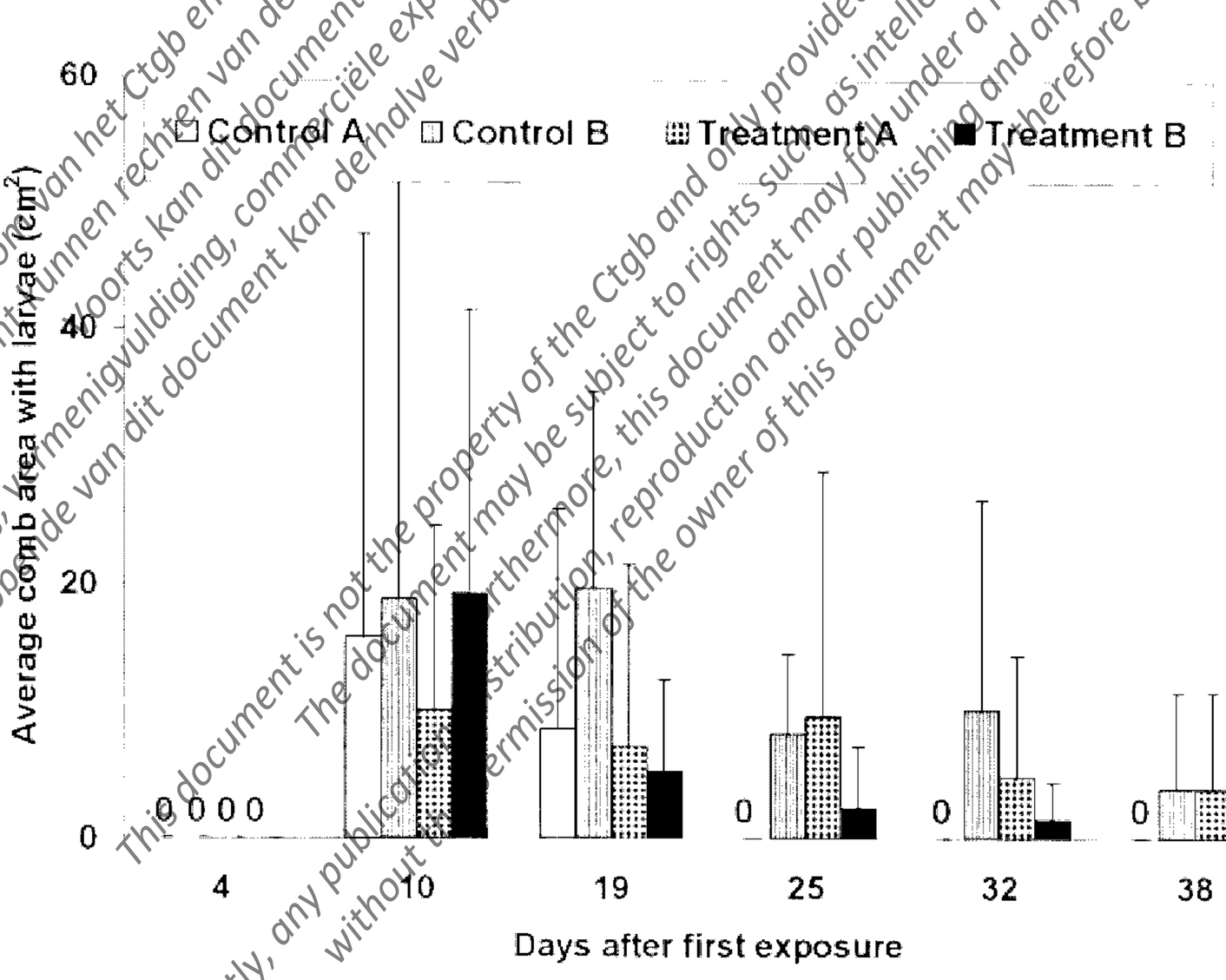


Figure 10: Abundance of honeybee larvae (non-capped brood) over time in control and treatment.
Small bee colonies were fed with sunflower honey and maize pollen. Maize pollen was taken from plants which had received imidaclopride seed-dressing. Columns show the average comb area (mean ± s.d., n=4) where larvae were seen in the cells during evaluations taking into account the increase of the comb area over time (see appendix V).

