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Experimental study on the toxicity of imidacloprid given in syrup to honey bee (*Apis mellifera*) colonies

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Abstract: Two groups of eight honey bee colonies were fed with two different concentrations of imidacloprid in saccharose syrup during summer (each colony was given 1 litre of saccharose syrup containing 0.5 μg litre^{-1} or 5 μg litre^{-1} of imidacloprid on 13 occasions). Their development and survival were followed in parallel with control hives (unfed or fed with saccharose syrup) until the end of the following winter. The parameters followed were: adult bee activity (number of bee entering the hive and pollen carrying activity), adult bee population level, capped brood area, frequency of parasitic and other diseases, mortality, number of frames with brood after wintering and a global score of colonies after wintering. The only parameters linked to feeding with imidacloprid-supplemented saccharose syrup when compared with feeding with non-supplemented syrup were: a statistically non-significant higher activity index of adult bees, a significantly higher frequency of pollen carrying during the feeding period and a larger number of capped brood cells. When imidacloprid was no longer applied, activity and pollen carrying were re-established at a similar level for all groups. Repeated feeding with syrup supplemented with imidacloprid did not provoke any immediate or any delayed mortality before, during or following the next winter, whereas such severe effects are described by several French bee keepers as a consequence of imidacloprid use for seed dressing in neighbouring cultures. In any case, during the whole study, mortality was very low in all groups, with no difference between imidacloprid-fed and control colonies. Further research should now address several hypotheses: the troubles described by bee keepers have causes other than imidacloprid; if such troubles are really due to this insecticide, they may only be observed either when bees consume contaminated pollen, when no other sources of food are available, in the presence of synergic factors (that still need to be identified), with some particular races of bees or when colonies are not strong and healthy.

Keywords: honey bee; *Apis mellifera*; imidacloprid; seed dressing; oral toxicity

1 INTRODUCTION

During the last few years, bee keepers in France have reported more frequently than previously weaknesses of honey bee colonies (*Apis mellifera* L, Hymenoptera: Apidae), and a decrease in honey production related to honey bee population depletions or disappearance.[1] Winter survival of colonies has been jeopardised and, in the most severe situations, there is a complete loss of colonies before winter. Wilson and Menapace[2,3] called this disorder the 'disappearing disease'. Identifying the original cause of this problem is a difficult challenge, as many factors interact with bee population level: environmental conditions including weather and pollutants, sanitary status of the apiary, bee keeper practices such as the use of bee races that may be more or less adapted to local conditions, sufficiency of winter feedings, and appropriateness of the use of toxicants for controlling the development of parasites and pathogens.[4,5] Because population weaknesses do not generally exhibit symptoms of acute intoxication, chronic intoxication may be suspected as the initial cause. Indeed, in 1982 the newly commercialised Decis^{NT}, whose active ingredient is deltamethrin, had been involved in several inquiries on hive weaknesses in the south-west of France.[6] Several pesticides at sub-lethal doses have been shown to be toxic to *A mellifera*: parathion, parathion-methyl, diflubenzuron, carbofuran, malathion and diazinon.[7]

Since 1994 many bee keepers in France have claimed that hive weaknesses of a new type were

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occurring following sunflower honey flow.[8] They have been describing various symptoms ranging from acute to chronic massive mortality that would appear within the few days of the first visits of foragers to sunflowers or during the following winter. In the Vendée département (the administrative unit surrounding the town of La Roche-sur-Yon in the west of France), the phenomenon had started in 1994 and coincided with the use of sunflower seeds coated with the systemic insecticide imidacloprid (Gaucho^{NT}) and increased with the extension of this use since this date. Both imidacloprid and its metabolites have been suspected of being responsible for hive depletion.[9,10] This insecticide, which belongs to the neonicotinoid family, is highly toxic to bees following a bimodal dose-mortality model; an unexpected mortality peak at a very low dose may be explained by the high toxicity of metabolites.[11] Moreover, several behavioural alterations have been described on bees exposed to various imidacloprid concentrations: changes in foraging and food collecting,[12] loss of ability to communicate the location of food sources, ie waggle dance,[13] and learning abilities.[14]

Since imidacloprid is considered to be a systemic insecticide, and notwithstanding its use as a seed coating, it was hypothesised that imidacloprid could migrate into nectar as other systemic insecticides such as thiometon, dimethoate and aldicarb do.[15] In fact, small quantities of this product have been detected in nectar of *Phacelia tanacetifolia* Benth[16] and other plants such as sunflower and canola[17] (see the exhaustive review by Doucet-Personeni *et al*[18]).

As toxicity results obtained on isolated bees in laboratory or in semi-field conditions cannot be directly transposed to the fields,[7] the present study was designed to assess on colonies kept in field conditions the possible toxic effect of imidacloprid exposure through nectar. For mimicking the natural consumption of contaminated nectar by bees, several honey bee colonies were repetitively fed with one of two concentrations of imidacloprid (0.5 and 5 μg litre^{-1}) in saccharose syrup during summer. These concentrations were chosen as covering the range of imidacloprid titres measured in the nectar of sunflower or canola from dressed seeds. Following Doucet-Personeni *et al*,[18] the mean titres of 1.9 μg kg^{-1} given by Stork[19] for sunflower nectar and the range of 0.5–0.85 μg kg^{-1} for canola nectar[20] should be considered valid. These artificially fed bee colonies were then followed in parallel with control hives until the end of the following winter.

2 EXPERIMENTAL METHODS

2.1 General protocol

Several honey bee colonies were fed with one of two different concentrations of imidacloprid in saccharose syrup during summer. Their development and survival were followed in parallel with control hives until the end of the following winter by experimenters who were not informed of which treatment was assigned to each group of colonies (groups were coded). Imidacloprid and one of its derivatives, 6-chloronicotinic acid, were thereafter searched for in bees and honey of all colony groups.

2.2 Apiary

Experiments were conducted on 33 10-frame Langstroth hives equipped with crown board feeders. These colonies were selected as the strongest in our laboratory's experimental apiary. Bees were a small black local race of *A mellifera mellifera*. They were transferred to new foundation combs on the 29 May 2000. The foundation comb wax originated from three batches purchased at Ickowicz (F-84502 Bollene) which were submitted to a multi-residue analysis before use (see Section 2.7). Hives were randomly distributed into four groups (of eight or nine hives) which were all installed at the same site in the premises of the laboratory at Sophia-Antipolis, Alpes-Maritimes, France. To limit drifting as much as possible, the distance between hives of different groups was more than 30 m and all hives of the same group were given the same orientation, with a different orientation (south, south-south-east, south-east and east) for each group. A white cloth was spread in front of the hives to facilitate the observation of sick or dead bees.

An extra colony was added to each group as an 'environmental sentinel' for testing any possible intercurrent source of pollutants in the pollen carried to the hive by bees. For this purpose, these four colonies were prepared in the same way as the 33 above-mentioned ones and were equipped with a pollen trap. They were not transferred on new foundation combs, nor visited or fed as will be described for the 33 other colonies.

2.3 Experimental feeding

In July, when all colonies were well established, one of the following treatments was randomly assigned to each group: no feeding during the experiment (group Gno), feeding with one of the three following syrups: saccharose syrup (50 g of saccharose + 50 ml water) alone (group G0), saccharose syrup with 0.5 μg litre^{-1} imidacloprid (group G0.5), saccharose syrup with 5 μg litre^{-1} imidacloprid (group G5).

The feeding started on the 12 July 2000. Each colony was given 1 litre of syrup precisely measured with a graduated cylinder. Feeding was repeated three times per week until 14 August, making a total of 13 distributions (total volume = 13 litres) of syrup per colony. To avoid robbing, feeders were filled with syrup at dusk after sunset. The period 12 July–14 August will be designated as the feeding period, and groups G0, G0.5 and G5 as the syrup-fed groups.

