Risk posed to honeybees (*Apis mellifera* L, Hymenoptera) by an imidacloprid seed dressing of sunflowers

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Abstract: In a greenhouse metabolism study, sunflowers were seed-treated with radiolabelled imidacloprid in a 700 g kg^{-1} WS formulation (Gaucho® WS 70) at 0.7 mg AI per seed, and the nature of the resulting residues in nectar and pollen was determined. Only the parent compound and no metabolites were detected in nectar and pollen of these seed-treated sunflower plants (limit of detection <0.001 mg kg^{-1}). In standard LD_{50} laboratory tests, imidacloprid showed high oral toxicity to honeybees (*Apis mellifera*), with LD_{50} values between 3.7 and 40.9 ng per bee, corresponding to a lethal food concentration between 0.14 and 1.57 mg kg^{-1}. The residue level of imidacloprid in nectar and pollen of seed-treated sunflower plants in the field was negligible. Under field-growing conditions no residues were detected (limit of detection: 0.0015 mg kg^{-1}) in either nectar or pollen. There were also no detectable residues in nectar and pollen of sunflowers planted as a succeeding crop in soils which previously had been cropped with imidacloprid seed-treated plants.

Chronic feeding experiments with sunflower honey fortified with 0.002, 0.005, 0.010 and 0.020 mg kg^{-1} imidacloprid were conducted to assess potential long-term adverse effects on honeybee colonies. Testing end-points in this 39-day feeding study were mortality, feeding activity, wax/comb production, breeding performance and colony vitality. Even at the highest test concentration, imidacloprid showed no adverse effects on the development of the exposed bee colonies. This no-adverse-effect concentration of 0.020 mg kg^{-1} compares with a field residue level of less than 0.0015 mg kg^{-1} (= limit of detection in the field residue studies) which clearly shows that a sunflower seed dressing with imidacloprid poses no risk to honeybees. This conclusion is confirmed by observations made in more than 10 field studies and several tunnel tests.

Keywords: imidacloprid; *Apis mellifera*; sunflower; pesticide; seed dressing; depopulation; disappearance disease

1 INTRODUCTION

Imidacloprid (Gaucho®), used as a sunflower seed-dressing, is a chloronicotinyl insecticide which has a highly specific affinity to the insect acetylcholine receptor.[1–4] The sunflower seed-dressing was first launched on the French market in 1994. Due to its excellent pest-control performance,[5–7] the treated area in the sunflower crop increased appreciably during the following years. In 1996 (one possible case had been reported in 1994) a novel bee malady was reported from Central and Western France which became aggravated in the following years. The characteristic symptoms of this bee malady are given by Schmuck.[8] Due to the temporal co-incidence of the market launch of Gaucho and the first appearance of the bee problem, some beekeepers assumed that imidacloprid, the active ingredient of this seed-dressing formulation, or its metabolites might be linked to this bee malady. Extensive research activities in 1998 found no conclusive evidence of such a link.[8] In an attempt to address any final concerns, a further extended inter-disciplinary research programme was set up to re-examine whether there was any indication of a link between the reported bee malady and the seed dressing of sunflowers with imidacloprid.

First, the level and identity of metabolite residues was to be examined in pollen and nectar of sunflower plants which had been raised from imidacloprid-dressed sunflower seeds. The objective of this metabolism study was to examine whether metabolites of imidacloprid could be detected in sunflower pollen and nectar that might potentially be more toxic to honeybees than the parent compound. It was intended that any metabolite of imidacloprid found during this study in either matrix would be tested for its toxicity to honeybees. For the determination of residue concentrations under practical use conditions, field-collected samples of pollen and nectar from imidacloprid seed-treated sunflowers were analyzed. In addition, imidacloprid-free seeds were drilled in soils with a history of

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imidacloprid treatment to examine whether sunflowers planted as a succeeding crop in soils previously planted with imidacloprid seed-treated crops could have ecotoxicologically relevant imidacloprid residue levels in pollen and nectar during flowering. The potential absorption of imidacloprid soil residues had been stressed by beekeepers when the novel bee malady continued even in those districts where imidacloprid was no longer used on sunflower crops.

Second, it was to be investigated at what residue concentration imidacloprid and/or its metabolites affected honeybees in a way which fitted with the reported symptoms of the novel bee malady. These studies would examine whether imidacloprid residues had an impact on bee colonies when the latter were continously exposed to the compound at a field-relevant concentration range. A comparison of the detected field residue levels with the highest observed no-adverse-effect level from this chronic feeding experiment should finally give a scientifically unequivocal answer to the question whether an imidacloprid seed-dressing of sunflowers could be involved in the bee malady reported from some departments in France.

2 MATERIAL AND METHODS

2.1 Metabolism of imidacloprid in sunflower plants

2.1.1 Seeding and plant growth

The sunflower metabolism study was performed in a greenhouse with 22 potted plants, sub-divided into two rows of 11 plants each. Prior to seeding, sunflower seeds (*Helianthus annuus* L cv Fleury) were dressed with [*methylene*-^{14}C]imidacloprid formulated as the commercial 700 g kg^{-1} WS (Gaucho® WS 70; Bayer AG, D-51368 Leverkusen, Germany). The seed was dressed at the maximum recommended application rate of 0.7 mg AI per seed. Sunflower plants were grown from the treated seeds (one plant per 34-litre pot, loamy sand soil substrate) under climatic conditions optimized for sunflower growth. Flowering started 62–66 days after seed dressing and sowing. During the flowering period of 2 weeks, nectar and pollen were sampled from the sunflower plants. Pollen trickled freely into plastic boxes which were fixed underneath the inflorescences. Nectar was collected daily from the female florets (stigma visible) with the aid of glass capillaries. Pollen and nectar samples of both plant rows were sampled, stored and analyzed separately (two replicates).

2.1.2 Analysis

Pollen (2 g) was soaked in methanol + water (30 ml; 3 + 1 by volume) for 30 min. Subsequently, the suspension was macerated with a Polytron (Kinematica) for *c* 3 min and vacuum filtered. The extraction was repeated twice more with methanol alone. The extracts were combined and rotary evaporated (40 °C) to the aqueous remainder. Nectar was diluted only with deionized water (1 + 1 by weight) before being purified. The diluted nectar and the aqueous remainder of the pollen extract were purified by solid phase extraction: diluted nectar (*c* 1.7 ml), or the whole amount of the aqueous remainder of the pollen extract, was placed on a 6-g Oasis® adsorbent cartridge (Waters), which was conditioned with methanol (30 ml) and water + formic acid (30 ml; 90 + 10 by volume). The cartridge was washed with deionized water (30 ml) and subsequently eluted with methanol (30 ml). The methanol eluates were concentrated on a rotary evaporator and then further concentrated under nitrogen for analysis. The ^{14}C-recovery of this purification procedure was 97.3 (±2.5)% (pollen) and 98.1 (±14.8)% (nectar).

Radioactivity in the samples was determined by liquid scintillation counting. The chemical composition of the radioactive residues in the methanol eluates was analysed by two-dimensional thin-layer chromatography using TLC plates (Kieselgel 60 F254, Merck no 1.05715; solvent systems in first direction, ethyl acetate + toluene + methanol + acetic acid [80 + 20 + 20 + 1 by volume]; solvent system in second direction, ethyl acetate + propan-2-ol + water [65 + 23 + 12 by volume]; no chamber saturation; developing distance, 10 cm in each direction) and by automated multiple development (AMD) using HPTLC plates (Kieselgel 60 WR F254S, Merck no 1.15 552). Details of the AMD method are given in Tables 1a and b. (HP)TLC plates were evaluated using an BAS 2000 Bio Imaging System (Fuji).

2.2 Toxicity of imidacloprid to honeybees

The oral toxicity of the residues identified in the nectar and pollen of sunflower plants during the metabolism study was tested on the honeybee *Apis mellifera* L var *carnica* according to EPPO test guideline no 170.[9] Honeybees originated from beehives housed near to the respective testing facility. Three facilities, located in the UK (A) The Netherlands (B) and Germany (C), were used in the tests. In these tests, groups of ten bees per dose (4–6 doses) were fed with sucrose + water (0.2 ml; 0.5 g + 1 ml) which contained known concentrations of imidacloprid either as technical grade material (purity >98%) or as formulated ready-to-use products (700 g kg^{-1} WG; 200 g litre^{-1} SC imidacloprid; Bayer AG, D-51368 Leverkusen). Due to their trophallactic behavior, individual bees share the food in these tests and so receive comparable doses. After complete consumption of the imidacloprid-containing sucrose solution, bees were provided with untreated sucrose solution and were further observed for a total period of 48 h.