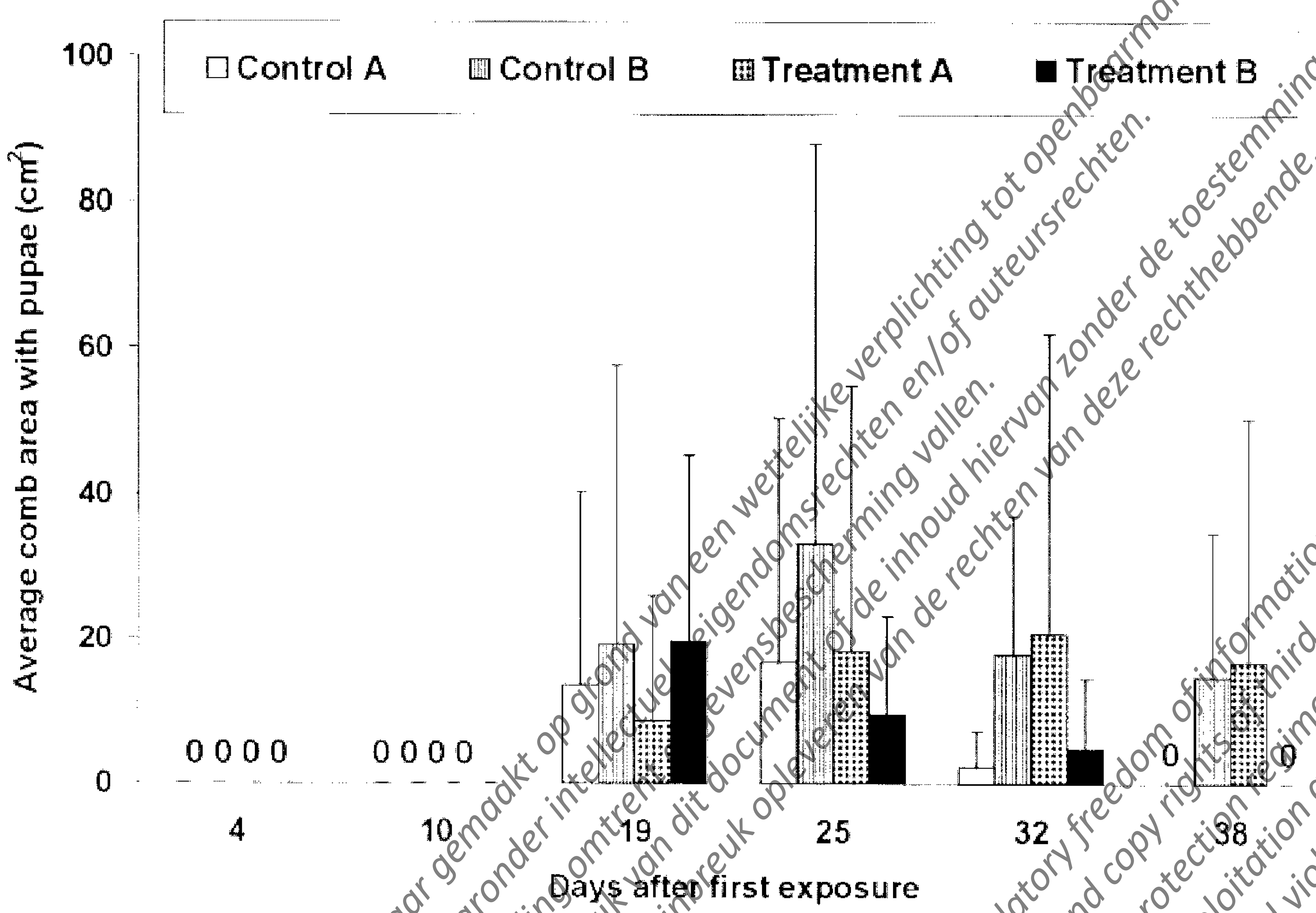


Figure 11: Abundance of honeybee pupae (= capped brood) over time in control and treatment.

Small bee colonies were fed with sunflower honey and maize pollen. Maize pollen was taken from plants which had received imidaclopride seed-dressing. Columns show the average comb area (mean ± s.d., n=4) where capped cells were seen during evaluations taking into account the increase of the comb area over time (see appendix V).

APPENDICES

APPENDIX I: Summary of the Findings of the Analytical Examination for Contaminants of the Maize Pollen Fed During the Study : Overview of Results

Data from appendix XV (MR study MR-111/01)

SAMPLE NAME	Hydroxy-Imidacloprid [mg/kg]	Olefin-Imidacloprid [mg/kg]	Imidacloprid [mg/kg]
Pollen Control 1 A	n.d.	n.d.	n.d.
Pollen Control 1 B	n.d.	n.d.	n.d.
Pollen Treated 2 A 1	n.d.	n.d.	< LOQ
Pollen Treated 2 A 2	n.d.	n.d.	< LOQ
Pollen Treated 2 B 1	n.d.	n.d.	< LOQ
Pollen Treated 2 B 2	n.d.	n.d.	< LOQ

Limit of quantitation: 0.005 mg/kg for imidacloprid and hydroxy-metabolite, 0.01 mg/kg for the olefin-metabolite
< 0.005 and < 0.010 = Residues below the limit of quantitation (< LOQ)
Limit of detection: 0.0015 mg/kg for imidacloprid and hydroxy-metabolite, 0.003 mg/kg for the olefin-metabolite,
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	22	69-100*
1	9	26	60-100
2	8	26	51-100*
3	9	30	50-100*
4	9	27	52-100*
5	9	27	51-100*
6	12	22	81-100*
7	9	22	72-100*
8	8	25	61-100*
9	9	24	59-100*
10	9	26	55-100*
11	11	23	72-100*
12	11	22	73-100*
13	10	20	69-100*
14	10	20	77-100*
15	10	25	47-100
16	11	17	91-100
17	11	23	68-100
18	12	27	64-100
19	15	26	68-100
20	13	27	60-100
21	13	30	47-100
22	13	33	45-100
23	14	29	50-100
24	12	28	57-100
25	13	24	71-100
26	11	18	80-100
27	12	17	93-100
28	10	23	65-100*
29	10	24	65-100*
30	11	16	95-100*
31	10	19	n.a.
32	9	25	n.a.
33	10	20	83-98*
34	7	22	65-100
35	12	21	68-100
36	12	28	55-100
37	12	20	n.a.
38	12*	n.a.	n.a.

* The thermohygrograph data suggest that the correct function of this measuring device was temporarily not given; according to the data, the air moisture would have been above 100% on some days. In these case, the values in question are marked with an asterisk (*).
n.a. = not assessed

APPENDIX III: Activity Pattern of Foraging Honeybees in Control and Treatment.

The table gives the average number of foraging honeybees which were recorded during the daily 5 minutes observation periods either at the pollen feeder, the honey feeder or at the tent roof.

Study day	Number of honeybees recorded at the pollen feeder			
	Control A	Control B	Treatment A	Treatment B
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	0	0	0	0
7	0	0	0	0
8	0	0	0	0
9	0	0	0	0
10	0	0	6	1
11	0	1	1	0
12	0	n.a.*	n.a.*	n.a.*
14	0	0	0	0
15	0	0	2	0
17	0	0	1	0
18	0	1	3	0
21	0	1	2	0
22	0	0	2	0
23	1	0	1	1
24	0	0	0	0
25	0	3	5	0
28	0	1	1	0
29	0	1	2	0
30	n.a.*	n.a.*	n.a.*	n.a.*
32	0	5	1	0
35	0	2	2	0
Total	**1**	**15**	**29**	**2**

* No assessment possible due to inappropriate weather conditions.

APPENDIX III: cont´d.

The table gives the average number of foraging honeybees which were recorded during the daily 5 minutes observation periods either at the pollen feeder, the honey feeder or at the tent roof.

Study day	Number of honeybees recorded at the honey feeder			
	Control A	Control B	Treatment A	Treatment B
1	2	1	1	1
2	1	1	2	1
3	3	3	1	1
4	6	5	1	1
7	13	15	10	13
8	14	9	14	10
9	15	14	16	14
10	22	18	25	26
11	6	5	6	3
12	2	n.a.*	n.a.*	n.a.*
14	2	1	3	2
15	16	17	17	19
17	5	2	2	4
18	22	24	23	21
21	21	19	23	21
22	23	22	25	20
23	10	12	11	10
24	11	10	10	12
25	17	9	15	16
28	14	16	17	14
29	21	22	23	20
30	n.a.*	n.a.*	n.a.*	n.a.*
32	11	15	16	15
35	12	13	13	11
Total	**267****	**253**	**274**	**255**

* No assessment possible due to inappropriate weather conditions.
** Day 12 not considered

APPENDIX III: cont´d.

The table gives the average number of foraging honeybees which were recorded during the daily 5 minutes observation periods either at the pollen feeder, the honey feeder or at the tent roof.