Imidacloprid was provided as a powder by Cluzeau Info Labo (Sainte-Foy-La-Grande, Gironde, France). From an initial 100 mg imidacloprid litre^{-1} solution in acetone, we prepared two standard solutions at 0.5 and 5 mg litre^{-1} of imidacloprid in acetone that were stored at 4 °C and used for the extemporal preparation

of enriched syrups. These syrups were obtained by mixing, with a magnetic agitator during 5 h, 10 litres of the initial saccharose solution with 10 ml of the solution at 0.5 mg litre^{-1} (to give a syrup containing 0.5 µg litre^{-1} imidacloprid) or 10 ml of the solution at 5 mg litre^{-1} (to give a syrup containing 5 µg litre^{-1} imidacloprid). For protecting imidacloprid from direct sunlight, the whole processing was performed in a dim room and flasks were wrapped in aluminium sheets. For imidacloprid titration, syrup aliquots of the first feeding were frozen either immediately after preparation or after 24 h at ambient temperature under protection from direct sunlight.

In autumn 2000, colonies of group Gno appeared too weak for a comfortable wintering, so they were fed with candy sugar (2.5 kg per colony) on the 22 September 2000.

2.4 Bee activity and mortality

From 12 July to 18 December 2000, bee activity was evaluated by visual counting of bees entering the colony during 1 min. Counting always started at 14.00 hours and was always done on hives successively in the same order to maintain the same time interval between day-to-day observations of the same hives. For the whole apiary, the total counting duration never exceeded 45 min.

Presence or absence of pollen brought back by workers was also observed and coded as '1' or '0' respectively.

Any symptoms observed on bees and the presence and aspect of dead bees at the flight hole were recorded. Dead bees outside entrances were collected with a portable vacuum cleaner. From 13 July 2000 to 1 September 2000, mortalities were assessed daily except for weekends. Dead bees were immediately counted, then frozen and stored at −20 °C until multi-residue, imidacloprid and 6-chloronicotinic acid analyses and disease research tests were performed. From 1 September 2000 onwards, only abnormal mortalities were reported by checking the white cloth placed in front of the hives three times per week.

2.5 Colony weight and honey production

Colonies were weighed early in the morning, before the departure of foragers, using an electronic balance (accuracy = ±0.05 kg). From the beginning, the whole hive was weighted with one honey chamber as a tare until the time when a super was adjusted onto the hives. Weighing was done every week from 12 July 2000 to 2 October 2000. From the latter date, as the bee activity decreased, the frequency of weighing was decreased. The last weighing was done on the 16 February 2001. Honey (from the honey chamber only) was extracted on the 17 August 2000, and imidacloprid and 6-chloronicotinic acid titration was performed (one pool of honey per batch). After honey removal, no tare was used for weighing.

2.6 Visits to colonies

Colonies were visited on 11 and 24 July, 7 and 21 August, 4 September, 25 October 2000 and 21 March 2001. Before opening the hive, the entrance was only lightly smoked so as not to unsettle the cluster, and then, after removing the crown board feeder and the inner cover, the population was rapidly evaluated by counting the number of inter-frames occupied by adult bees. When the hive was equipped with a super, the number of occupied inter-frames was evaluated as 11 plus the number of occupied inter-frames in the super. Brood quality, presence of eggs, queen cells and any specific symptoms of diseases were noted.

During the first six visits, the area of capped brood cells was measured using the graphic software Photoshop® on numeric photographs of honeycombs. Both sides of the honeycombs with capped brood were identified with a label and photographed. A reference area (a coloured pin) was fastened on each frame for scaling. The reproducibility of the method had been previously assessed on other frames (for example, seven independent repeated measures of the brood area of a frame gave a mean area of 76.76 cm^2 with a coefficient of variation of 0.53%).

On the last visit (21 March 2001), frames with capped or non-capped brood cells were counted, but the brood area was not measured. After this final visit, the strength of colonies was qualitatively evaluated as a bee-keeper would do: one of the experimenters (ignoring the meaning of the code given to each colony group) attributed to each colony a score ranging between 0 and 5. This experimenter attributed an initial score of 2, 3, 4 or 5 to colonies with 3 to 4, 5 to 7, 8, and 8 to 9 frames with brood combined with 5 to 8, 5 to 9, 8 to 10 and 11 to 12 inter-frames occupied by adult bees respectively. This initial score was decreased by one if the brood was not compact, and by one again if disease symptoms were observed in adult bees or in the brood.

2.7 Chemical analyses

Multi-residue analyses were performed in the AFSSA Sophia-Antipolis laboratory on foundation wax, dead bees from experimental hives and pollen loads from the extra colonies. Multi-residue analysis was performed by gas chromatography (Autosystem XL, Perkin-Elmer) using an electron-capture detector for organochlorine and synthetic pyrethroids and using a nitrogen–phosphorus detector (NPD) for organophosphorus. The pesticides searched for and their limits of detection are listed in Table 1.

Imidacloprid and 6-chloronicotinic acid analysis in syrup, bees, pollen loads and honey was conducted in another laboratory (GIRPA, Angers, France) using GC/MS/MS respectively. The 6-chloronicotinic acid titration method included a preliminary oxidation of all imidacloprid residues according to Placke and Weber.[21] Therefore, the 6-chloronicotinic acid titres obtained in analysed samples revealed the presence of any imidicaloprid residues (not necessarily only

Table 1. Pesticides detected using the multi-residues method[a]

Organochlorines ($0.001-0.01\,mg\,kg^{-1}$)	Organophosphorus ($0.01-0.3\,mg\,kg^{-1}$)	Synthetic pyrethroïd ($0.005\,mg\,kg^{-1}$)	Others ($0.05-0.1\,mg\,kg^{-1}$)
Dieldrin	Azinphos-ethyl	Cypermethrin	Bromopropylate
α-Endosulfan	Azinphos-methyl	Cyfluthrin	Dicofol
β-Endosulfan	Chlorpyriphos-ethyl	Deltamethrin	Captafol
γ-HCH	Diazinon	Fenvalerate	Captan
Heptachlor	Diethion or ethion	Fluvalinate	Folpet
	Disulfoton	Lambda-cyhalothrin	Methoxychlor
	Fenitrothion	Permethrin	Sulfur
	Fenthion		Tetradifon
	Fonofos		Thionazin
	Malathion		
	Methidathion		
	Parathion-ethyl		
	Parathion-methyl		
	Phosalone		

[a] Detection limits are given in brackets.

6-chloronicotinic acid). The limits of quantification (LQ) were 8 and $5\,\mu g\,kg^{-1}$ for imidacloprid and 6-chloronicotinic acid respectively and the limits of detection (LD) approximated to 3 and $1\,\mu g\,kg^{-1}$. Imidacloprid analysis in the syrup was also performed by LC/MS/MS in AFSSA Maisons-Alfort (France) ($LQ = 0.25\,\mu g\,kg^{-1}$; $LD = 0.08\,\mu g\,kg^{-1}$). Except for two samples of syrup sent to the AFSSA Maisons-Alfort laboratory (one sample of syrup without imidacloprid and one sample of the syrup with the expected titre of $5\,\mu g$ imidacloprid $litre^{-1}$) and pollen loads, all samples of syrup, bees and honey had been coded before been sent for analysis.

2.8 Pathologies

Surveyed pathologies were acarapisosis, nosemosis, varroosis, American foulbrood, European foulbrood, chalkbrood and chronic bee paralysis (CBPV). Acarapisosis and nosemosis were diagnosed according to approved OIE methods.[22,23] According to the number of spores observed in the microscope field, nosemosis was coded as nul (no spores), light (1 to 9 spores), weak (10 to 49 spores), medium (50 to 100 spores) or heavy (more than 100 spores). Clinical and bacterioscopic techniques were used for detecting chalkbrood and foulbrood diseases.[24] SBV was diagnosed by agarose gel immunodiffusion (AGID).[25] Unapparent infection of adult bees by CPBV was revealed by RT-PCR, and the symptomatic form by both AGID and RT-PCR.[26,27] Both tests were systematically performed on bees sampled inside all colonies on the 11 July and on the 25 October 2000 and on bees sampled at the hole entrance from all hives on the 21 July 2000. When symptoms of chronic paralysis were suspected during the experimentation, adult bees were sampled for analysis by AGID. When found on the white cloth placed in front of the hives, sick or dead bees were considered to belong to the corresponding group of hives, not to a precise hive, contrary to bees collected inside or at the entrance of hives.