2.3 Field residue studies with sunflowers

The field residue studies were performed on the farms Höfchen (approximately 1 km from Burscheid, Germany, 205 m above sea level) and Laacher Hof (approximately 3 km S of Monheim, Germany, 41 m above sea level). Twenty soil samples were taken from each study site to determine the soil characteristics of

Table 1. AMD method for imidacloprid and metabolites

(a) General parameter and solvents

Device	AMD2 (CAMAG)
Volume per development step	6000 µl
Vacuum test approved with final pressure	<10.0 mbar
Vacuum test approved with pressure drop	<10.0 mbar min^{-1}
Number of flushings before development	1
Additional volume in tubes	0 µl
Chamber saturation	Water + formic acid (95 + 5 by volume)
HPTLC plate	Merck no. 15552, cleaned with 2-propanol
Solvents	
S1	Methanol + formic acid (99.75 + 0.25 by volume)
S2	Acetonitrile + formic acid (99.75 + 0.25 by volume)
S3	Dichloromethane
S5	Acetonitrile

(b) Gradients used in AMD method

Run number	*Preconditioning*	*S1 (%$_{vol}$)*	*S2 (%$_{vol}$)*	*S3 (%$_{vol}$)*	*S5 (%$_{vol}$)*	*Distance (mm)*	*Dry (min)*
1	No	10	90	0		10.0	2.0
2	No	10	90	0		10.0	2.0
3	No	10	90	0		10.0	2.0
4	No		77	23		13.0	2.0
5	No		63	37		16.0	2.0
6	No		50	50		19.0	2.0
7	No		46	54		22.0	2.0
8	No		43	57		25.0	2.0
9	No		39	61		28.0	2.0
10	No		35	65		31.0	2.0
11	No			66	34	34.0	2.0
12	No			67	33	37.0	2.0
13	No			67	33	40.0	2.0
14	No			68	32	43.0	2.0
15	No			69	31	46.0	2.0
16	No			70	30	49.0	2.0
17	No			71	29	52.0	2.0
18	No			71	29	55.0	2.0
19	No			72	28	58.0	2.0
20	No			73	27	61.0	2.0
21	No			74	26	64.0	2.0
22	No			75	25	67.0	2.0
23	No			76	24	70.0	2.0
24	No			76	24	73.0	2.0
25	No			77	23	76.0	2.0
26	No			78	22	79.0	2.0
27	No			90	10	82.0	2.0
28	No			100	0	85.0	2.0

the study plots. At each farm, study plots with different histories of imidacloprid treatment were selected (Table 2). After the standing crop had been destroyed by glyphosate (360 g $litre^{-1}$; 4 litre ha^{-1}; Glyfos) and subsequent ploughing, all study plots (8 m × 30 m) were drilled with sunflower seed (0.5 U ha^{-1}; 1 U = 150 000 seeds; seed variety: Fleury) on 10th and 12th May 1999 in Höfchen and Laacher Hof, respectively. The control plots and all plots which had received an imidacloprid treatment during 1996–1998 (Table 2) were drilled with imidacloprid-free sunflower seed (treated only with a combined fungicide of carbendazim, metalaxyl and copper oxyquinolate). At each study site one plot which was free of historical imidacloprid treatment was drilled with sunflower seed dressed with the standard combined fungicide and imidacloprid 700 g kg^{-1} WS (150 g AI U^{-1}). The drilling distance was 50 cm between rows and 22.8 cm in-row. Before sowing the drilling machine was adjusted according to the preconditions (eg seed density).

Immediately before sowing, 20 soil cores (5 cm diameter, depth of 30 cm) were taken along the plot diagonals with equal distances between sampling points, and analysed for imidacloprid residues.

Shortly before full sunflower blossom, gauze cages

Table 2. Plot history regarding imidacloprid treatments of field plots drilled with either treated or untreated sunflower seed

Study location	Field	Imdacloprid treatments between 1996 and 1998 ($g\,AI\,ha^{-1}$)	Soil residue level of imidacloprid before drilling of sunflower seed (0–30 cm) ($mg\,kg^{-1}$ dry weight)
Höfchen	Control	None	nd
	Field 1	Sept 98: barley [52].	0.0178
	Field 2	Oct 97: wheat [59], April 99: sprayed [50]	<LOQ[a]
	Field 3	May 99: sunflower [45][b]	nd
Laacher Hof	Control	None	nd
	Field 1	April 96: sugar beet [111], Oct 98: barley [49]	0.0127
	Field 2	April 98: sugar beet [105], Oct 98: wheat [76]	0.0143
	Field 3	Oct 97: wheat [77], April 99: sprayed [50]	0.0157
	Field 4	May 99: sunflower [45][b]	nd

[a] LOQ (Limit of quantitation) = 0.006 mg kg^{-1}.
[b] Drilled after soil analyses.

(10 m × 5 m, 3 m high; 2 mm × 2 mm mesh) were installed on each study plot. One small bee colony (Mr Josef Gilli, Reinartzstrasse 25, D 53925 Kall) with three combs (2000–3000 honeybees) each was placed on the left of the entrance on each study plot between 21st and 26th July 1999. Bee colonies remained in the tunnels till 5th August 1999.

2.3.1 Sampling of foraging honeybees

On days 2 and 3 following hive installment, approximately 100 honeybees were sampled with glass tubes from sunflower heads within the tunnel. Sampled honeybees were killed by placing the sampling glass tubes immediately into a container with dry ice. Samples of honeybees (1 g) were analyzed for residues of imidacloprid or its metabolites.

2.3.2 Sampling of sunflower nectar and pollen

On days 2, 4 and 8 after study initiation, freshly collected sunflower nectar (1 ml) was sampled from the combs of each colony. On day 4, there were also sufficient pollen stores to take a pollen sample (approximately 5 ml). Additional pollen was sampled between day 1 and day 9 by directly shaking pollen out of the sunflower heads.

2.3.3 Sampling of sunflower florets and leaves

Male and female sunflower florets (20 g) and the youngest leaves (100 g) were sampled from the sunflower plants during the peak flowering period.

Immediately after sampling, all samples (ie soil, honeybees, florets, leaves, nectar and pollen) were stored on dry ice in the field. At the end of each sampling day at the latest, samples were transferred into a freezer (−20 °C) where they were retained until residue analysis.

2.3.4 Analysis of soil samples

Soil samples (25 g) were extracted in Soxhlet (Tecator) extraction devices with boiling methanol for 1 h and then placed in rinse position for 30 min until the extraction was terminated. The extracts were evaporated to dryness in a Turbo-Vap evaporator (Zymark) at 50 °C and reconstituted in acetonitrile + water (1 + 1 by volume; 2 ml). The residues were analyzed for imidacloprid by HPLC with a Hewlett Packard 1090 liquid chromatograph, using a LiChrospher 60 RP-Select B (Merck, 5 µm, 125 mm × 4 mm) analytical column. Sodium dihydrogen phosphate dihydrate + water (1 g $litre^{-1}$) was used as solvent A and acetonitrile as solvent B. Sample volume was set at 25 µl, oven temperature at 40 °C and flow rate at 1.5 ml min^{-1}. Detection was at a wavelength of 270 nm. The gradient for the HPLC-UV measurement at times 0, 10, 13, 18, 20 and 30 min, was 10, 25, 90, 90, 10 and 10% of solvent B, respectively. The retention time of imidacloprid was 6.4 minutes. Within each series of analyses the identity of imidacloprid was confirmed additionally by LC-MS-MS (see Section 2.3.5). Therefore, one standard sample (recovery experiment), one control sample and one sample from the trials were analysed for the characteristic mass-to-charge ratio of imidacloprid.

Samples were analyzed in duplicate. The limit of quantification (LOQ) and the limit of detection (LOD) for soil analyses were 0.006 and 0.002 mg kg^{-1} imidacloprid, respectively. For the method of LOQ/LOD determination see Appendix I.

