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Learning performances of honeybees (*Apis mellifera* L) are differentially affected by imidacloprid according to the season

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Abstract: To establish the sublethal concentrations domain, acute and chronic oral tests were conducted on caged honeybee workers (*Apis mellifera* L) using imidacloprid and a metabolite, 5-OH-imidacloprid, under laboratory conditions. The latter showed a 48-h oral LD_{50} value (153 ng per bee) five times higher than that of imidacloprid (30 ng per bee). Chronic feeding tests indicated that the lowest observed effect concentrations (LOEC) of imidacloprid and of 5-OH-imidacloprid on mortality of winter bees were 24 and 120 $\mu g\,kg^{-1}$ respectively. Behavioural effects of imidacloprid and 5-OH-imidacloprid were studied using the olfactory conditioning of proboscis extension response at two periods of the year. Winter bees surviving chronic treatment with imidacloprid and 5-OH-imidacloprid had reduced learning performances. The LOEC of imidacloprid was lower in summer bees (12 $\mu g\,kg^{-1}$) than in winter bees (48 $\mu g\,kg^{-1}$), which points to a greater sensitivity of honeybees behaviour in summer bees, compared to winter bees.

Keywords: honeybee; *Apis mellifera*; imidacloprid; sublethal effect; learning; season; chronic toxicity; proboscis extension response

1 INTRODUCTION

Imidacloprid (Gaucho®), is a chloronicotinyl insecticide specifically targeting the nicotinic acetylcholine receptors of insects.[1] It shows systemic properties and it is used against soil pests and aphids.[2] Imidacloprid is metabolised more or less completely over time, depending on plant species.[3] One of the main metabolites of imidacloprid is 5-OH-imidacloprid (Fig 1), which also has insecticidal properties.[4] When used on melliferous plants, such as sunflower, the question of the possible side-effects of imidacloprid or its metabolites on pollinating insects arises. Despite the fact that imidacloprid has proved highly toxic to honeybees (*Apis mellifera* L) in acute laboratory tests,[5] several semi-field and field tests indicated that seed dressing with imidacloprid posed no risk during the period of sunflower flowering.[6,7] However, it was thought that imidacloprid could reduce sunflower honey production, since this product or its metabolites could migrate into nectar and induce deleterious effects in foraging bees after ingestion of contaminated nectar.[8] Furthermore, it was suspected that the induced effects were due to an alteration of the foraging behaviour, rather than to lethal effects.

Under natural conditions, foraging behaviour relies on learning and memory processes. While collecting nectar or pollen, foragers memorise floral cues, among which odours play a major role in flower recognition during the following trips.[9] Therefore, the study of olfactory learning performances as an endpoint in

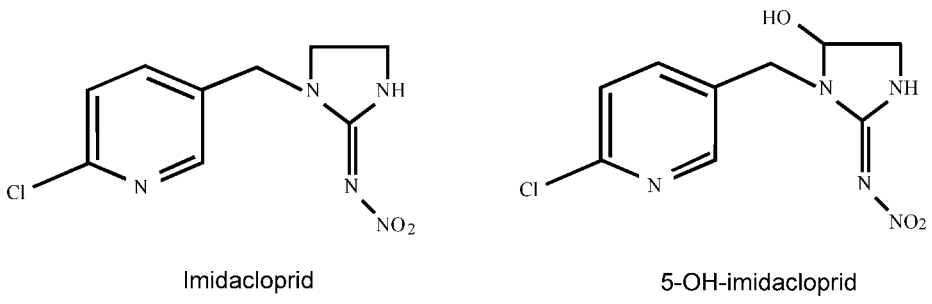

Figure 1. Chemical structures of imidacloprid and 5-OH-imidacloprid.

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ecotoxicity tests is ecologically relevant, since individual learning alteration may indirectly account for changes in the colony survival.