Varroa destructor Anderson & Trueman (Acari: Varroaidae) was preventively controlled using amitraz (Apivar®) from 21 March to 29 May 2000. Following the observation of bees with deformed wings in front of several hives, the same treatment was repeated from 22 August to 6 November 2000. Varroas were collected in all groups, every 1, 2 or 3 days during the three-week period that followed the amitraz treatment of 22 August. Coumaphos (Perizin®) was applied on the 7 November 2000 as the last treatment before winter.

Fumagillin (Fumidil B®), an antibiotic recommended for controlling *Nosema apis* Zand (Microsporidia) infection, was applied on the 3, 9 and 22 January 2001.

All these treatments were carried out on the whole apiary, including the four extra hives.

2.9 Meteorology and environment

Temperature, rainfalls and relative humidity were obtained from the neighbouring meteorological stations of Valbonne and Nice airport. The weather was typically that of a Mediterranean climate with a dry hot summer and a cool and wet winter. From May to August 2000 monthly mean temperatures ranged from 19.00 to 24.15 °C and rainfall was very scarce (mean: 2.2 mm per month). Sunshine was maximum during July (mean: 11.21 h per day). Temperature began to decrease in September 2000: the lowest mean temperature during the period was 9 °C in January 2001. Monthly maximum mean rainfall (13.42 mm) occurred in November 2000. Mean temperature and insulation started to increase again in February 2001.

The apiary was closely surrounded by typical Mediterranean forest (*Quercus suber* L and *Q ilex* L) and meadows. Therefore, in addition to Mediterranean oaks, sources of pollen, nectar and honeydew were from a large variety of wild plants such as *Dorycnium pentaphyllum* Scop, sages (*Salvia* sp), thyme (*Thymus* sp), rosemary (*Rosmarinus officinalis* L), heather (*Erica arborea* L and *E* sp), arbutus (*Arbutus unedo* L), *Inula viscosa* Aiton and *Solidago* sp.

2.10 Statistical analysis

Means of continuous variables for which we could make the assumption that they came from normal distributions were compared between groups according to the one-way analysis of variance method with the appropriate module of JMP® software (SAS Institute Inc, Cary, NC, USA 27513). This was the case for the measurement of the capped brood area and the weight of hives. Following such analyses, multiple comparisons of group means were performed on all pairs of means using the Tukey–Kramer honestly significant difference test[28] included in the same software. As those parameters were measured repetitively on the same colonies, the more accurate method was to perform the analyses on the individual variation of this parameter between the beginning and the time of measurement.

Crude activity data have been transformed into logarithms for normalisation and then compared between groups using the multivariate model with repeated measures module of the same JMP software.

In most cases, the nature of the data did not allow any assumption to be made on their underlying distribution, and non-parametric methods had to be applied. Results were organised into contingency tables and interpreted with the help of the StatXact® statistical package,[29] (CYTEL Software Corporation, Cambridge, MA 02139, USA) which provides for many non-parametric tests an exact calculation of *P*-values based on computational permutations of contingency tables. According to the characteristics of the data, these tests were:

- the Fisher exact test[30] applicable to 2×2 contingency tables. We used this test for comparing the frequency of pollen carrying in each group during and after the feeding period
- the Pearson's χ^2 test for independence[30] was used for comparing frequency between all groups when responses and treatments had not to be considered in any intensity order. This was the case for comparing the frequency of swarming or diseases between groups
- the Kruskal–Wallis test[31] applicable to tables in which responses (but not the treatments) are naturally ordered. This test was used when assessing the contingency within all groups of responses such as the number of days when bees were seen carrying pollen into the hive, the number of inter-frames occupied by bees, the number of frames occupied by capped brood, the strength scores attributed to hives
- the Jonckheere–Terpstra test,[32] applicable to doubly ordered tables, was used for assessing the contingency between progressively increasing dose of imidacloprid in syrup and increasing response. Therefore, this test was applied for comparing the same types of response as for the previous test but when syrup-fed groups only were considered during and after feeding periods (not before)
- the Cochran–Armitage trend test[33] applicable for testing any trend in the response rate when comparing several binomial populations. We used this test for assessing the possibility that bees of groups fed with the highest imidacloprid doses were or were not more (or less) susceptible to becoming infected by CPBV

For all tests, the precise *P*-value has been given and for homogeneity, indicated with the same symbol (α). Following the common use, we considered as statistically significant any result with α equal to or less than 0.05.

3 RESULTS

3.1 Collection of syrup by bees

Every day following feeding, the apiary was inspected: no robbing occurred, which indirectly indicated that no feeder had leaked. Some feeders were directly observed and bees were seen collecting the syrup. When replenishing the feeders, we always observed that in the interval all had been emptied and several bees were still cleaning the place.

3.2 Egg laying

On the 11 July 2000 (ie on the day before the first experimental feeding), a queen of group Gno was accidentally killed. As all the hives present at the same site were included in this experiment, no other queen was available for an immediate replacement. Another hive of the same group and two hives of group G0.5 were found without eggs, a probable consequence of unobserved recent swarmings. During the two following visits which were carried out during the feeding period, no eggs were found in the first hive (logically), but eggs were observed in the three other above-mentioned hives. However, we observed that another hive of group Gno and three hives of group G0 had swarmed. After the feeding period, another colony of group Gno was transitorily found without eggs and one of the above-mentioned colonies of group G0.5 swarmed in September. After wintering (21 March 2001), all hives in all groups were found with eggs, whereas the queen of one colony of group G0 became a drone-layer.

As a summary, during and after the feeding period, the number of colonies that surely or probably swarmed was 2, 3, 1 and 0 in groups Gno, G0, G0.5 and G5, respectively. There is no statistically significant difference in this frequency between groups (calculated $\chi^2 = 3.6$ with 3 degrees of freedom (*df*): $\alpha = 0.45$).

3.3 Activity

The mean activity index during the study is described by Fig 1a. At the beginning of the experiment, the activity index of colonies ranged between 5 and 55 bees entering the hive per minute (mean = 27 bees per minute), with no statistically significant difference between groups ($F = 2.16$ with 3 and 29 *df*: $\alpha = 0.11$).

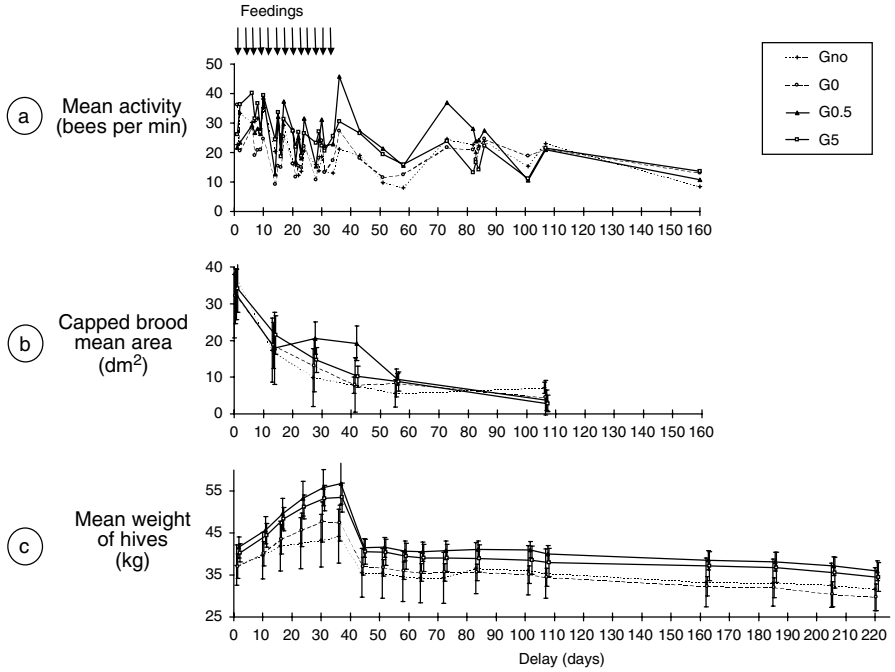

Figure 1. Mean activity, mean size of the capped brood area and mean weight of hives of group Gno, G0, G0.5 and G5 during and after the feeding period.