2.3.5 Analysis of biological samples

Biological samples were analyzed for imidacloprid and the structurally related olefin and hydroxy-metabolites. Although not detected in the metabolism studies, the samples were simultaneously checked for these metabolites, since they may have been formed in preceding crops and reached the soil with non-harvested crop material. In addition, the analyzed leafy material had not been examined in the metabolism study but may have contained these metabolites.

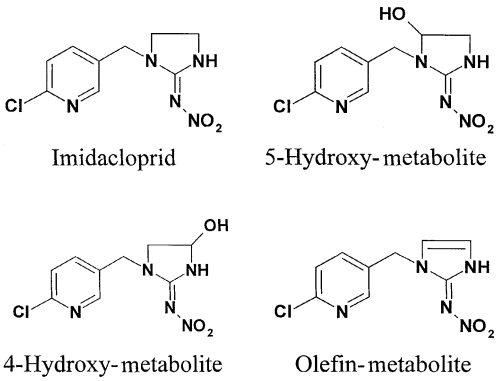

Figure 1. Chemical structures of imidacloprid and structurally related metabolites.

The chemical structures of imidacloprid and these metabolites are given in Fig 1.

For sample preparation, methanol + water (30 ml; 3 + 1 by volume) was added to sample material (2 g), allowed to soak for 30 min and blended for another 1 min in an Ultra-Turrax blender (IKA). The resulting suspension was vacuum filtered and washed with methanol + water (30 ml; 3 + 1 by volume). The residual solvent was squeezed from the solids using a glass rod with a rubber tip. The remaining solids were discarded. One half of the methanol/water extract was concentrated on a rotary evaporator (50 °C) to the aqueous remainder (5–10 ml). Water was added to give a final total volume of 20 ml, placed on a ChemElut® CE 1020 (20 ml volume) column fitted with a disposable stainless steel needle, and allowed to distribute uniformly over the column for 15 min. The residues were eluted from the column with dichloromethane (140 ml), concentrated to dryness on a rotary evaporator (40 °C), re-dissolved in toluene + ethyl acetate (2 ml; 85 + 15 by volume), and poured onto a 0.5-g silica gel column (Varian; flow rate 1 ml min^{-1}). The residues were eluted with acetonitrile (5 ml, flow rate 1 ml min^{-1}), again concentrated to dryness on a rotary evaporator (40 °C), and re-dissolved in acetonitrile + water (1 ml; 2 + 8 by volume). Since the olefin-metabolite is photo-sensitive (*c* 50% degradation per day under natural daylight), the solutions were protected from light and stored in a cool and dark place during the entire procedure. The residues were quantified by HPLC-MS-MS with a Hewlett Packard HP 1100 liquid chromatograph equipped with a Gilson 233 XL injector, using a Luna C18 (2) (Phenomenex, 5 µm, 150 × 4.6 mm) analytical column. Water + acetonitrile + acetic acid (90 + 10 + 0.01 by volume) was used as solvent A and acetonitrile + acetic acid (100 + 0.01 by volume) as solvent B. Sample volume was set at 50 µl, oven temperature at 40 °C and flow rates at 1.0 ml min^{-1} and 0.15 ml min^{-1} on the column and to the MS, respectively. The gradient for the HPLC-MS-MS measurement samples at 0, 10, 10.1, 15, 15.1 and 19 min was 11.1, 11.1, 90.0, 90.0, 11.1 and 11.1% of solvent B, respectively. The retention times were 4.6, 5.5, and 9.1 min for the olefin-metabolite, the hydroxy-metabolites (both forms) and imidacloprid, respectively. The mass spectra were obtained with a triple Quadrupol LC-MS/MS mass spectrometer (Perkin-Elmer Sciex Instruments), fitted with an electrospray interface (Turbo Ion Spray, potential: +4400 V) operated in the positive ion mode under MRM (Multiple Reaction Monitoring Mode).

The mass spectrometer was tuned by infusing a standard solution of imidacloprid (0.5 mg $litre^{-1}$) and its metabolites (dissolved in water + acetonitrile + acetic acid (8 + 2 + 0.01 by volume) at a flow rate of 10–20 µl min^{-1}. Mass axis was calibrated using 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 between 0.8 and 1.0 DA.

Optimized collision-activated dissociation (CAD) conditions for fragmentation of imidacloprid and its metabolites were as follows: gas used, nitrogen (99.999% purity, Nitrogen 5.0, Linde); collision gas, 0.87 litre min^{-1} (collision gas density approximately 1.46×10^{15} atoms cm^{-2}); nebulizer gas, 1.48 litre min^{-1}; curtain gas, 1.44 litre min^{-1}; turbo gas, 6.0 litre min^{-1}; temperature, 400 °C; collision offset, −19 eV (imidacloprid), −21 eV (hydroxy-metabolites), −13 eV (olefin-metabolite).

The mass spectrometer operating parameters are given in Table 3. Samples were analyzed in duplicate. The limits of quantitation in the analyses of the biological samples were 0.005 mg kg^{-1} for imidacloprid and the hydroxy-metabolites and 0.01 mg kg^{-1} for the olefin-metabolite. The limits of detection were 0.0015 mg kg^{-1} for imidacloprid and the hydroxy-metabolites and 0.003 mg kg^{-1} for the olefin-metabolite. For the method of LOQ/LOD determination see Appendix I.

Table 3. Mass spectrometer operating parameters

Compound[a]	*Precursor ion Q1 mass (amu)*	*Product ion Q3 mass (amu)*	*Dwell time (ms)*	*Collision energy (eV)*
Olefin-metabolite (^{37})	256	238	250	−13
Olefin-metabolite (^{35})	254	236	250	−13
Hydroxy-metabolites (^{37})	274	191	250	−21
Hydroxy-metabolites (^{35})	272	191	250	−21
Imidacloprid (^{37})	258	211	500	−19
Imidacloprid (^{35})	256	209	500	−19

[a] The Cl^{37} isotope of all substances was detected to build the isotope ratio.

2.3.6 Observations on honeybees

All behavioural anomalies eg exaggerated motility, discoordinated movements (trembling, shaking, apathy) of the honeybees foraging on the study plots were recorded. In addition, foraging intensity, ie the number of bees foraging on 100 randomly selected sunflower heads was recorded on days 0, 1, 2, 3, 7 and 8 after first exposure. In front of the hive nuclei, linen sheets (60 cm × 50 cm) were placed on the ground to trap the dead bees removed from the beehives. In addition, the numbers of dead honeybees along the cage margins were counted as an indication of whether a higher number of bees tried to leave the tunnel or failed to return to the hive.

2.4 Chronic feeding study to examine concentration-related effects of imidacloprid on developing beehive colonies

Batches (2 kg) of sunflower honey (Honig Müngersdorff, Cologne, Germany) were fortified with 0, 0.002, 0.005, 0.010 and 0.020 mg kg^{-1} of technical imidacloprid (for the rationale of selecting honey as carrier material see Section 4). Before fortification, the sunflower honey was analyzed for background contamination. According to these analyses, the sunflower honey was free of imidacloprid (LOD = 0.0015 mg kg^{-1}) and free of pyrethroids (LOD = 0.01 mg kg^{-1}) and organophosphates (LOQ = 0.02 mg kg^{-1}).

After fortification, five samples (1 g) were taken from each preparation for an analytical verification of the target concentrations and the homogeneity of distribution. Sampling spots were on the left and right sides of the top and bottom position and the centre of the honey surface within the glass containers (1 litre volume, 10 cm filling height). The analytically verified average residue concentrations (±SD) in % of the target concentration were 115.6 (±5.8)%, 110.4 (±5.2)% and 103.5 (±2.0)% for the 0.005, 0.010 and 0.020 mg kg^{-1} preparations, respectively. No detectable residues were found in the control batches and residues below the LOQ but above the LOD were found in the 0.002 mg kg^{-1} preparation.

On 19th and 20th of May 1999, seven 50-m^2 gauze cages (10 m × 5 m, 3 m high; 2 mm × 2 mm mesh) were installed on an oat field (drilled at 150 kg ha^{-1}; cv Jumbo; seed-treated with 150 g dtonne^{-1} Sibutol plus adhesive) in the vicinity of Euskirchen-Billig (Germany), adjacent to the area Billiger Wald. There were no pesticidal treatments of the oat field throughout the study.