Study day	Number of honeybees recorded at the tent roof			
	Control A	Control B	Treatment A	Treatment B
1	2	2	5	4
2	5	5	5	5
3	5	5	5	5
4	2	5	2	2
7	10	20	20	10
8	2	5	5	5
9	5	5	5	5
10	20	25	30	35
11	2	1	2	2
12	0	n.a.*	n.a.*	n.a.*
14	0	0	0	0
15	10	15	15	20
17	2	5	2	2
18	20	20	20	10
21	20	10	10	10
22	20	20	5	10
23	5	5	5	5
24	10	10	10	10
25	5	5	5	5
28	10	10	10	10
29	10	10	10	10
30	n.a.*	n.a.*	n.a.*	n.a.*
32	10	15	20	15
35	5	5	5	5
Total	**180**	**203**	**196**	**185**

* No assessment possible due to inappropriate weather conditions.
** Day 12 not considered

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

Days after test start (Duration of exposition to the bees)	Quantity of Collected Honey [g]			
	Control A	Control B	Treatment A	Treatment B
7 (7)	18	27	22	35
7 (3)	24	42	31	39
8 (1)	24	31	20	28
9 (5)	17	10	17	23
9 (2)	24	20	35	36
10 (2)	34	47	34	41
11 (2)	25	31	32	47
14 (3)	30	28	36	43
14 (2)	34	40	37	44
15 (5)	22	41	27	39
17 (3)	24	29	28	54
21 (6)	43	50	45	45
21 (4)	39	44	43	39
22 (4)	46	44	47	45
24 (3)	42	51	41	46
25 (3)	40	48	50	39
28 (4)	37	35	34	36
28 (3)	37	40	37	33
32 (6/7*)	33	40	44	36
32 (4)	25	25	23	22
35 (5)	37	39	43	32
35 (3)	32	39	37	28
38 (3)	23	30	29	27
38 (3)	26	22	27	20
Total	**736**	**853**	**819**	**877**

* Different refilling date in control A.

APPENDIX IV: Quantity of Pollen and Honey Collected by the Foraging Honeybees in Control and Treatment (cont'd.).

Days after test start (Duration of exposition to the bees)	Quantity of Collected Pollen [g] Control A	Control B	Treatment A	Treatment B
9 (9)	6	9	4	9
9 (9)	0	0	0	0
11 (2)	n.a.	n.a.	1	n.a.
14 (5)	0	1	n.a.	1
14 (3)	n.a.	n.a.	1	n.a.
15 (1)	n.a.	n.a.	1	n.a.
15 (6)	3	7	2	9
18 (3)	n.a.	n.a.	1	n.a.
18 (4)	1	1	n.a.	1
19 (4)	2	8	0	4
19 (1)	n.a.	1	1	n.a.
21 (2)	n.a.	1	4	n.a.
22 (1)	n.a.	1	5	n.a.
24 (2)	n.a.	2	3	n.a.
24 (6)	1	n.a.	n.a.	1
25 (1)	n.a.	3	3	n.a.
25 (3)	n.a.	4	n.a.	n.a.
25 (6)	0	4	0	1
28 (3)	n.a.	4	3	n.a.
29 (1)	n.a.	4	4	n.a.
29 (5)	2	n.a.	n.a.	0
32 (7)	0	0	0*	0
32 (3)	0	n.a.	4	0
35 (3)	n.a.	4	3	n.a.
38 (3)	n.a.	3	3	n.a.
38 (6)	0	1	0	0
38 (6)	1	n.a.	n.a.	0
Total	**16**	**58**	**43**	**26**

n.a. = not assessed
* Contaminated by mould.

APPENDIX V: Comb Production in Control and Treatment.

The table gives 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 of this geometric form into a rectangular form. The second and third values in each column 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.

Study day	Increase in comb area [cm²]											
	Control A			Control B			Treatment A			Treatment B		
4	**144**	12	6	**88**	11	4	**80**	10	4	**100**	10	5
	66	11	3	**0**	0	0	**24**	6	2	**48**	6	4
	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: 210			**Total: 88**			**Total: 104**			**Total: 148**		
10	**192**	12	8	**132**	11	6	**120**	10	6	**154**	11	7
	154	11	7	**126**	9	7	**154**	11	7	**154**	11	7
	12	3	2	**126**	9	7	**100**	10	5	**154**	11	7
	0	0	0	**0**	0	0	**0**	0	0	**0**	0	0
	Total: 358			**Total: 384**			**Total: 374**			**Total: 462**		
19	**192**	12	8	**192**	12	8	**192**	12	8	**192**	12	8
	192	12	8	**192**	12	8	**192**	12	8	**192**	12	8
	66	11	3	**154**	11	7	**132**	11	6	**168**	12	7
	30	5	3	**30**	5	3	**16**	4	2	**30**	5	3
	Total: 480			**Total: 568**			**Total: 532**			**Total: 582**		
25	**192**	12	8	**192**	12	8	**192**	12	8	**192**	12	8
	192	12	8	**192**	12	8	**192**	12	8	**192**	12	8
	110	11	5	**154**	11	7	**168**	12	7	**192**	12	8
	0	0	0	**40**	5	4	**20**	5	2	**48**	8	3
	Total: 494			**Total: 578**			**Total: 572**			**Total: 624**		
32	**192**	12	8	**192**	12	8	**192**	12	8	**192**	12	8
	192	12	8	**192**	12	8	**192**	12	8	**192**	12	8
	110	11	5	**154**	11	7	**168**	12	7	**192**	12	8
	0	0	0	**70**	7	5	**80**	8	5	**48**	8	3
	Total: 494			**Total: 608**			**Total: 632**			**Total: 624**		
38	**192**	12	8	**192**	12	8	**192**	12	8	**192**	12	8
	192	12	8	**192**	12	8	**192**	12	8	**192**	12	8
	192	12	8	**154**	11	8	**168**	12	7	**192**	12	8
	30	5	3	**80**	8	5	**108**	9	6	**88**	11	4
	Total: 606			**Total: 618**			**Total: 660**			**Total: 664**		

APPENDIX VI: Size of Honey and Pollen Stores over Time in Control and Treatment.

The table gives the proportion of comb areas (four combs) where stored honey and pollen was recorded during evaluation. The first values in each column give the mean values of both comb sides (in bold). The second and third values give the single values of the front and back side of each comb.
The total value refers to the absolute area in cm² which contained honey and pollen taking into account the values of the newly produced comb area from appendix V.