This index decreased in all groups reaching a mean of 20 bees per minute at the end of the feeding period (ie 34 days later). At this time, the mean activity indexes per group were significantly heterogeneous ($F = 2.94$ with 3 and 29 *df* at the 0.05 level). However, when excluding the colonies that swarmed during the period, differences between groups is no longer significant: $F = 2.79$ with 3 and 25 *df*: $\alpha = 0.06$. The study of the feeding period as a whole, using multivariate model on repeated measures, confirmed (a) a significant overall decrease in activity index for all groups (time effect: $F = 9.8$ with 20 and 10 *df*: $\alpha = 0.0004$), and (b) no significant heterogeneity between groups when excluding the colonies that swarmed during the period (group effect: $F = 2.0$ with 3 and 25 *df*: $\alpha = 0.14$).

After the feeding period, the activity index varied simultaneously for all groups: a sharp increase in mid-August, then a decrease leading to a minimum in September and a new increase in late September. The general trend of the period was a decrease. At the end of the experiment (early December), no difference between groups was observed: the mean index was 11.5 bees per minute. Using the multivariate model on repeated measures for studying the general trend during the post-feeding period confirmed (a) the persistence of the decrease in activity index for all groups (time effect: $F = 9.47$ with 11 and 19 *df*: $\alpha < 0.0001$), and (b) no significant difference in activity index between groups (group effect: $F_{G0/G0.5/G5} = 0.8$ with 2 and 22 *df*: $\alpha = 0.46$; $F_{G0/Gno} = 0.04$ with 1 and 15 *df*: $\alpha = 0.84$).

3.4 Pollen carrying

The frequency of pollen carrying by bees is summarised in Fig 2. During the feeding period, the number of days when bees were seen carrying pollen loads to the hive ranged between 5 and 21 (mean: 15.5 days out of 21 days of observation) with means of 14.0, 14.3, 16.0 and 18.0 days for groups Gno, G0, G0.5 and G5, respectively. When considering all groups, the differences do not appear significant (Kruskal–Wallis test: $\alpha = 0.10$), but when considering syrup-fed groups, the higher the imidacloprid concentration in syrup, the more frequent were the days when bees were seen carrying pollen (Jonckheere–Terpstra test: $\alpha = 0.03$).

After the feeding period, the number of days when bees were seen carrying pollen loads to the hive ranged between 8 and 12 (mean: 10.7 days out of 12 days of observation) with means of 10.0, 10.8, 10.8 and 11.4 days for groups Gno, G0, G0.5 and G5, respectively. These differences are not significant (all groups, Kruskal–Wallis test: $\alpha = 0.11$; syrup-fed groups, Jonckheere–Terpstra test: $\alpha = 0.24$).

From the first to the second period, the frequency of pollen carrying increased significantly in all groups: from 67 to 87%, 68 to 90%, 76 to 90% and 86 to 95% for groups Gno, G0, G0.5 and G5, respectively (the Fisher test gives the following probabilities: 0.00, 0.00, 0.01 and 0.02, respectively).

3.5 Adult bee population

The distribution of colonies according to the number of inter-frames occupied by bees is given by Fig 3. At the beginning of the experiment, hives had 6–11 occupied inter-frames (mean = 9.8) with no significant difference between groups (Kruskal–Wallis test: $\alpha = 0.18$). Until August, adult bee population of colonies increased to a mean number 11.8 occupied inter-frames. Then, the population decreased to the

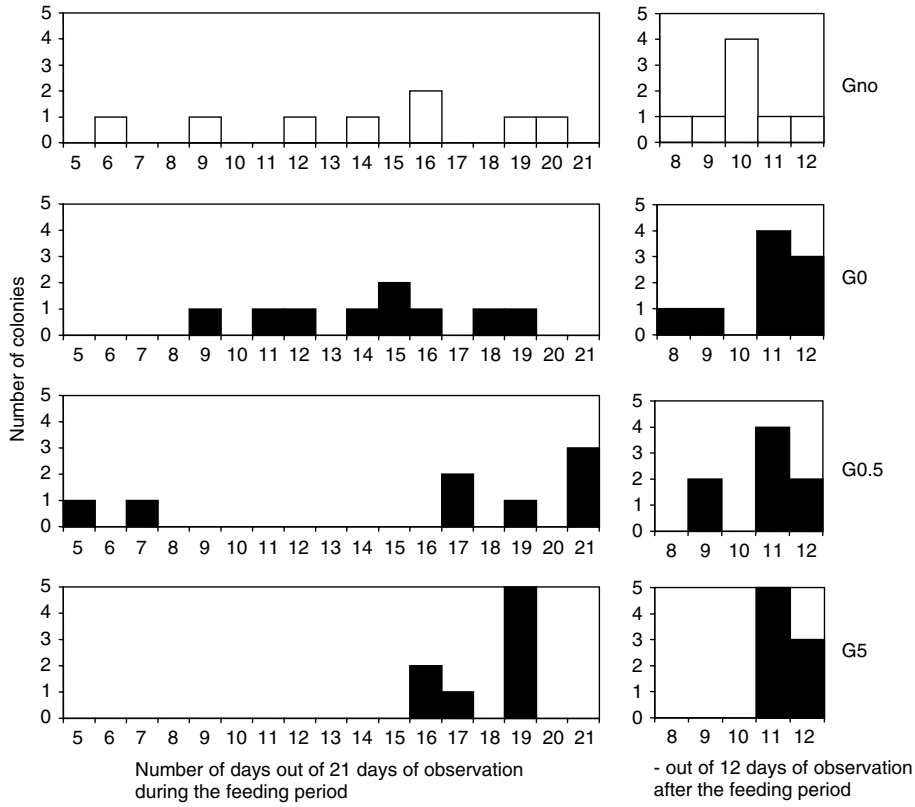

Figure 2. Number of bee colonies whose at least one forager was seen carrying pollen load to its hive during a given number of days during the period.