Preparation of the hive colonies used for the test started on 27th May 1999. Honeybees were purchased from one large commercially managed beehive (Mr Josef Gilli, Reinartzstrasse 25, D-53925 Kall). In the standard LD_{50} test (see Section 2.2) bees from this apiary showed a sensitivity to imidacloprid which was in the range of the toxicity values given in Table 4. Honeybees sampled from the hive were divided into sub-samples (70 g) which were equivalent to approximately 500 honeybees. Each sub-sample was placed into one of seven multiple-comb-fertilization cages which contained four native comb strips (13 cm × 2 cm), ie only comb matrices. One sister queen in egg-laying mode was added to each of these hive colonies within a separate and closed cage. On the next day, the colonies were allocated to one of the seven tunnel cages, using a random list, and the queen cage disclosed. Two days later (May 30, 1999), the queen cage was removed. At this time, all queens had started to lay eggs in the small hives.

Four colonies were fed throughout the study with the fortified sunflower honey (0.002, 0.005, 0.010 or 0.020 mg imidacloprid kg^{-1}). The two control colonies received untreated sunflower honey. One further group was fed with pieces of honey combs from a small bee colony (about 1500 bees) which had been fed during the previous year over 7 days with sucrose solution fortified with 0.010 mg imidacloprid kg^{-1}. At the study termination, the six honey combs contained in this small colony were removed. From each of these six honey combs, 20 comb cells were impartially selected and the honey taken for residue analysis. The honey combs were then stored at −18 °C until the initiation of the 1999 study. Residue analysis of the collected honey samples, showed that five out of the six honey combs contained imidacloprid residues between 0.005 and 0.008 mg kg^{-1}, and one had residues below the LOQ (0.005 mg kg^{-1}).

The fortified honey samples, or the comb pieces, were offered in an elevated and sheltered glass Petri dish which was positioned on the tunnel end opposite to the entrance. The honey was provided in such portions that about 10% remained until the next portion was offered (each third day). The remaining old portion was removed and reweighed.

The bee colonies were further supplied with pollen from the Mediterranean Bush (*Rosmarinus officinalis* L) which was harvested 2 months before study initiation in Spain (except for control 2 which received freshly collected maize pollen). Before use, the pollen was analyzed for background contamination, which showed that the pollen was free of imidacloprid, pyrethroids and organophosphates (for LOQ/LOD values, see honey analyses, above). The pollen was ground and provided in sub-samples (10–30 g) 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.

The sunflower honey and the ground pollen was stored before use in a refrigerator between +6 and +9 °C.

At each food replenishment event, and finally on day 39, the amounts of honey and pollen collected were 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 reported here represents an underestimate of the total amount of collected pollen.

All anomalies in the development and behaviour of the exposed honeybee colonies were recorded. In front of the colony hives, cotton sheets (60 cm × 50 cm) were spread on the ground. Dead bees were collected from these sheets daily except during weekends. Any dead bee found along the tunnel margins was also sampled and counted. The area within the oat strip was also checked for dead bees but the figure was only recorded if a conspicous difference from the control plots was evident. The increase in the comb cell area was assessed on days 3, 7, 15, 19, 27, 32 and 39 after study initiation. 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 estimated with a ruler (length × width). The amount of stored sunflower honey was assessed in two different ways. The weight increase of the small colonies which reflects mainly the amount of stored honey was recorded on days 5, 11, 15, 21, 28, 34 and 39. In addition to these weight records, the percentage of comb cells filled with honey was estimated on days 3, 7, 15, 19, 27, 32 and 39 along with the percentage of comb cells which contained brood (eggs, larvae or pupae) and the percentage of comb cell area covered by honeybees (colony strength). The percentage values were converted into absolute areas by taking into account the actual comb cell area at the time of evaluation. The number of bees foraging on the honey and pollen feeder during a 5-min observation period was recorded daily, except at weekends, along with the number of honeybees encountered on the tunnel roof. The latter can give an indication of possible disorientation or repellent/antifeedant phenomena. Whenever observed, behavioural anomalies were recorded with the date and time of observation. In particular, honeybees were observed for exaggerated motility, discoordinated movements (trembling, flight incapability), apathy and lethargic behaviour.

3 RESULTS

3.1 Metabolism of imidacloprid in sunflower plants

In total, 3.5 g nectar and 9.6 g pollen were harvested from the sunflower plants (total of 22 plants). The total radioactive residues (TRR) in the samples were very low and almost identical for both rows: 0.0039 (±0.001) mg kg^{-1} (pollen), and 0.0019 (±0.001) mg kg^{-1} (nectar). These low residues were measurable only because a radiolabelled compound was used (limit of detection <0.001 mg kg^{-1}). The major portion of radioactivity in pollen (85.8 (±1.3)% of the TRR, 0.0033 (±0.001) mg kg^{-1}) was extractable with methanol + water (3 + 1 by volume) and methanol while 14.2 (±1.3)% of the TRR (0.0006 (±0.001) mg kg^{-1}) remained non-extractable in the solids. The residues in the nectar (0.0019 (±0.001) mg kg^{-1}) and pollen extract (0.0033 (±0.001) mg kg^{-1}) consisted entirely of imidacloprid and no known or unknown imidacloprid metabolites were detected.

Table 4. Acute toxicity to honeybees (*Apis mellifera*) of imidacloprid as technical grade material (>98%) or as formulated product[a]

	48-h LD_{50} (ng AI per bee)[b]	
Research Facility	*Oral*	*Contact*
A (technical grade)	3.7 (2.6–5.3)	81.0 (55.0–119.0)
B (technical grade)	>21.0	230.3
C (technical grade)	40.9	nt[c]
C (WG 70)	11.6 (7.3–18.3)	242.6 (173.3–353.4)
C (SC 200)	21.2 (15.0–29.6)	59.7 (39.1–92.7)

[a] Tests were performed by three different research facilities (A–C) according to EPPO testing guideline no. 170. LD_{50} values were calculated using probit analysis.[10]
[b] Values in parentheses give the 95% CI (not determined for tests where highest mortality rate was <60%).
[c] nt = not tested.

3.2 Toxicity to honeybees of imidacloprid residues detected in sunflower pollen and nectar

The acute toxicity (LD_{50}, 48 h) of imidacloprid was tested by three different research facilities. The oral LD_{50} was determined to be between 3.7 and 40.9 ng per bee. The contact toxicity in these tests was between 59.7 and 242.6 ng per bee (Table 4).

3.3 Findings of the field residue studies

The soil characteristics of the study plots are given in Table 5. The analytically verified imidacloprid soil residues in the study plots are shown in Table 2. The recovery of imidacloprid at fortification levels of 0.006 and 0.060 mg kg^{-1} in the soil matrix was 93.5 (±1.6)%. The mean coefficient of variation from duplicate samples was 6.2%. During cultivation of the study plots, temperature and precipitation events were continuously recorded by weather stations located adjacent to the study sites (within 3 km distance). During the sampling period, climatic conditions within the tunnels were recorded during each evaluation. Both sets of climatic records are given in Tables 6a and b.

According to the soil analyses and the climatic records, the two field study plots differed significantly in soil characteristics (Table 5), imidacloprid soil residue levels (nd–0.018 mg kg^{-1}; Table 2) and climatic conditions (Table 6). Nevertheless, the results of the two studies were identical. Residue levels of imidacloprid and its structurally related metabolites (hydroxy- and olefin-metabolites) were all below the limits of detection (0.0015 mg kg^{-1} and 0.003 mg kg^{-1} for the parent compound/hydroxy-metabolites and the olefin-metabolite, respectively) in all bee-relevant sunflower parts (nectar, pollen), in the florets and in the exposed honeybees. In these analyses, the specific recoveries of imidacloprid and its metabolites as a

Study location	Soil sampling date	Particle size fractions (%)	pH (KCl)	Soil organic carbon (%)	Soil water holding capacity (g 100g^{-1} dry soil)
Höfchen	8th December 1998	Sand, 7.1 Silt, 83.9 Clay 9.1	6.72	1.95	64.47
Laacher Hof	8th December 1998	Sand, 78.5 Silt, 19.2 Clay 2.3	6.08	0.75	39.08

Table 5. Soil characteristics of the study plots

percentage of the nominal concentrations at a fortification level of 0.005 mg kg^{-1} were as shown in Table 7.