Study day	Honey deposition area [%combs with honey/total = cm² combs with honey]											
	Control A			Control B			Treatment A			Treatment B		
4	**5**	5	5	**0**	0	0	**0**	0	0	**0**	0	0
	0	0	0	**0**	0	0	**0**	0	0	**0**	0	0
	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: 7			**Total: 0**			**Total: 0**			**Total: 0**		
10	**20**	20	20	**73**	70	75	**43**	50	35	**43**	50	35
	35	30	40	**8**	10	5	**33**	30	35	**23**	20	25
	0	0	0	**3**	0	5	**70**	75	65	**63**	60	65
	0	0	0	**0**	0	0	**0**	0	0	**0**	0	0
	Total: 92			**Total: 110**			**Total: 172**			**Total: 199**		
19	**23**	25	20	**38**	35	40	**65**	65	65	**45**	40	50
	43	40	45	**10**	10	10	**30**	30	30	**48**	45	50
	0	0	0	**30**	35	20	**83**	80	85	**83**	80	85
	0	0	0	**0**	0	0	**0**	0	0	**0**	0	0
	Total: 127			**Total: 138**			**Total: 291**			**Total: 318**		
25	**40**	45	35	**50**	50	50	**75**	75	75	**60**	70	50
	83	80	85	**20**	20	20	**25**	25	25	**63**	60	65
	50	50	50	**38**	40	35	**88**	90	85	**88**	90	85
	0	0	0	**0**	0	0	**0**	0	0	**0**	0	0
	Total: 291			**Total: 193**			**Total: 340**			**Total: 405**		
32	**78**	75	80	**60**	60	60	**90**	90	90	**90**	90	90
	80	90	70	**20**	20	20	**30**	25	35	**75**	75	75
	45	40	50	**40**	40	40	**65**	65	65	**73**	75	70
	0	0	0	**23**	25	20	**55**	70	40	**0**	0	0
	Total: 353			**Total: 231**			**Total: 384**			**Total: 457**		
38	**88**	90	85	**63**	70	55	**95**	95	95	**90**	90	90
	65	65	65	**40**	45	35	**48**	50	45	**85**	80	90
	73	75	70	**13**	10	15	**40**	65	15	**33**	40	25
	0	0	0	**45**	50	40	**70**	65	75	**0**	0	0
	Total: 434			**Total: 254**			**Total: 417**			**Total: 399**		

APPENDIX VI: Size of Honey and Pollen Stores over Time in Control and Treatment (cont'd).

The table gives the proportion of comb areas (four combs) where stored honey and Pollen was recorded during evaluation. The first values in each column give the mean values of both comb sides (in bold). The second and third values give the single values of the front and back side of each comb.
The total value refers to the absolute area in cm² which contained honey and pollen taking into account the values of the newly produced comb area from appendix V.

Study day	Pollen deposition area [%combs with honey/total = cm² combs with pollen]											
	Control A			Control B			Treatment A			Treatment B		
4	**0**	0	0	**0**	0	0	**0**	0	0	**0**	0	0
	0	0	0	**0**	0	0	**0**	0	0	**0**	0	0
	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**		
10	**0**	0	0	**5**	5	5	**3**	5	0	**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
	0	0	0	**0**	0	0	**0**	0	0	**0**	0	0
	Total: 0			**Total: 7**			**Total: 4**			**Total: 5**		
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
	0	0	0	**0**	0	0	**0**	0	0	**0**	0	0
	Total: 0			**Total: 0**			**Total: 0**			**Total: 0**		
25	**0**	0	0	**3**	0	5	**0**	0	0	**0**	0	0
	0	0	0	**0**	0	0	**0**	0	0	**0**	0	0
	0	0	0	**3**	5	0	**0**	0	0	**0**	0	0
	0	0	0	**0**	0	0	**0**	0	0	**0**	0	0
	Total: 0			**Total: 10**			**Total: 0**			**Total: 0**		
32	**0**	0	0	**0**	0	0	**0**	0	0	**0**	0	0
	0	0	0	**3**	5	0	**0**	0	0	**0**	0	0
	0	0	0	**5**	5	5	**3**	5	0	**0**	0	0
	0	0	0	**5**	5	5	**0**	0	0	**0**	0	0
	Total: 0			**Total: 17**			**Total: 5**			**Total: 0**		
38	**0**	0	0	**5**	5	5	**0**	0	0	**0**	0	0
	0	0	0	**3**	0	5	**0**	0	0	**0**	0	0
	0	0	0	**5**	5	5	**0**	0	0	**0**	0	0
	0	0	0	**0**	0	0	**3**	5	0	**0**	0	0
	Total: 0			**Total: 23**			**Total: 3**			**Total: 0**		

APPENDIX VII: Weight Increase of Bee Hives in Control and Treatment.

Study day	Total Hive Weight [g]			
	Control A	Control B	Treatment A	Treatment B
0	865	910	925	875
7	865	935	955	925
14	830	925	970	975
21	850	925	975	975
29	930	980	1060	1030
38	950	970	1040	1020
Total weight gain [g]	**85**	**60**	**115**	**145**
Total weight gain [%]*	**9.8**	**6.6**	**12.4**	**16.6**

* In relation to the initial weight

APPENDIX VIII: Population Growth in Control and Treatment.

The table shows the proportions of comb area (four combs) which was occupied by adult honeybees during the evaluations. The first values (in bold) give the mean values of both comb sides. The second and third values give the single values of the front and back side of each comb.
The total value refers to the absolute area in cm² covered by honeybees taking the values of the newly produced comb area from appendix V.

Study day	Population density [% occupied combs/total=cm² occupied comb area]											
	Control A			Control B			Treatment A			Treatment B		
4	**70**	80	60	**50**	50	50	**60**	50	70	**75**	80	70
	70	70	70	**0**	0	0	**55**	80	30	**45**	50	40
	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: 147			**Total: 44**			**Total: 61**			**Total: 97**		
10	**50**	30	70	**70**	60	80	**65**	40	90	**43**	35	50
	45	50	40	**90**	90	90	**85**	90	80	**50**	50	50
	45	80	10	**30**	30	30	**78**	80	75	**80**	80	80
	0	0	0	**0**	0	0	**0**	0	0	**0**	0	0
	Total: 171			**Total: 142**			**Total: 287**			**Total: 266**		
19	**65**	50	80	**55**	60	50	**80**	90	70	**55**	40	70
	65	80	50	**70**	90	50	**70**	90	50	**50**	60	40
	70	50	90	**75**	90	60	**65**	80	50	**50**	60	40
	30	30	30	**30**	30	30	**10**	10	10	**35**	50	20
	Total: 305			**Total: 365**			**Total: 375**			**Total: 296**		
25	**60**	70	50	**35**	30	40	**60**	40	80	**40**	50	30
	40	40	40	**65**	80	50	**50**	60	40	**50**	30	70
	55	60	50	**40**	30	50	**35**	40	30	**55**	70	40
	0	0	0	**20**	30	10	**0**	0	0	**5**	5	5
	Total: 253			**Total: 262**			**Total: 270**			**Total: 281**		
32	**65**	70	60	**45**	40	50	**40**	30	50	**40**	50	30
	50	60	40	**70**	70	70	**50**	50	50	**65**	50	80
	70	80	60	**20**	20	20	**30**	30	30	**40**	50	30
	0	0	0	**15**	20	10	**20**	20	20	**65**	50	80
	Total: 298			**Total: 252**			**Total: 239**			**Total: 310**		
38	**40**	30	50	**40**	30	50	**35**	35	35	**50**	50	50
	55	30	80	**55**	60	50	**55**	40	70	**50**	70	30
	45	30	60	**30**	30	30	**25**	30	20	**28**	25	30
	35	30	40	**25**	30	20	**35**	30	40	**20**	10	30
	Total: 279			**Total: 249**			**Total: 253**			**Total: 263**		

APPENDIX IX: Mortality in Control and Treatment.