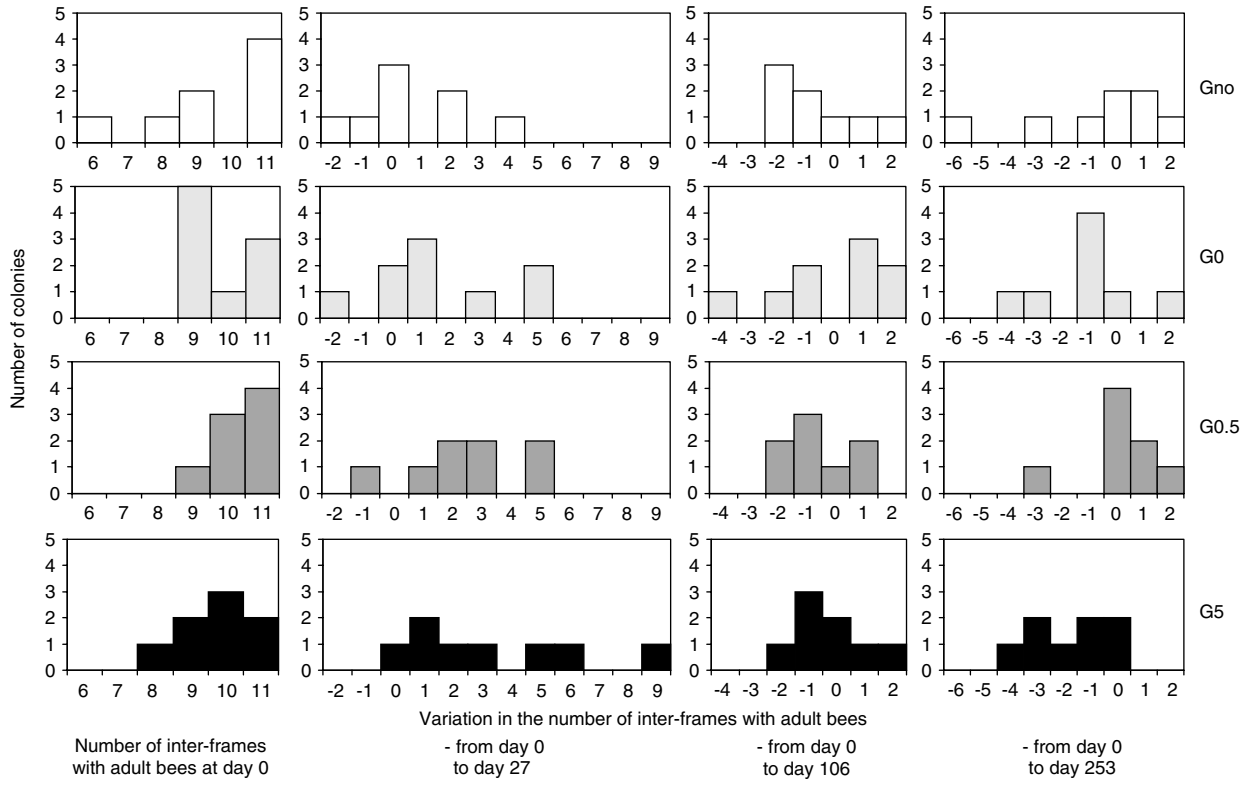

Figure 3. Number of colonies with a given number of inter-frames occupied by adult bees in the experimental groups Gno, G0, G0.5 and G5.

value of 9.5 occupied inter-frames in late October 2000. The mean variation in the number of occupied inter-frames was +0.6, +1.6, +2.5 and +3.4 from the beginning to the end of the feeding period, and −0.6, −0.1, −0.6 and −0.25 from the beginning of the experiment to late October for groups Gno, G0,

G0.5 and G5, respectively. Whatever time interval is considered (from the beginning of the experiment to August or to late October), no difference between groups is statistically significant (comparisons between all groups, Kruskal–Wallis test: $\alpha > 0.23$ and 0.87 respectively for both periods; for syrup fed groups only, Jonckheere–Terpstra test: $\alpha = 0.09$ and 0.35).

After wintering (21 March 2001), the mean number of occupied inter-frames was 8.9 with no difference between groups (comparisons between all groups, Kruskal–Wallis test: $\alpha > 0.69$; for syrup fed groups only, Jonckheere–Terpstra test: $\alpha = 0.41$). This is consistent with the fact that the observed variations of occupied inter-frames from the beginning of the experiment to the next spring (equal to −0.75, −1.1, +0.125 and −1.75 for groups Gno, G0, G0.5 and G5 respectively) are not statistically different (comparisons between all groups, Kruskal–Wallis test: $\alpha > 0.62$; for syrup fed groups only, Jonckheere–Terpstra test: $\alpha = 0.29$).

3.6 Brood

Mean capped brood area variation during the study is described for all groups by Fig 1b. At the beginning of the experiment, the mean areas were 38.0, 32.1, 32.5 and 34.1 dm^2 for groups Gno, G0, G0.5 and G5, respectively, with no significant difference between groups ($F = 0.37$ with 3 and 29 *df*: $\alpha = 0.78$). During the feeding period, the mean area of the capped brood diminished by 28.1, 19.0, 11.9 and 19.4 dm^2 (day 27). Then 21 days after the end of the feeding period (day 55), these means had again decreased by 4.3, 4.7, 11.3 and 5.9 dm^2, respectively, for the same group. Whether we consider the interval from the beginning of the experiment until day 27 or until day 55, the variation in the area of the capped brood was never significantly different between groups ($F = 1.66$ with 3 and 29 *df*: $\alpha = 0.20$ and $F = 1.52$ with 3 and 29 *df*: $\alpha = 0.23$ respectively). The same result is obtained when tests are performed at intermediate delays (day 13 and day 41).

However, from August to October (ie from day 27 to day 106), the overall mean decrease of the capped brood area became statistically different between groups: 2.8, 8.7, 16.9 and 11.9 dm^2 ($F = 5.21$ with 3 and 29 *df*: $\alpha = 0.005$). The Tukey–Kramer test allows us to state that group Gno experienced the smallest decrease in capped brood area and G0.5 the largest, but this test does not allow us to differentiate groups G0 and G5 from the others.

An overall decrease of capped brood area was observed during the whole season (ie from day 0 to day 106): the capped brood area ranged between 10.5 and 79.8 dm^2 in July 2000 (mean: 34.1 dm^2) then ranging between 0 and 19.8 dm^2 in late October (mean: 4.5 dm^2).

After wintering (21 March 2001), the number of frames with brood ranged between 3 and 9, with mean values of 6.6, 7.0, 8.0 and 4.6 for groups Gno, G0, G0.5 and G5, respectively. Whereas the Kruskal–Wallis test does not confirm any significant difference when all groups are considered ($\alpha = 0.33$), the Jonckheere–Terpstra test shows a significant heterogeneity between the syrup-fed groups ($\alpha = 0.02$), with more frames with brood in G0.5 and less in G5 than in G0 ($\alpha = 0.03$ and $\alpha = 0.004$ respectively).

3.7 Colony weight

The mean hive weight for all groups during the study is described by Fig 1c. At the beginning of the experiment, the mean weights were 37.3, 37.1, 41.6 and 40.2 kg for groups Gno, G0, G0.5 and G5, respectively, with no significant difference between groups ($F = 2.53$ with 3 and 29 *df*: $\alpha = 0.08$). The weights of all hives increased steadily from the 12 July to 16 August 2000, when honey was harvested. The individual hive increase in weight ranged from 2.45 to 22.8 kg (mean: 11.38 kg). The mean increases per group were 6.89, 10.31, 15.21 and 13.25 kg for groups Gno, G0, G0.5 and G5, respectively. Heterogeneity between all groups was statistically significant ($F = 5.32$ with 3 and 24 *df*: $\alpha = 0.006$), whereas when comparing syrup-fed groups only, there was no longer any statistically significant heterogeneity between groups ($F = 0.97$ with 2 and 18 *df*: $\alpha = 0.40$).

As the heavier colonies logically experienced the highest weight gain (correlation is significant: weight increase = 0.45 × initial weight − 6.2; $F = 6.3$ with 1 and 31 *df*: $\alpha = 0.02$), we had to assess whether the interaction between the initial weight and group was non-significant in both previous analyses. This was confirmed: $F = 0.076$ with 3 and 24 *df*: $\alpha = 0.97$; and $F = 0.067$ with 2 and 18 *df*: $\alpha = 0.94$. In other words, the final heterogeneity in mean weight gain between groups did not originate from any bias related to the initial weight of hives: hives were randomly distributed between groups according to this parameter.

On the 16 August, the weight of the honey body ranged between 7.6 and 23.6 kg (mean: 12.1 kg). The mean weights of the honey bodies were 8.7, 10.9, 16.1 and 12.8 kg for groups Gno, G0, G0.5 and G5 respectively. Heterogeneity between all the four groups was statistically significant ($F = 3.59$ with 3 and 29 *df*: $\alpha = 0.025$), whereas there was no statistically significant heterogeneity between syrup-fed groups ($F = 2.12$ with 2 and 22 *df*: $\alpha = 0.14$).