Likewise no residues of imidacloprid or the hydroxy- or olefin-metabolite could be detected in the nectar and pollen of plants raised from imidacloprid-dressed seed. Residues were not even detected in leaves of the sunflowers planted as succeeding crop (only the youngest leaves analyzed). Only in the leaves of plants which were raised from imidacloprid-dressed seed were traces of imidacloprid (0.007 mg kg^{-1}) and of the hydroxy-metabolites (<LOQ; LOQ = 0.005 mg kg^{-1}) detected.

No treatment-related behavioural impacts (eg apathy, exaggerated motility, discoordinated movements), increased mortality or depressed foraging activity were observed on the honeybees used for collecting sunflower nectar and pollen.

3.4 Chronic feeding study to examine the effects of imidacloprid on developing bee hive colonies

Because of the large scale of the feeding experiment, it was technically not possible to replicate the five treatment levels. However, potential adverse effects of the test compound should have been detected by a concentration-related increase of the specific impact.

Climatic conditions were recorded within one control cage with a thermohygrograph. Air tempera-

Table 6. Climatic records during the field residue studies

(a) Cultivation period

Study location	Month	Rain (mm)	Minimum air temperature at 2m (°C)	Maximum air temperature at 2m (°C)	Soil temperature at 0cm (°C)	Energy input (kJ cm^{-2})
Höfchen	April	70.6	0.1–10.9	4.9–20.9	0.1–12.7	38.7
	May	39.5	3.7–15.3	12.5–27.6	9.5–21.6	56.7
	June	80.3	6.8–15.0	13.3–28.1	11.8–19.3	54.5
	July	29.7	11.0–18.4	17.0–30.4	13.8–28.7	60.7
	August	86.6	7.8–18.6	15.9–30.1	12.2–29.9	46.4
Laacher Hof	April	70.9	1.2–12.4	7.4–22.7	3.8–13.4	39.9
	May	49.6	6.3–17.9	14.8–30.4	12.3–25.9	54.5
	June	71.8	9.2–17.2	15.6–30.3	13.0–26.6	52.4
	July	41.1	13.1–20.7	18.8–33.0	16.2–30.0	59.1
	August	77.8	8.6–19.9	18.7–32.4	14.1–28.1	46.0

(b) Sampling period

Study location	Day after first exposure	Rain (mm)	Air temperature (°C)	Soil temperature (°C)	Remarks
Höfchen	0	0	24	32	Clear sky, slightly windy
	1	0	23	32	Clear sky, slightly windy
	2	0	26	28	Clear sky, slightly windy
	3	0	27	39	Clear sky, slightly windy
	7	0	29	37	40% cloudy, calm
	8	0	30	34	40% cloudy, calm
Laacher Hof	0	0	24	32	Clear sky, slightly windy
	1	0	23	32	Clear sky, slightly windy
	2	0	26	28	Clear sky, slightly windy
	3	0	27	39	Clear sky, slightly windy
	7	0	29	37	40% cloudy, calm
	8	0	30	34	40% cloudy, calm

Test substrate	*Imidacloprid (%)*	*Hydroxy-metabolites (%)*	*Olefin-metabolite (%)*
Nectar	104–105	89–104	90–97
Pollen	97–108	86–91	91–94
Honeybees	101	96	99
Florets	89–96	95–96	82–89
Leaves	113	99	100

Table 7. Recoveries of imidacloprid and its metabolites as percentage of nominal concentrations at fortification level of 0.005 mg kg^{-1}

tures during the study fluctuated between 9 and 34 °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 speed was slight to modest throughout the study period.

As shown in Fig 2, activity patterns of foraging honeybees did not differ in relation to treatment. On average, the same number of foraging honeybees were encountered on either the honey or the pollen feeder. There was no higher number of honeybees on the tent roof after exposure to imidacloprid residues. The latter end-point was recorded as an indicator of an anti-feedant response or disorientation.

Figure 3 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 rates. All hives started immediately with the production of new comb cells. No treatment-related difference was found for this testing endpoint either (Fig 4). This shows that residue levels of up to 0.020 mg kg^{-1} imidacloprid in the honey do not influence the wax production of young worker bees.

The amount of the honey stores fluctuated considerably with time and treatment (Fig 5). These fluctuations are most presumably associated with the varying breeding activity of the hive nuclei. However, no dose-response relationship could be established for this end-point, and it is, therefore, concluded that imidacloprid residue concentrations up to 0.020 mg kg^{-1} do not adversely affect the food storage rate. Pollen was not stored within the combs, since it was partly offered directly within the hive nuclei. However, from the breeding performance it is evident that honeybees of all treatment groups collected and fed sufficient pollen to allow an increase of population strength.

A more precise figure for honey storage and comb cell production is derived from the hive weight development. As shown in Fig 6 there was no treatment-related difference in this end-point.

Figure 7 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 7). Mortality was not related to treatment (Fig 8) which demonstrates that the tested imidacloprid residue levels had no impact on honeybee longevity.

The egg-laying cycle of the queen differed between the treatments, but the overall laying activity was broadly comparable (Fig 9). Thus, it can be concluded that the treatment had no influence on the reproductive capacity of the hive nuclei.

The difference in egg-laying cycle is also evident from the abundance of larval and pupal stages in the nuclei combs (Figs 10 and 11). However, the absolute amount of pre-imaginal stages produced up to study termination was broadly comparable between the treatment groups (as expressed by the colony strength development).

4 DISCUSSION

Although imidacloprid is typically degraded in plants

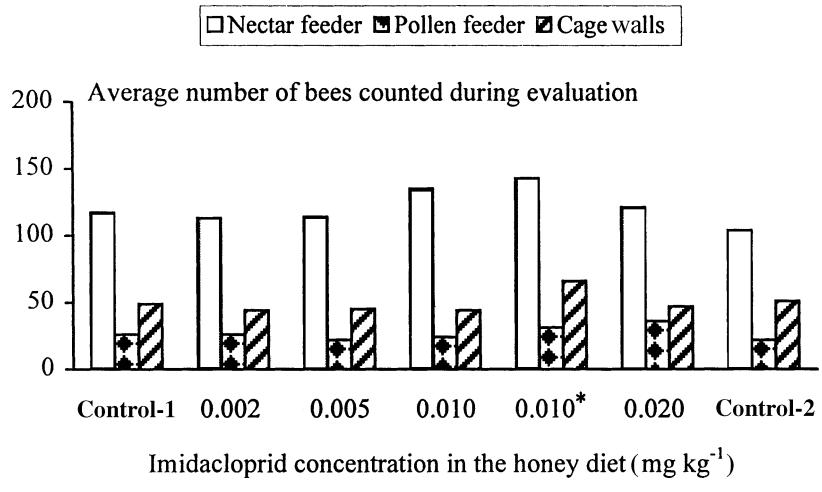

Figure 2. Activity pattern of foraging honey bees in relation to treatment. In a cage study, small bee hives (500 worker bees) were fed with sunflower honey fortified with different concentrations of imidacloprid (* with pieces of honey combs fortified at 0.010 mg kg^{-1}), and uncontaminated pollen from *Rosmarinus officinalis*. Bars give the average number of foraging honeybees which were recorded per day either on the pollen feeder, the honey feeder or at the cage walls.