The table gives 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 edges.

Study day	Number of dead honeybees found in front of the bee hives			
	Control A	Control B	Treatment A	Treatment B
1	1	3	3	0
2	0	2	0	7
3	0	0	1	0
4	1	0	0	1
7	1	0	1	1
8	2	0	0	1
9	0	0	0	0
10	1	2	0	0
11	0	0	0	0
12	0	n.a.*	n.a.*	n.a.*
14	1	0	0	0
15	1	0	1	1
17	11	4	1	1
18	1	1	0	3
21	4	6	2	4
22	3	1	0	2
23	0	1	1	3
24	0	1	1	1
25	0	0	0	0
28	1	0	2	3
29	1	0	2	2
30	n.a.*	n.a.*	n.a.*	n.a.*
32	1	1	5	0
35	2	5	0	0
Total	**32**	**27**	**20**	**30**

* No assessment possible due to inappropriate weather conditions.

APPENDIX IX: cont´d.

The table gives 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 edges.

Study day	Number of dead honeybees found at the tent edges			
	Control A	Control B	Treatment A	Treatment B
1	2	3	1	2
2	0	7	3	11
3	4	3	3	1
4	2	1	18	8
7	5	3	9	13
8	1	4	3	3
9	2	7	4	0
10	3	9	7	8
11	3	2	4	7
12	n.a.*	n.a.*	n.a.*	n.a.*
14	4	3	6	5
15	3	6	3	11
17	17	13	5	6
18	7	9	1	4
21	22	17	9	13
22	13	11	4	8
23	7	7	13	16
24	15	8	5	11
25	14	4	4	1
28	5	7	2	2
29	3	3	4	11
30	n.a.*	n.a.*	n.a.*	n.a.*
32	6	6	17	6
35	8	8	14	4
Total	**146****	**141**	**139**	**151**

* No assessment possible due to inappropriate weather conditions.
** Day 12 not considered

APPENDIX X: Queen Egg-Laying Activity in Control and Treatment.

This table shows the proportions of comb area (four combs) where an egg was found during evaluation. The first values of a column give the mean values of both comb sides (in bold). The second and third values give the single values of the front and back side of each comb. The total value refers to the absolute area in cm² which contained eggs taking into account the values of the newly produced comb area from appendix V.

Study day	Egg deposition activity [% combs with eggs/total=cm² combs with eggs]											
	Control A			Control B			Treatment A			Treatment B		
4	**15**	10	20	**0**	0	0	**0**	0	0	**35**	20	50
	0	0	0	**0**	0	0	**0**	0	0	**0**	0	0
	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: 22			**Total: 0**			**Total: 0**			**Total: 35**		
10	**43**	45	40	**0**	0	0	**38**	35	40	**25**	20	30
	38	40	35	**25**	15	35	**38**	35	40	**48**	50	45
	0	0	0	**73**	70	75	**0**	0	0	**5**	10	0
	0	0	0	**0**	0	0	**0**	0	0	**0**	0	0
	Total: 141			**Total: 124**			**Total: 104**			**Total: 112**		
19	**28**	25	30	**40**	45	35	**20**	20	20	**15**	10	20
	50	50	50	**28**	25	30	**33**	35	30	**20**	30	10
	0	0	0	**50**	45	55	**0**	0	0	**0**	0	0
	0	0	0	**0**	0	0	**0**	0	0	**0**	0	0
	Total: 150			**Total: 208**			**Total: 102**			**Total: 67**		
25	**3**	5	0	**23**	35	10	**18**	15	20	**8**	5	10
	8	10	5	**10**	10	10	**18**	15	20	**5**	5	5
	0	0	0	**13**	15	10	**0**	0	0	**0**	0	0
	0	0	0	**0**	0	0	**0**	0	0	**0**	0	0
	Total: 21			**Total: 83**			**Total: 69**			**Total: 25**		
32	**0**	0	0	**13**	15	10	**0**	0	0	**0**	0	0
	0	0	0	**23**	20	25	**10**	10	10	**3**	0	5
	0	0	0	**0**	0	0	**0**	0	0	**0**	0	0
	0	0	0	**3**	0	5	**0**	0	0	**0**	0	0
	Total: 0			**Total: 71**			**Total: 19**			**Total: 6**		
38	**0**	0	0	**13**	15	10	**0**	0	0	**0**	0	0
	5	5	5	**20**	10	30	**8**	5	10	**5**	5	5
	5	5	5	**0**	0	0	**0**	0	0	**3**	5	0
	0	0	0	**0**	0	0	**0**	0	0	**3**	5	0
	Total: 19			**Total: 63**			**Total: 15**			**Total: 18**		

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

This table shows the proportions of comb area (four combs) where a larva was seen during the evaluations. The first values in a column give the mean values of both comb sides (in bold). Thesecond and third values give the single values of the front and back side of each comb.

The total value refers to the absolute area in cm² which contained larvae taking into account the increase of the comb area over time (see appendix V).