From the 16 August 2000 until the end of October, the mean weight of hives decreased only slightly (mean variation: −0.67 kg), with a large individual variation (range: −4.4 and +3.2 kg). From late October, the weight decreased more markedly. Eventually, from 16 August 2000 until 2 February 2001, the individual hive weight variation ranged from −10.45 to −0.55 kg (mean: −5.33 kg). This overall decrease is highly significant statistically ($F = 5.5$ with 11 and 14 *df*: $\alpha < 0.002$). The mean variation per group was −3.96, −6.61, −4.63 and −6.14 kg for groups Gno, G0, G0.5 and G5, respectively, with no statistically significant difference between groups ($F = 2.98$ with 3 and 24 *df*: $\alpha = 0.052$).

3.8 Diseases and parasites

At the beginning of the experiment, *N apis* infestation levels were not evenly distributed between groups (Kruskal–Wallis test: $\alpha = 0.04$) (Fig 4). Leaving out the group Gno (all of whose colonies were *Nosema* free), infestation levels were not different in the other groups (Kruskal–Wallis test: $\alpha = 0.62$). After the feeding period, four colonies of group Gno were infested, and among the syrup-fed groups, colonies of group G0.5 were the less frequently and the less severely infested (Kruskal–Wallis test: $\alpha = 0.04$).

Results of RT-PCR specific to CBPV are summarised in Table 2. At the beginning of the experiment the virus was latent in eight (all), four, four and five colonies of groups Gno, G0, G0.5 and G5, respectively (Calculated $\chi^2 = 6.7$ with 3 *df*: $\alpha = 0.10$). On the 25 October (ie four months after the feeding period), one colony of group Gno had apparently become CBPV free. Among the syrup-fed groups, this event occurred for one and two colonies of groups G0.5 and G5 respectively, while five, three, and one colonies that were RT-PCR negative at day 0 were found positive on the 25 October (groups G0, G0.5 and G5 respectively). This apparent trend (ie groups fed with syrup with the higher imidacloprid concentration would had been less prone to become latently CPBV infected) is not statistically significant (Cochran–Armitage test: $\alpha = 0.35$).

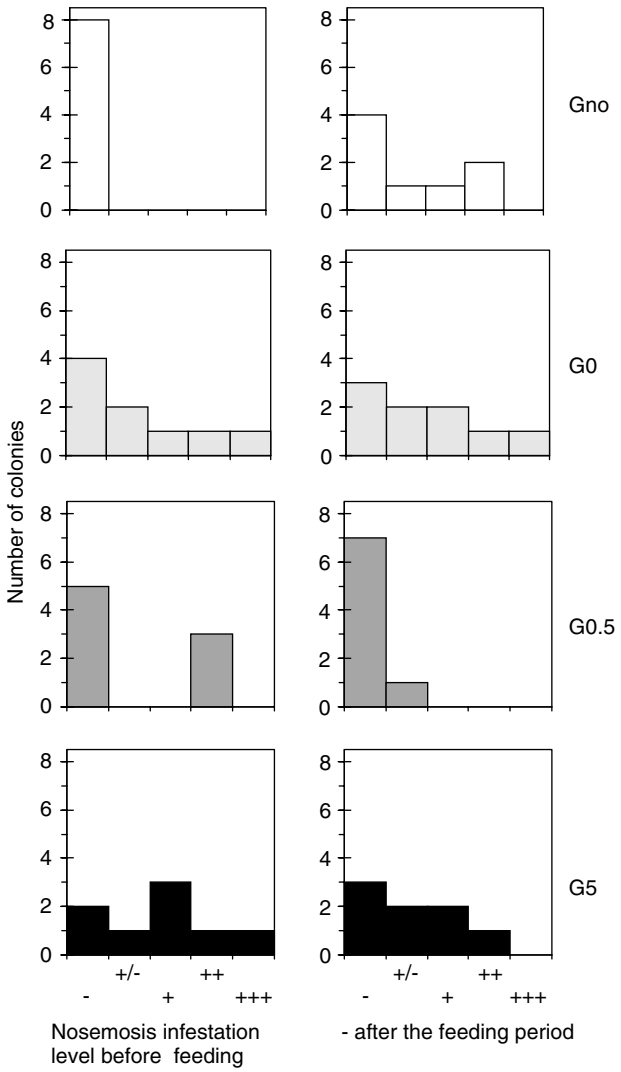

Figure 4. Number of colonies with a given level of infestation by *Nosema apis* in the experimental groups Gno, G0, G0.5 and G5.

Table 2. Result of the RT-PCR specific to chronic bee paralysis virus (number of bee colonies in groups Gno, G0, G0.5 and G5)

	Tests performed on 25 October 2000							
Tests performed on 11 July 2000 that were	Gno		G0		G0.5		G5	
	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos
Negative	0	0	0	5	1	3	2	1
Positive	1	7	0	4	1	3	2	3

Symptoms of chronic paralysis (dead or trembling bees on the white cloth or at the entrance) were observed in all groups and their aetiology was confirmed by AGID.

Despite the application of amitraz in spring, typical symptoms of *Varroa* infestation (bees with atrophied wings) were seen in front of colonies of group Gno, G0.5 and G5 during the feeding period. The same symptoms were seen again in front of hives of group G0 and G5 after this period. The total number of varroas collected after the feeding period (Fig 5) ranged between 3 and 1244 per colony with a geometric mean of 51.4. No significant difference was observed between groups ($F = 1.64$ with 3 and 29 *df*: $\alpha = 0.20$).

3.9 Mortality

Mortality was low during the whole experiment: the highest mortality was observed on the 20 July with 38 dead bees per colony in group G5. The total number of dead bees per group collected in front of the hives amounted to 791, 1268, 1101 and 855 during the feeding period, then 137, 231, 229 and 31 during the 16 days that followed the feeding period for groups Gno, G0, G0.5 and G5, respectively. For the same groups and periods respectively, these numbers

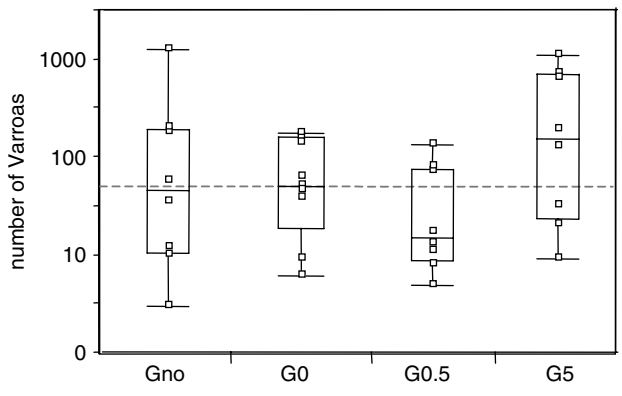

Figure 5. Number of varroas collected from the bee colonies of the experimental groups Gno, G0, G0.5 and G5. The limits of vertical lines give the minimal and maximal observed values, the lower and higher sides of the vertical boxes indicate the 25 and 75% percentiles, and the inner line of boxes indicate the median.

correspond to daily means of 3.1, 4.4, 4.3 and 3.3, and then 1.1, 1.6, 1.8 and 0.2 bees found dead per colony. All these rates are very low and no statistical analysis is required.

In December, some dead bees and bees with symptoms evoking *Nosema* infestation (bees crawling on the ground but without dysentery) were observed firstly in group G0, then in all other groups within less than one week. This required the application of a specific treatment in January (see Section 2.8). From 2 September until the following spring, no other abnormal mortality was observed in any colony.

3.10 Scores of colonies after wintering

The individual scores given to colonies ranged from 2 to 5 (mean = 3.6), with the means 3.6, 3.6, 4.4 and 2.9 for groups Gno, G0, G0.5 and G5, respectively. These means are not statistically different (comparisons between all groups, Kruskal–Wallis test: $\alpha > 0.06$; for syrup-fed groups only, Jonckheere–Terpstra test: $\alpha = 0.11$).

3.11 Chemical analyses

Multi-residue analysis of foundation wax revealed the presence of *tau*-fluvalinate in all the three batches (0.141, 0.431 and 2.005 mg kg^{-1}) and sulfur in two batches (0.756 and 1.126 mg kg^{-1}). Multi-residue analyses performed in pollen loads (from the sentinel hives) and bees (from all groups) revealed none of the products listed in Table 1.