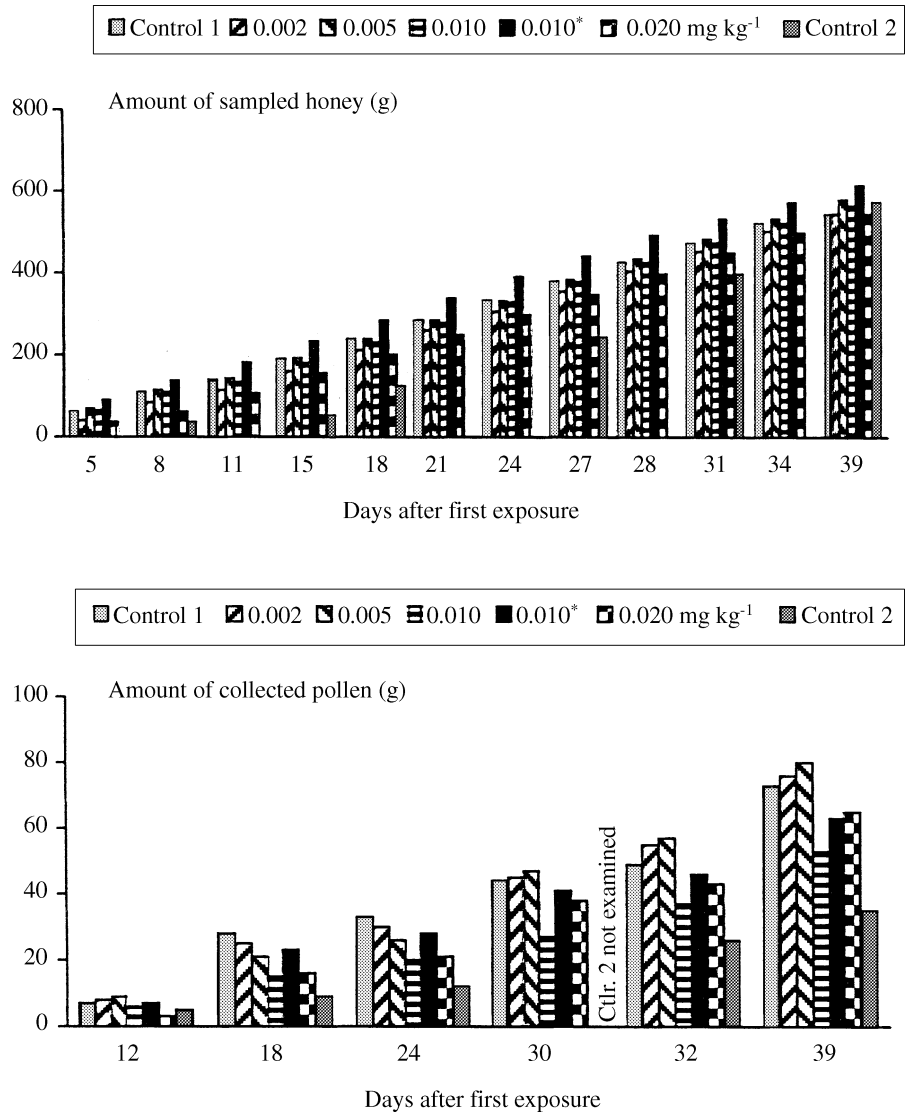

Figure 3. Honey (upper graph) and pollen (lower graph) foraging rate of honeybees in relation to treatment. Study carried out as described in Fig 2. Bars show the cumulative quantity of honey and pollen which was collected by the foraging honeybees. Control 2 was examined with a lower frequency to check for potential handling-related biases.

into various metabolites,[11,12] only the parent compound was detected in the sunflower metabolism study in the bee-relevant part of the sunflower, ie nectar and pollen. Accordingly, if the use of Gaucho® sunflower seed-dressing is involved in the development of the bee malady reported in France, this has to

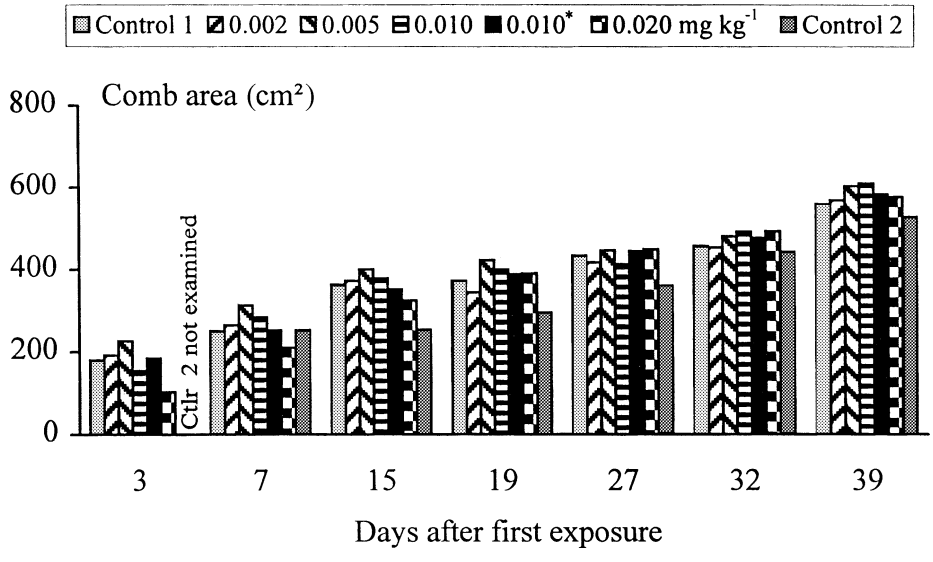

Figure 4. Development of the comb area over time in relation to treatment. Study carried out as described in Fig 2. Bars give the total comb cell area of four combs (cm^2).

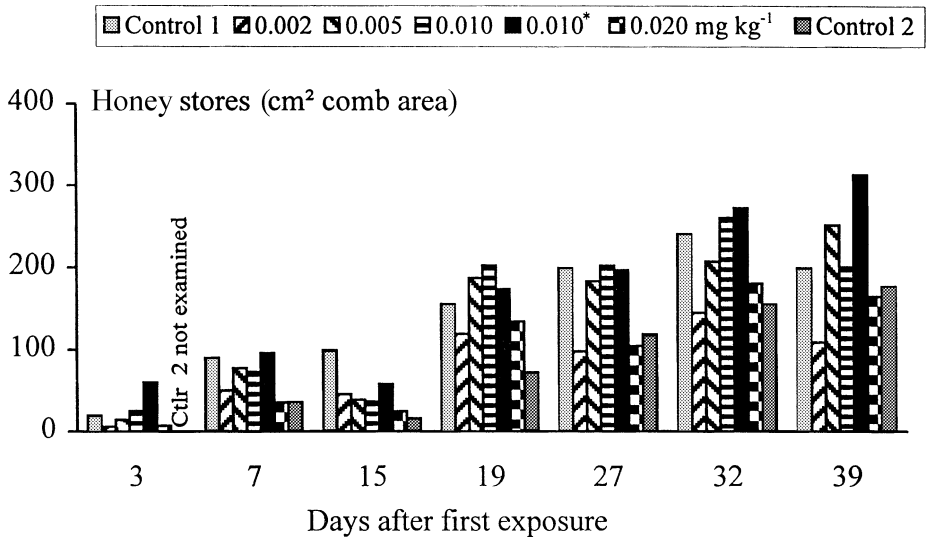

Figure 5. Amount of the honey stores over time in relation to treatment. Study carried out as described in Fig 2. Bars show the size of honey stores as comb area (cm^2) which contained cells filled with honey.

be attributed to imidacloprid, the active ingredient of this product.

In the standard LD_{50} study according to EPPO 170, imidacloprid showed an oral LD_{50} value between 3.7 and 40.9 ng per bee, which is considered to be highly toxic to honeybees.[13] In these tests, the oral doses were diluted in 20 µl sucrose solution per honeybee. From this figure the acute LD_{50} values can be converted into lethal food concentrations using the formula

$$a = [b/(20 \times 1.3)] \times 1000$$

where a = lethal concentration in mg kg^{-1} and b = oral dose in µg per bee. The corresponding lethal food concentrations (LC_{50}) for imidacloprid would be between 0.142 and 1.573 mg kg^{-1}, with a no-observed-effect concentration of 0.046 mg kg^{-1} (lowest NOED = 1.2 ng per bee).