Study day	Abundance of larvae [% combs with larvae/cm² combs with larvae]											
	Control A			Control B			Treatment A			Treatment B		
4	**0**	0	0	**0**	0	0	**0**	0	0	**0**	0	0
	0	0	0	**0**	0	0	**0**	0	0	**0**	0	0
	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**		
10	**33**	30	35	**0**	0	0	**8**	0	15	**25**	25	25
	0	0	0	**60**	70	50	**20**	20	20	**25**	25	25
	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: 63			**Total: 76**			**Total: 40**			**Total: 77**		
19	**18**	20	15	**18**	15	20	**0**	0	0	**3**	5	0
	0	0	0	**15**	20	10	**15**	15	15	**8**	10	5
	0	0	0	**10**	10	10	**0**	0	0	**0**	0	0
	0	0	0	**0**	0	0	**0**	0	0	**0**	0	0
	Total: 35			**Total: 79**			**Total: 29**			**Total: 21**		
25	**0**	0	0	**8**	0	15	**0**	0	0	**0**	0	0
	0	0	0	**5**	5	5	**20**	20	20	**5**	5	5
	0	0	0	**5**	5	5	**0**	0	0	**0**	0	0
	0	0	0	**0**	0	0	**0**	0	0	**0**	0	0
	Total: 0			**Total: 33**			**Total: 38**			**Total: 10**		
32	**0**	0	0	**3**	0	5	**0**	0	0	**0**	0	0
	0	0	0	**18**	10	15	**10**	5	15	**3**	0	5
	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: 40**			**Total: 19**			**Total: 6**		
38	**0**	0	0	**0**	0	0	**0**	0	0	**0**	0	0
	0	0	0	**8**	10	5	**8**	10	5	**3**	5	0
	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: 15**			**Total: 15**			**Total: 6**		

APPENDIX XII: Abundance of Honeybee Pupae (capped brood) in Control and Treatment.

This table shows the proportions of comb area (four combs) where capped cells was seen during evaluation. The first values in a column give the mean values of both comb sides (in bold). The second and third values give the single values of the front and back side of each comb.

The total value refers to the absolute area in cm^2 which contained larvae taking into account the increase of the comb area over time (see appendix V).

Study day	Abundance of pupae [% combs with pupae/ total=cm^3 combs with pupae]											
	Control A			Control B			Treatment A			Treatment B		
4	**0**	0	0	**0**	0	0	**0**	0	0	**0**	0	0
	0	0	0	**0**	0	0	**0**	0	0	**0**	0	0
	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**		
10	**0**	0	0	**0**	0	0	**0**	0	0	**0**	0	0
	0	0	0	**0**	0	0	**0**	0	0	**0**	0	0
	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**		
19	**28**	25	30	**0**	0	0	**0**	0	0	**28**	30	25
	0	0	0	**40**	40	40	**18**	15	20	**13**	10	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
	Total: 54			**Total: 77**			**Total: 35**			**Total: 79**		
25	**35**	35	35	**5**	5	5	**0**	0	0	**5**	5	5
	0	0	0	**60**	60	60	**38**	40	35	**15**	20	10
	0	0	0	**5**	5	5	**0**	0	0	**0**	0	0
	0	0	0	**0**	0	0	**0**	0	0	**0**	0	0
	Total: 67			**Total: 133**			**Total: 73**			**Total: 38**		
32	**5**	5	5	**10**	15	5	**0**	0	0	**0**	0	0
	0	0	0	**23**	20	25	**43**	50	35	**10**	10	10
	0	0	0	**5**	5	5	**0**	0	0	**0**	0	0
	0	0	0	**0**	0	0	**0**	0	0	**0**	0	0
	Total: 10			**Total: 71**			**Total: 83**			**Total: 19**		
38	**0**	0	0	**5**	5	5	**0**	0	0	**0**	0	0
	0	0	0	**23**	25	20	**35**	35	35	**0**	0	0
	0	0	0	**3**	5	0	**0**	0	0	**0**	0	0
	0	0	0	**0**	0	0	**0**	0	0	**0**	0	0
	Total: 0			**Total: 58**			**Total: 67**			**Total: 0**		

APPENDIX XIII: Copy of the GLP Certificate

[redacted]

APPENDIX XIII: (continued):

[redacted]

APPENDIX XIV: Quality Assurance Statement

Referat GLP
Quality Assurance Statement

[redacted]

APPENDIX XV: Analytical Report on Pollen Analysis

Study Title

Effects of Residues of Imidacloprid in Maize Pollen from Dressed Seeds on Honey Bees (*Apis mellifera*)

Author

[redacted]

Testing Facility

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

Analytical Completion Date

2001-03-05

MR Study No. MR-111/01

STUDY TITLE

Effects of Residues of Imidacloprid in Maize Pollen from Dressed Seeds on Honey Bees (*Apis mellifera*)

Author

[redacted]

Testing Facility

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

Analytical Completion Date

2001-03-05

Study Number

E 319 1912-6

CERTIFICATION OF AUTHENTICITY

[redacted]

Author and
Responsible Analyst

2001-03-05
Date

Study Director

2001-04-12
Date

Inquiries should be directed to

[redacted]

TABLE OF CONTENT

1 INTRODUCTION

Maize pollen samples obtained from a France trial station were analysed for residues of imidacloprid and its olefin- and hydroxy metabolites. The results are summarized in the table below. Extraction, sample clean up and determination of imidacloprid, hydroxy- and olefin-metabolite by HPLC-MS/MS were performed according to method 00537/E001 (MR-568/99). The limit of quantitation was 0.005 mg/kg for imidacloprid and the hydroxy-metabolite and 0.01 mg/kg for the olefin-metabolite. The limit of detection was 0.0015 mg/kg for imidacloprid and the hydroxy-metabolite and 0.003 mg/kg for the olefin-metabolite.

2 TIME SCHEDULE

The experimental work was performed during the following time period:

Signature of study protocol: 2000-08-15
Start of analytical phase: 2000-08-17
End of analytical phase: 2000-08-21

3 RESULTS OF POLLEN:

SAMPLE NAME	Hydroxy-Imidacloprid [mg/kg]	Olefin-Imidacloprid [mg/kg]	Imidacloprid [mg/kg]
Pollen Control 1 A	n.d.	n.d.	n.d.
Pollen Control 1 B	n.d.	n.d.	n.d.
Pollen Treated 2 A 1	n.d.	n.d.	< LOQ
Pollen Treated 2 A 2	n.d.	n.d.	< LOQ
Pollen Treated 2 B 1	n.d.	n.d.	< LOQ
Pollen Treated 2 B 2	n.d.	n.d.	< LOQ