Titration of imidacloprid is summarised in Table 3. Titration by AFSSA Maisons-Alfort of imidacloprid in an aliquot (frozen just after preparation) of the syrup given to group G5 resulted in a titre of 4.65 µg litre^{-1}, a value very close to the expected titre of 5 µg litre^{-1}. Quantification by GIRPA of imidacloprid and 6-chloronicotinic acid in the syrup given to group G5 confirmed the presence of both products in the aliquot frozen immediately after preparation and in the aliquot kept for 24 h at ambient temperature. Titres obtained on the syrups given to group G0.5 and group G0 were lower than the LD. Imidacloprid and 6-chloronicotinic acid were detected by GIRPA in bees of group G0.5 but not in bees of the other groups (Gno, G0 and G5). Both products were found in the honey collected in hives of group G5, but not in the honey from the other groups (Gno, G0 and G0.5). The results obtained independently on honey by the other laboratory confirmed the presence of imidacloprid in honey from group G5 with a titre of 2.95 µg kg^{-1}. No residues of imidacloprid or 6-chloronicotinic acid were detected in pollen loads.

4 DISCUSSION AND CONCLUSION

4.1 Main outcomes of the study

(a) Before feeding, none of the groups of colonies was statistically different in respect of the weight of hives, the number of adult bees, the number of

Table 3. Imidacloprid titration in the syrup given to bee colonies of group G0, G0.5 and G5, in dead bees and honey[a]

	Syrup (first feeding)			Bees		Honey	
	Imidacloprid		6-Chloronicotinic acid GIRPA	Imidacloprid GIRPA	6-Chloronicotinic acid GIRPA	Imidacloprid	6-Chloronicotinic acid GIRPA
Group	AFSSA-MA	GIRPA				AFSSA-MA GIRPA	
Gno	na	na	na	nd	nd	<LD <LD	<LD
G0	<LD	<LD	<LD	nd	nd	<LD <LD	<LD
G0.5	nd	<LD	<LD	tr	tr	<LD <LD	<LD
G5	After prepn: 4.65 µg litre^{-1}	Frozen after prepn: tr	tr	<LD	<LD	2.95 µg kg^{-1} tr	tr
	After 24 h at ambiant temp: nd	After 24 h at ambient temp: tr	tr				

[a] na: not applicable; nd: not determined; LD: limit of detection; tr: traces.

bees returning to the hives, the daily frequency of pollen carrying by bees, the size of the capped brood or the health status.

(b) During the feeding period, all the syrup provided in the feeders was removed by bees. This was directly observed and is confirmed by the significant difference of weight gain experienced by hives whose colonies were syrup-fed compared with hives of group Gno. The mean increase in weight was the same whether the syrup contained imidacloprid or not, and whatever its concentration. Just after the feeding period, the mean difference in weight gain was 5.93 kg for the syrup-fed hives compared with the non-fed ones. As this difference in weight gain can be considered as the result of the feeding with 13 litres or 15.98 kg of syrup per colony, we must conclude that during the feeding period, the main part of the syrup had been stored (and 2.7 times concentrated) and probably partly consumed by bees.

It has been shown that imidacloprid has a negative action on the feeding behaviour of several insect species: *Rhagoletis pomonella* Walsh, the apple maggot fly,[34] *Reticulitermes flavipes* Koll, a subterranean termite[35] and the aphids *Myzus persicae* Sulzer and *Aphis gossypii* (Glover).[36] Neither of the two imidacloprid concentrations tested in the present experiment had an antifeedant effect, although much higher concentrations of imidacloprid in saccharose syrup (500 and 1000 $\mu g\,kg^{-1}$) have been shown to be repellent for honey bees.[37] No abnormal mortality was observed in groups fed with syrup supplemented with imidacloprid (nor in any other group).

The only parameter with a statistically significantly link to feeding with imidacloprid-supplemented saccharose syrup was the frequency of pollen carrying that seems to increase with the concentration of imidacloprid in the syrup. However, the semi-quantitative measurement of this parameter leads us to use this result with care.

(c) After the feeding period until the end of the winter, the previously observed differences in pollen carrying frequency disappeared. The activity, the size of the adult bee population and the weight of hives remained statistically homogenous between fed groups whatever the syrup they were given. The only significant differences were observed regarding the size of the capped brood: the number of capped brood cells decreased less in colonies fed with a low imidacloprid concentration than in colonies fed with non-supplemented syrup (logically as a consequence of a more intense egg laying by queens of group G0.5). However, this phenomenon was not observed in colonies fed with the higher imidacloprid concentration.

(d) During summer, autumn and winter, while no diseases (including nosemosis diagnosed in all groups during early winter) had a significant impact on the colonies, apparent mortality was very low in all groups, with no difference between imidacloprid-fed and control colonies.

(e) After winter, all hives were found with eggs, with a large and similar number of adult bees and no statistically significant difference in the weight of hives. All groups of colonies obtained similar qualitative scores based on their strength and health. Logically following the difference observed before winter, more frames with capped brood were observed in the group of hives that had been fed with a low imidacloprid concentration than in hives fed with non-supplemented syrup, and this was again more than in hives fed with the highest imidacloprid content syrup. However, after wintering, the brood area was evaluated semi-quantitatively by using the number of frames occupied by brood (instead of the precise measurement of the brood area done before winter): this limits the accuracy of this observation. Moreover, the differences between groups remain low and, as the number of frames with brood in the control group is comprised between groups G0.5 and G5, this heterogeneity is probably not related to this factor.

As a summary, it is striking that the only statistically significant differences between syrup-fed groups originated from tests performed on two semi-quantitative data sets: pollen-carrying activity and the number of frames with brood after winter. No differences were significant regarding all other semi-quantitative or quantitative parameters related to activity, adult bee population, capped brood area, frequency of parasitic and other diseases, mortality or global score of colonies after wintering.

Dealing with the first difference, it would be surprising that, if an enhanced motility were a result of intoxication, it would be not uncoordinated but converted into pollen carrying, a very elaborate and biologically meaningful activity. Dealing with the second observed difference, influence of imidacloprid on insect fecundity has not been widely studied. Micro-colonies of *Bombus terrestris* L whose larva were fed with syrup and pollen supplemented with imidacloprid at sub-lethal doses (respectively 1.99 and 0.159 ng per worker per day) produced less adults than control ones,[15] whereas a sub-lethal spraying of imidacloprid has been shown to increase egg production of *Tetranychus urticae* Koch (Acari: Tetranychidae).[38] In the present study, the slower decrease in capped brood area observed in colonies fed with the 0.5 $\mu g\,litre^{-1}$ imidacloprid syrup (with no significant effect obtained with the 5 $\mu g\,litre^{-1}$ syrup) compared with the control colonies may correspond to the same paradoxically positive phenomenon.

The present experiment did not reveal any inter-relation of imidacloprid and diseases. Toxicity of

pesticides is liable to influence honey bee sensitivity to pathogens. Four to eight weeks following pesticide-induced losses, bee colonies more frequently suffer outbreaks of European foulbrood, sacbrood and chilled brood.[39] Imidacloprid is known to enhance the susceptibility of *R flavipes* (Isoptera: Rhinotermitidae)[35] and of *Blatella germanica* (L) (Dictyoptera: Blatellidae)[40] to *Metarhizium anisopliae* Sorokin, a fungal entomopathogen. A synergy of imidacloprid at sub-lethal doses with *M anisopliae* and *Beauveria bassiana* (Bals) Vuill has been demonstrated on *Diaprepes abbreviatus* L (Coleoptera: Curculionidae).[41] However, no evidence was found that imidacloprid interfered with a virus (the *Heliothis* single embedded nucleopolyhedrovirus) tested as an alternative way to control pests.[42]

Overall, all the parameters studied during this experiment followed the seasonal patterns usually recorded in healthy apiaries. During and after feeding with imidacloprid-supplemented syrup during summer, bee colonies did not show any immediate or delayed counter-productive or severe problems until the end of the observation period, ie until the beginning of the following spring.