Under field growing conditions, no residues of imidacloprid were detected in either pollen or nectar of Gaucho® seed-dressed sunflowers (limit of detection = 0.0015 mg kg^{-1}). Only in the leaves of seed-dressed plants were residues of imidacloprid and traces of hydroxy-imidacloprid found (only the youngest leaves were analyzed). When planted as a succeeding crop in soils with imidacloprid residue levels up to 0.018 mg kg^{-1}, sunflowers did not build up any detectable residues, either in leaves (only youngest leaves analyzed) or in pollen and nectar. The difference in foliar residues between seed-dressed sunflowers and sunflowers planted as a succeeding crop may be due to the more concentrated soil residues within the plant root zone in the case of seed dressing, or to the lower bioavailability of aged imidacloprid soil residues. The latter assumption is supported by a soil residue extraction with calcium chloride solution which yielded only a fraction of the residue amounts yielded by the standard hot methanol extraction method (Schramel O, unpublished). From these findings it is evident that honeybees are not exposed to residues of imidacloprid or structurally related imidacloprid metabolites when foraging on

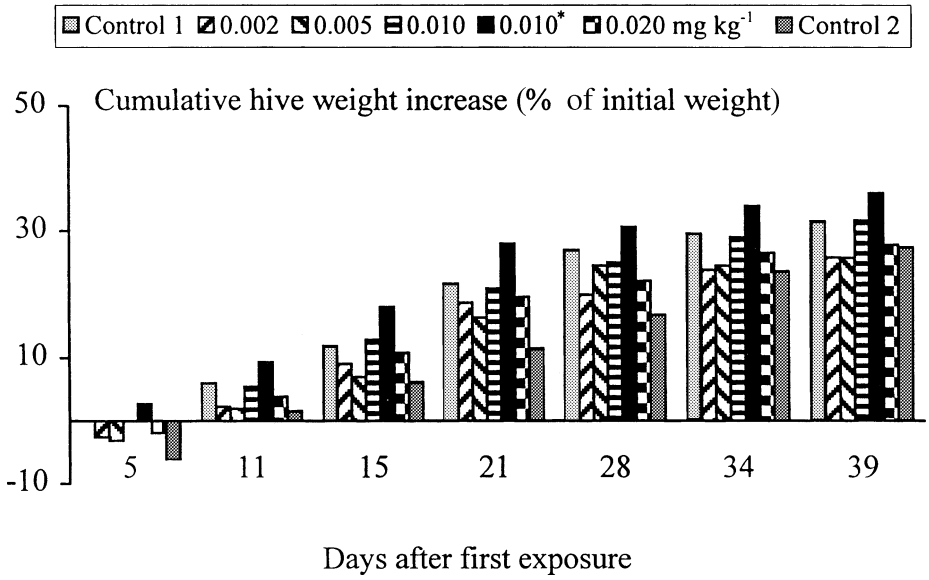

Figure 6. Weight increase of bee hives in relation to treatment. Study carried out as described in Fig 2. Bars show the weight increase as a percentage of the initial hive weight.

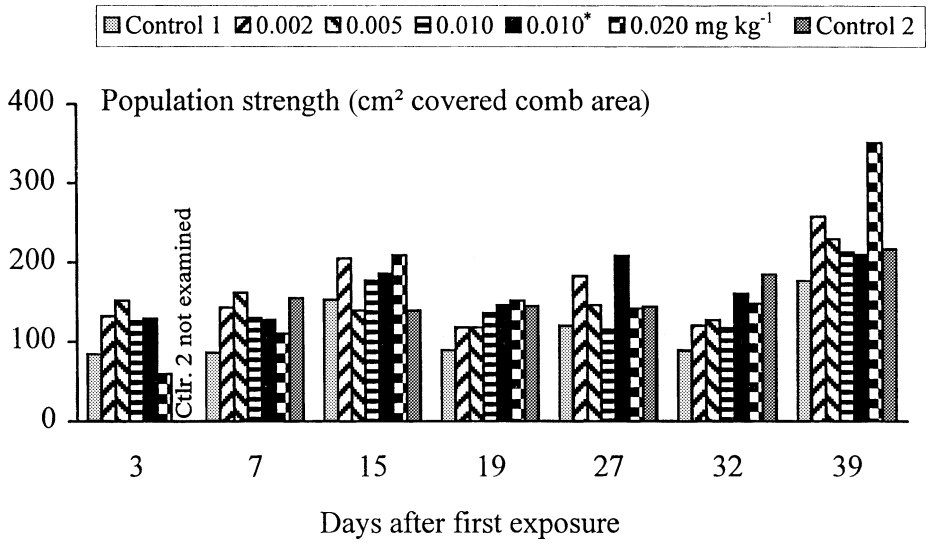

Figure 7. Population development in relation to treatment. Study carried out as described in Fig 2. Bars show the total comb area (four combs) covered by adult honeybees during evaluations, taking into account the increase of the comb area over time.

sunflower plants, irrespective of whether these plants had been cultivated on previously imidacloprid-treated soils or had been raised from imidacloprid-dressed seed.

In earlier studies it was demonstrated that feeding honeybee colonies with sugar solutions fortified with 0.02 mg kg^{-1} imidacloprid over several days had no impact on individual honeybees or honeybee colonies.[8] In the long-term feeding study with small bee colonies reported here, imidacloprid levels of 0.02 mg kg^{-1} in sunflower honey also had no adverse effects on the various testing end-points. Honey was selected as a carrier material since it is a highly attractive food source for foraging honeybees and it is the main energy substrate for the bee brood. Since the transformation of nectar into honey is mainly a process of water evaporation,[14] it would be expected that residues present in the sampled nectar are concentrated during honey formation. From this perspective, it seems more appropriate to feed an aqueous sugar solution instead of honey, since the bee population within the hive could then be exposed to higher final residue concentrations. Surprisingly, however, small colonies which were fed sugar solutions fortified with 0.01 mg kg^{-1} imidacloprid revealed imidacloprid residue concentrations of only 0.008 mg kg^{-1} or less in their honey stores (results of the 1998 feeding study from which pieces of honey combs were taken for this chronic feeding study). Likewise, Wallner *et al*[15] also found no difference in the imidacloprid residue concentration between sampled nectar and stored honey in a cage test where small honey bee hives were exposed to flowering *Phacelia tanacetifolia* Benth raised from imidacloprid-dressed seed. Although the mechanism of residue elimination is not clear, it appears justified from the findings of the 1998 study and of Wallner *et al*[15] to consider fortified sunflower honey as a field-relevant test substrate for examining potential long-term impacts of imidacloprid residues on honeybee populations. The end-points of our chronic feeding study included comb cell production allowing the evaluation of the potential impact of the test compound on the wax gland activity of the exposed honeybees, which starts typically about 13 days after

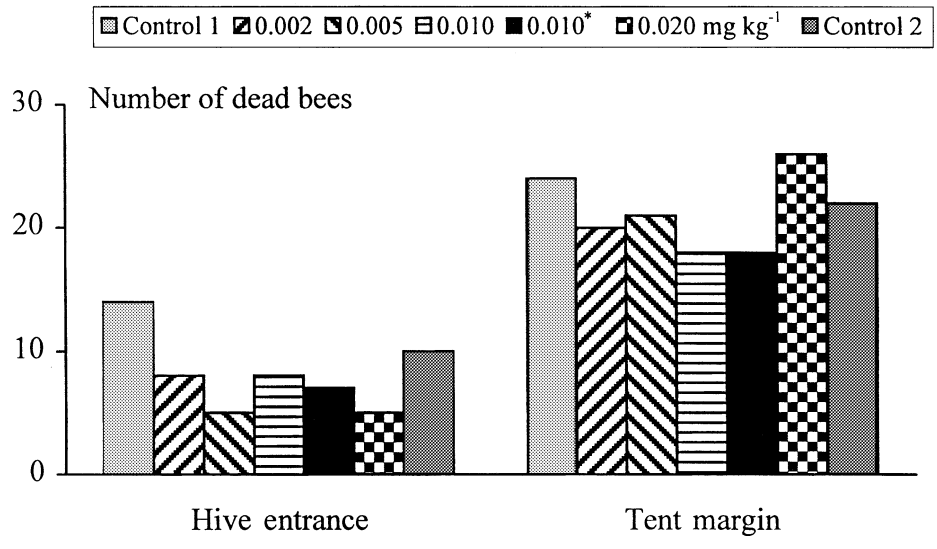

Figure 8. Mortality in relation to treatment. Study carried out as described in Fig 2 bush. Bars give the total number of dead honeybees (worker bees and drones) which were found either in front of the bee hives or at the tent margin.