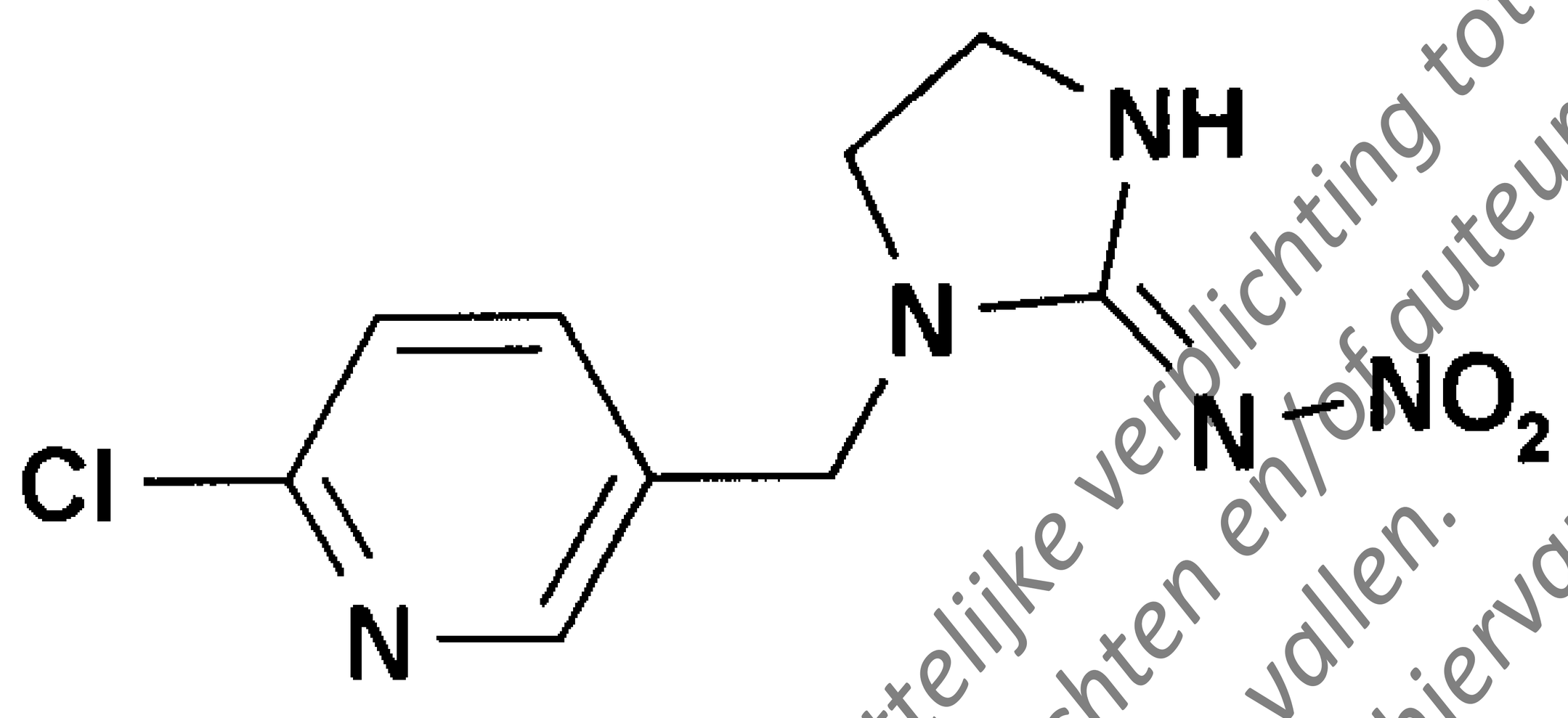
Limit of quantitation: 0.005 mg/kg for imidacloprid and hydroxy-metabolite, 0.01 mg/kg for the olefin-metabolite
< 0.005 and < 0.010 = Residues below the limit of quantitation (< LOQ)
Limit of detection: 0.0015 mg/kg for imidacloprid and hydroxy-metabolite, 0.003 mg/kg for the olefin-metabolite,
n.d.: Residues below the limit of detection

4 EXPERIMENTAL

4.1 Reference Substances

Imidacloprid

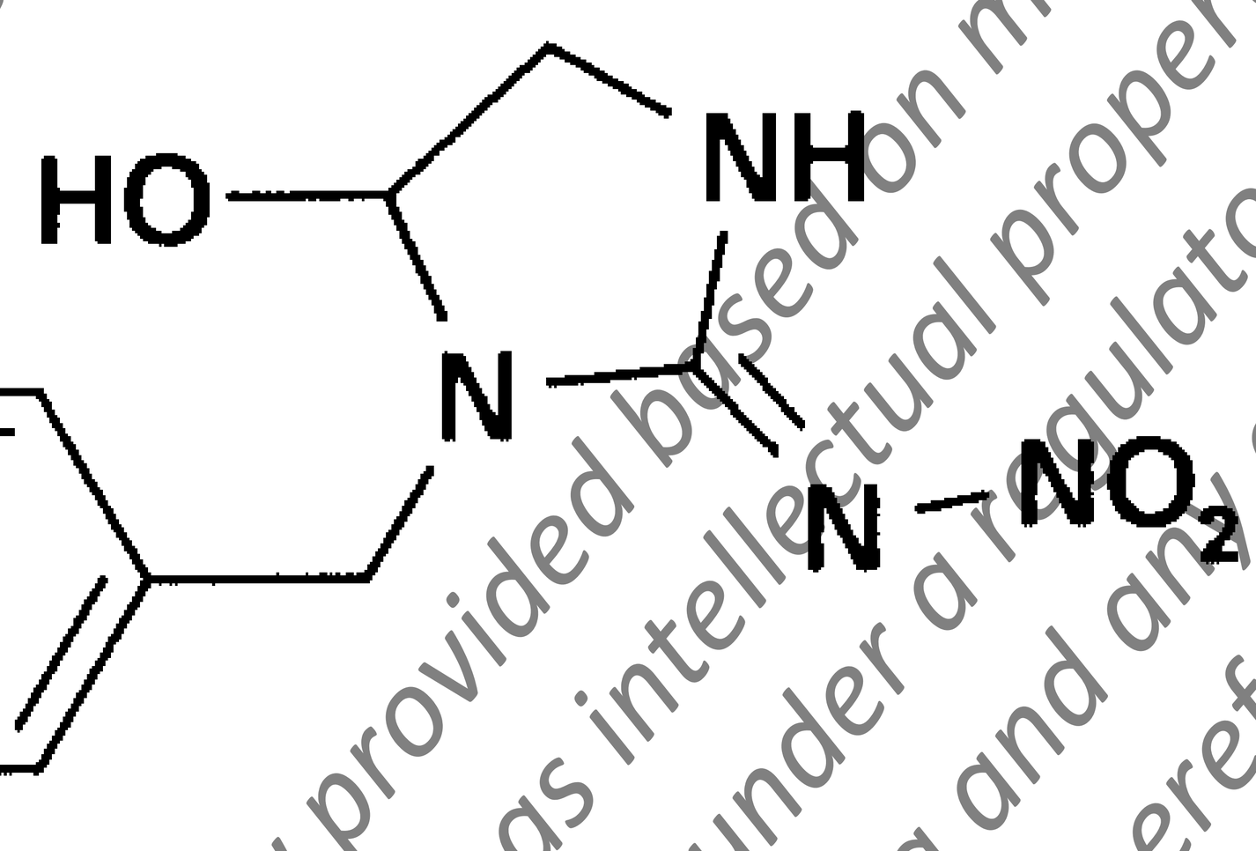
Structural formula:



Empirical formula: $C_9H_{10}ClN_5O_2$
Molecular weight: 255.7 g/mole
Certificate of analysis: M06693, 2000-01-11
Certified assay: 99.8 %
Expiry date: November 2001

Hydroxy-Imidacloprid (WAK 4103)

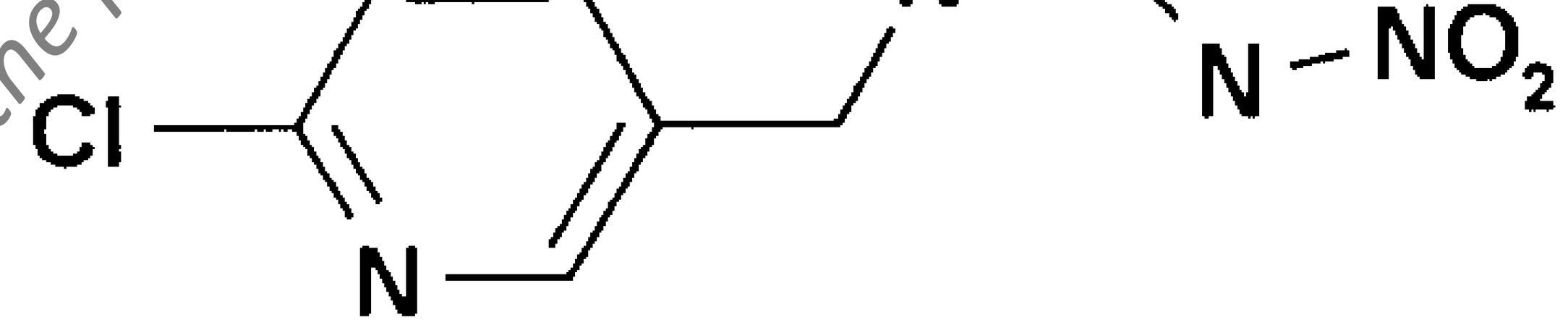
Structural formula:



Empirical formula: $C_9H_{10}ClN_5O_4$
Molecular weight: 271.7 g/mole
Certificate of analysis: 930323ELB03, 2000-05-11
Certified assay: 99.4 %
Expiry date: May 2005

Olefin-Imidacloprid (NTN 35884)

Structural formula:



Empirical formula: $C_9H_8ClN_5O_2$
Molecular weight: 253.6 g/mole
Certificate of analysis: M11453, 2000-07-28
Certified assay: 98.6 %
Expiry date: July 2002

4.2 Residue Analytical Methodology

4.2.1 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.

4.2.2 ChemElut® Column Clean-up

1. Add 5 to 10 mL water to the aqueous solution from 4.2.1 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.

4.2.3 Silica Gel Column Clean-up

1. Dissolve the residues from 4.2.2 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.

4.3 HPLC-MS/MS determination of Imidacloprid and Metabolites

4.3.1 Measuring equipment and HPLC conditions:

Instrument:	Hewlett Packard 1100
Column:	e.g.: Phenomenex, Luna C18 (2), 5 µm, 15 x 0.46 cm i.D. or Merck, Superspher, RP select-B, 4 µm, 12.5 x 0.4 cm i.D.
Solvent A:	Water/ACN (9/1, v/v) + 0.1 mL Acetic acid/L
Solvent B:	ACN + 0.1 mL Acetic acid/L
Oventemperature:	40 °C
Inject.volume:	50 µL
Flow:	1.0 mL/min
Split:	150 µL into MS from 1000 µL

Time Table	0 min	11 % B TO MS
	10 min.	11 % B
	10.1 min	90 % B
	11 min	90 % To Waste
	15 min	90 % B
	15.1 min	11 % B
	16 min	11 % B TO MS
	19 min	11 % B

Retention Times:	Olefin-Imidacloprid	approx. 4.9 min
	Hydroxy-Imidacloprid	approx. 5.7 min
	Imidacloprid	approx. 8.7min

4.3.2 MS/MS-Detection

The experiments were performed on a triple-quadrupole mass spectrometer 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, Hydroxy-Metabolite and Olefin-Metabolite (dissolved in acetonitrile / water (2/8, v/v) + 0.1 mL acetic acid per litre) at a flow rate of 5-10 µL/min. Mass axis calibration was done by infusing a polypropylene glycol solution. Unit mass resolution was established and maintained in each mass resolving quadrupole by maintaining a full widht 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, Hydroxy-Metabolite and Olefin-Metabolite were determined. These experiments were performed with nitrogen as collision gas with a collision offset of -19 and -23 eV for Imidacloprid, -23 eV for Hydroxy-Metabolite and –12 and -13 eV for Olefin-Metabolite at an approximate collision gas thickness of 1.56 x 10^{15} atoms/cm^2. Nebulization gas is set at 1.48 L/min, curtain gas is set at 1.44 L/min, CAD gas is set at 0.87 L/min and turbo gas is set at 7 L/min.

Detector:	Triple Quadrupol LC-MS/MS Mass Spectrometer PE Biosystems (Perkin-Elmer Sciex Instruments) API 365, Windows NT 4.0 System
Interface:	Electrospray, Turbo Ion Spray Potential: + 4800 V Temperature: 350° C, 400° C (Source)
Gas:	Nebulization gas: 1.48 L/min (liquid nitogen 5.0) Curtain gas: 1.44 L/min (liquid nitogen 5.0) Collision gas 0.87 L/min (liquid nitogen 5.0) Turbo gas: 7 L/min (liquid nitogen 5.0)
Scan Type:	MRM (Multiple Reaction Monitoring Mode)
Polarity:	Positive
Aquisition mode:	Profile

Mass spectrometer operating parameters

Compound	Precursor Ion Q1 Mass (amu)	Product Ion Q3 Mass (amu)	Dwell Time (msec)	Collision Energy (eV)
Imidacloprid (Cl 37)#	258	211	250	-19
Imidacloprid (Cl 35)	256	209	250	-19
Imidacloprid (Cl 37)#	258	175	250	-23
Imidacloprid (Cl 35)	256	175	250	-23
Hydroxy-Metabolite (Cl 37)#	274	191	250	-23
Hydroxy-Metabolite (Cl 35)	272	191	250	-23
Olefin-Metabolite (Cl 35)#	254	236	250	-12
Olefin-Metabolite (Cl 35)	254	207	250	-13

#= ^{37}Cl isotope of all substances were detected to use as qualifiers