The repeated feeding of several colonies with syrup supplemented with imidacloprid did not provoke any mortality within the few days following syrup absorption, nor any delayed mortality before, during or following the next winter, whereas such severe effects are described by many bee keepers as a consequence of the use of imidacloprid as seed dressing in neighbouring cultures.

4.2 Several hypotheses that may explain the discrepancy between these findings and other reports

Either the methodology was erroneous or our experimental conditions did not involve some biological parameter determinant for the outcome of such troubles.

4.2.1 Protocol validity

(a) Is it confirmed that imidacloprid was present in the supplemented saccharose syrups?

Imidacloprid had been found in the 5 μg litre^{-1} syrup independently by two laboratories (one of them had been given coded positive and negative samples). In addition, 6-chloronicotinic acid, a degradation derivative of imidacloprid, was found in this syrup. Imidacloprid and 6-chloronicotinic acid were also found in the same syrup kept 24 h at ambient temperature. Moreover, imidacloprid and 6-chloronicotinic acid had been found in the honey produced by colonies fed with the 5 μg litre^{-1} syrup. As the limit of detection for imidacloprid in the GIRPA laboratory was above the calculated titre of the 0.5 μg litre^{-1} syrup, it is logical that this insecticide was not detected in this syrup; however traces of imidacloprid and 6-chloronicotinic acid had been found in dead bees from colonies fed with it. Of course, in the worse case scenario, these bees could have drifted from colonies fed with the 5 μg litre^{-1} syrup and the syrup given to group G0.5 had no imidacloprid. This would mean that, when processing the 0.5 μg litre^{-1} syrup, the same error had been repeated 13 times for this syrup only, but not when preparing the 5 μg litre^{-1} syrup. This is rather improbable as the same person had processed both syrups according to parallel protocols.

(b) Was the number of hives sufficient for observing any significant difference between control and experimental colonies?

Eight to nine colonies per treatment were used in this experiment, which is more than the minimal number of three per concentration recommended by the EPPO guidelines when evaluating side-effects of insecticides.[43]

Moreover, because the symptoms described by bee keepers as a consequence of imidacloprid intoxication are very severe and involve a large number of bees, the number of hives used in the present experiment should had been sufficient to show them.

(c) Was the syrup given in feeders consumed by bees?

The imidacloprid given through feeders has not only been stored in the brood chamber but also in the honey chamber, similar to what is observed when drugs are administered by the same means to bees. This is confirmed by the titration of honey of group G5. All the nectar and the honey found during summer in the hive had been gathered or produced since spring, (the honey produced during the previous year had all been consumed). Therefore, since the beginning of the feeding, all bees of groups G0.5 and G5 were in contact with imidacloprid. This is particularly true for house bees who thermo-regulated the hive during summer and winter and consumed nectar and honey for this activity, which requires a high amount of energy.

To permit a continuous contamination of bees during winter, we did not harvest the honey from the brood chamber. We can assume that this allowed more severe conditions than occur in common apicultural practice, consisting of harvesting the honey in this part of the hive and replacing it by saccharose syrup.

This protocol should have allowed us to observe the immediate or delayed demise of a large number of house bees whose cadavers would had been found in front of the hives during summer, autumn or winter: this was never observed in this experiment. In addition, no population decrease was observed during the last visit performed at early spring.

(d) Was the present protocol appropriate for demonstrating the consequences of any perturbed

behaviour of foragers intoxicated by contaminated nectar?

Among the behavioural perturbations provoked by imidacloprid, Kirchner[13] within a short abstract wrote that bees fed with 20 ppb of imidacloprid in saccharose syrup performed less precise waggle dances, which may indicate a negative effect on orientation. Unfortunately, no other protocol guarantees a free uptake of toxicant by bees from complete colonies while keeping control of colonies in the same place as recommended by Oomen *et al*.[43] However, in the present protocol, foragers had access to the feeders. Before departure, they consume nectar or honey for ensuring the energy cost of flight, which is evaluated to 11.5 mg h^{-1}. One part of this need is covered by the meal taken before departure, and another part during foraging.[18]

Whereas this protocol did not allow the direct observation of dead bees (if any) in foraging places, any significant disappearance of foragers would had entailed a population loss during summer or autumn that would had been measured in this experiment; such a phenomenon is not sustained by the present data.

4.2.2 Biological significance of the results

To our knowledge, this is the first report of a controlled experiment of repeated exposure of whole colonies to imidacloprid in syrup at doses comparable with those found in nectar in the field. As this experiment failed to reproduce the troubles alleged by bee keepers to arise with this toxicant, several non-exclusive interpretations may be proposed:

(a) The troubles observed by the bee keepers are not provoked by imidacloprid, or not by imidacloprid alone or not by imidacloprid in the nectar alone.

Notwithstanding the fact that imidacloprid has been shown by many authors to be toxic to experimental groups of bees isolated from their colonies, the question remains on how this toxicant acts on whole colonies. Tasei *et al*[15] showed that the sensitivity of *B terrestris* workers to sub-lethal doses was age-dependant. Guez *et al*[44] hypothesised the existence of two different nicotinic receptors that have different affinities to imidacloprid and are differentially expressed in 7- and 8-day-old bees. Because a colony gathers all classes of age and has its own dynamic to respond to toxicant aggression (increase in egg-laying, for example), results obtained on samples of bees cannot be simply transposed to entire colonies, and trials on the latter are required and highly informative. In this experiment, if colonies reacted to imidacloprid during the feeding period, these reactions were so weak that they would had remained unnoticed in common apicultural practice.

As feeding free-ranging bees with pollen is not practical, bees were not fed with contaminated pollen in this protocol, and this could also explain why no lethal effect was observed. While not supported by any scientific reference, one may hypothesise that the combination of imidacloprid and some component of sunflower pollen or other parts of the plant may be more toxic to bees than imidacloprid.

(b) In the present protocol, colonies were in good physiological condition, which may explain why they sustained imidacloprid contamination.

In additional to genetic factors, physiological condition and health status determine the detoxification abilities of bees. In particular, pollen quantity and quality absorbed during the first days of life are determinant for these abilities during the whole life of bees.[45] Decourtye *et al*[46] hypothesised that the higher sensitivity of winter bees compared with summer bees of the same colony may be explained by the fact that they had no access to fresh pollen. It is interesting that sunflower pollen, which has a low protein concentration, does not ensure the optimal development of hypopharyngeal glands of workers as other pollens can do, and 'honey-bee colonies restricted to foraging on *Helianthus annuus* during its bloom period may suffer a slight loss of fitness'.[47] Compared with bees kept in mono-crop farming areas, bees of the present protocol had access to food sources which were certainly more rich and varied, and with no gap during the apicultural season. One can also argue that these colonies had not been culled year after year by repeated action of toxicants. All these factors could explain why the imidacloprid absorbed did not entail any lethal effect.

(c) The local bee race used in the present study may be less sensitive to imidacloprid than local races that prevail in the areas where bee keepers observed the problems that they relate to the use of this insecticide.

It has been known for many years that honey bee colonies vary widely in their ability to withstand insecticide exposures.[48,49] Moreover, compared with other insecticides, the acute toxicity of imidacloprid varies greatly in the honey bee: by oral route, LD_{50} ranges from 5.4 ng per bee[10] to 40.9 ng per bee,[17] by contact the range is from 6.7 to 230.3 ng per bee. Such large ranges may originate from various experimental conditions, including the choice of *A mellifera* subspecies.[10]

Further research should now address several hypotheses: the troubles described by bee keepers have other causes than imidacloprid; if such troubles are really due to this insecticide, they may only be observed when bees consume contaminated pollen, when no other sources of food are available, in the presence of synergic factors (that still need to be identified), with some particular races of bees, or when colonies are not strong and healthy.

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