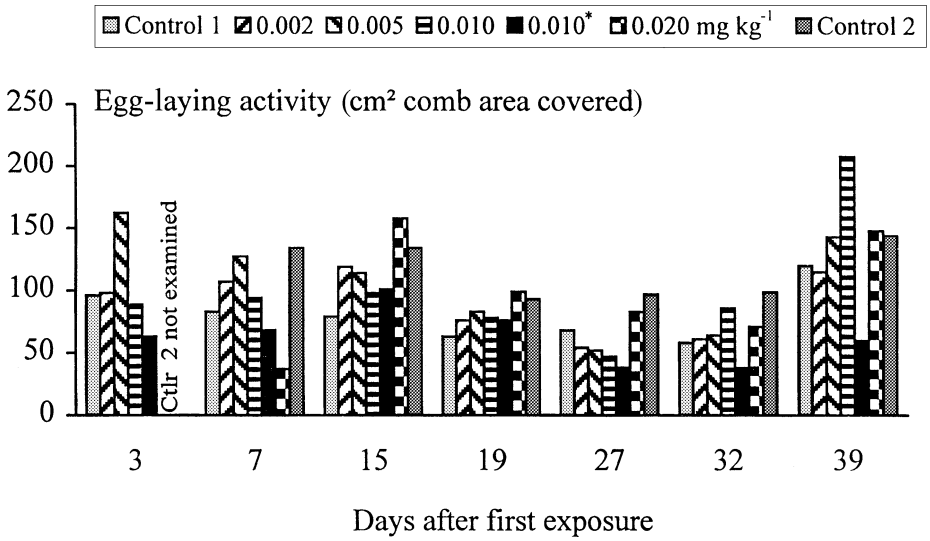

Figure 9. Egg-laying activity of the queens in relation to treatment. Study carried out as described in Fig 2. Bars show the total comb area (four combs) where a egg was seen during evaluations, taking into account the increase of the comb area over time.

ecdysis.[14] Another testing end-point addressed was breeding performance, which allows the evaluation of potential influences of the test compound on the egg-laying activity and the egg-fertilization rate of the queen. In addition, this end-point also addresses potential impacts on the development of the hypopharyngeal glands of young worker bees. The proper functioning of the hypopharyngeal glands of young worker bees is vital for their nursery activity between day 4 and 12 after ecdysis.[14] Finally, the evaluated colony strength integrates potential impacts of the test compound on breeding success, longevity and mortality of honeybees. None of these sensitive end-points were affected by imidacloprid up to a residue concentration of 0.02 mg kg^{-1}. Since residue levels in bee-relevant parts of imidacloprid-treated sunflowers were below the limit of detection (LOD = 0.0015 and 0.003 mg kg^{-1}) it is very unlikely that use of an imidacloprid seed-dressing on sunflowers, or planting of sunflowers in soils which contain traces of residues from previous croppings, will adversely affect bee colonies. This conclusion is supported by the fact that no impacts such as depopulation of hives, immobilized or disorientated bees or increased mortality could be observed in several tunnel and field studies on imidacloprid-treated sunflowers.[8] In more than ten field studies, performed under different climatic and soil conditions and with different sunflower varieties, there were no observations that resembled the symptoms reported by French beekeepers. Identical results came from three tests under tunnels during which no symptoms could be noted in the colonies placed on treated sunflower plots. Moreover, the bee malady in France was still continuing in 1999, although Gaucho® had been suspended for use in sunflowers since 1997. All the facts strongly contradict a causal link between seed dressing of sunflowers with imidacloprid and the reported bee malady.

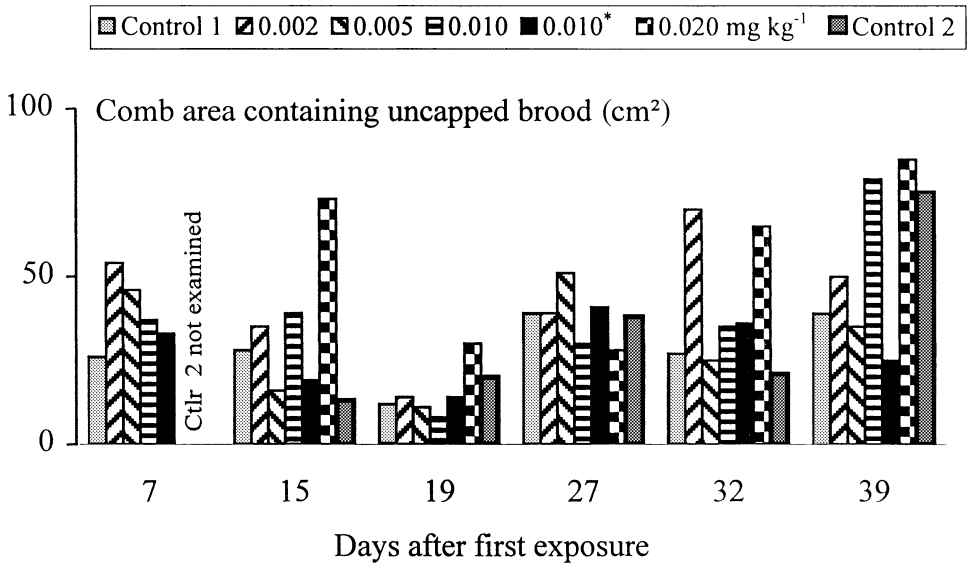

Figure 10. Abundance of honeybee larvae (non-capped brood) in relation to treatment. Study carried out as described in Fig 2. Bars show the total comb area (four combs) where a larva was seen during evaluations, taking into account the increase of the comb area over time.

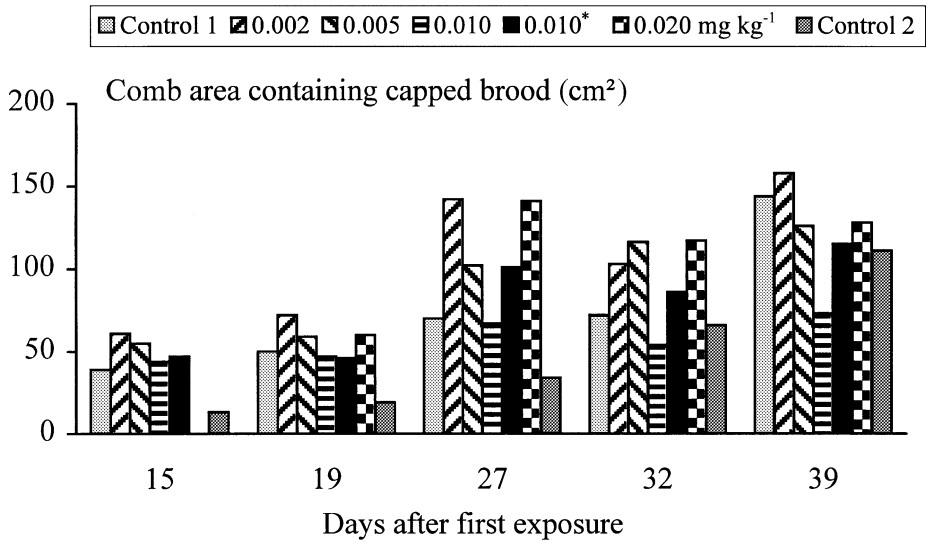

Figure 11. Abundance of honeybee pupae (capped brood) in relation to treatment. Study carried out as described in Fig 2. Bars show the total comb area (four combs) where capped cells were seen during evaluations, taking into account the increase of the comb area over time.

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APPENDIX I

Definition of the Limit of Quantitation (LOQ) and the Limit of Detection (LOD)

Limit of quantitation

The limit of quantitation (LOQ) is matrix-specific and defined as the lowest concentration of a compound in the investigated matrix which is quantifiable by the analytical method. In the present study the lowest fortification level at which an average recovery rate between 70 and 110% and a relative standard deviation of $\leq$20% could be achieved, defined the limit of quantitation. The signal in the control extract must not exceed 30% of the LOQ.

Limit of detection

The limit of detection (LOD) is defined as the lowest concentration of a compound producing a chromatographic peak which significantly differs from the control extract value. In this study, the LOD was set to one-third of the LOQ. The chromatographic peaks at this concentration in the present study exceeded by three times the signal of any background noise from the control extract, ie at the relevant retention times the signal-to-noise ratio was $>$3.

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