To study the foraging behaviour in honeybees, two main approaches have been used: observations of free-flying bees visiting natural or artificial food sources,[10,11] or the recording of conditioned proboscis extension response (PER) in restrained individuals.[12–15] Several neurotoxic insecticides have been shown to have effects on various aspects of foraging behaviour in free-flying bees. Thus, it has been reported that parathion disrupted the communication dance of foraging bees,[16,17] diazinon affected the onset and the duration of foraging, and the handling of nectar,[18] and permethrin and deltamethrin induced an abnormal behaviour pattern during the homing flight.[19,20] The PER assay has also been used to assess the effects of insecticides.[21–24] The classical odour conditioning of the PER is based on the temporal paired association of a conditioned stimulus and an unconditioned stimulus. During conditioning, the PER is elicited by contacting the gustatory receptors of the antennae with a sucrose solution (unconditioned stimulus), an odour (conditioned stimulus) being simultaneously delivered. The proboscis extension is immediately rewarded by the uptake of the sucrose solution constituting a food reward. Bees can exhibit the PER as a conditioned response to the odour alone after even a single pairing of the odour with a sucrose reward.[12–15] Some reports have indicated that good correlation can be found between olfactory responses in free-flying foragers and in individuals subjected to the PER paradigm.[25,26] Therefore, the PER assay could be a tool in studies on sublethal effects of pesticides, especially on foraging behaviour, since it guarantees good control of bee-rearing conditions and of exposure to chemicals, as well as standardised responses in the test.

In this study, we tested the hypothesis that imidacloprid ingestion would induce deleterious effects on bee learning abilities under laboratory conditions. The olfactory conditioning of PER was used to evaluate the long-term effect of feeding syrups contaminated with imidacloprid and with one of its main metabolites, 5-OH-imidacloprid. Prior to the study of behavioural effects, standard acute toxicity tests were carried out in order to establish the sublethal concentration domain and to evaluate the sensitivity of our biological material towards the test chemicals. In a first set of behavioural experiments, we tested the concentration-dependent effect of imidacloprid and of its metabolite on the olfactory learning performances in winter bees. In a second set of experiments, we checked for a season-dependent effect of imidacloprid, using summer bees.

2 MATERIALS AND METHODS

2.1 Acute toxicity

The median lethal doses (LD_{50}) of imidacloprid and 5-OH-imidacloprid were determined following the 'Commission des Essais Biologiques' method No 95 for risk assessment of pesticides to bees.[27] According to the official tests, bees are collected in summer and from a single hive. We introduced a slight modification to the guidelines by collecting and mixing worker bees from three different hives in order to reduce a potential hive effect.

2.1.1 Insecticides

Technical grade imidacloprid and 5-OH-imidacloprid (both 99.4% pure) were provided by Bayer AG (Leverkusen, Germany). Imidacloprid of 98% purity was also obtained from Cluzeau Info Labo, Sainte-Foy-La-Grande, France. Dimethoate of 96% purity was obtained from Calliope SA (Noguères, France).

The five concentrations of each insecticide tested were determined by preliminary experiments and information gained from the existing literature. They ranged from 0.2 to 3.2 mg litre^{-1} for imidacloprid (2–32 ng per bee), and from 1.25 to 20 mg litre^{-1} for 5-OH-imidacloprid (12.5–200 ng per bee), in a geometrical progression of factor 2. Dimethoate was used as a positive control at doses of 0.10 and 0.35 μg per bee.[28]

Stock solutions of each chemical were prepared in acetone. This solvent was chosen following the guidelines as it is a rather generalist solvent. Aliquots of the stock solutions were used to make each test solution at a specific concentration. The chemicals were added to a sucrose solution (500 g litre^{-1}). The final concentration of acetone in the sucrose solutions was 10 ml litre^{-1}. The effects of insecticide-containing solutions were compared with that of an untreated sucrose solution containing 10 ml litre^{-1} acetone.

2.1.2 Honeybees

The tests were carried out with *Apis mellifera* worker bees of unknown age. Bees from frames without a brood, to avoid the youngest bees, were caged in groups of 20. They were provided with a sugar solution (500 g litre^{-1} sucrose) and were maintained in an incubator (darkness, 25 (±2) °C, 40 (±10)% RH) overnight. Tests for acariosis, nosemosis, black disease, acute paralysis virus (AFSSA, Sophia Antipolis, France) and spiroplasmosis (INRA, Bordeaux, France) were performed in the three experimental hives. The negative results confirmed the good health of the colonies.

2.1.3 Protocol

The toxicity tests were conducted on late summer bees (August to October). Three replicates for each of the five concentrations and of the untreated control were undertaken simultaneously, and this was repeated at least three times over the experimental period. Twenty bees were used per replicate. The oral administration of chemicals was chosen because ingestion of contaminated nectar following sunflower treatment by seed dressing is the main potential exposure route.

After a 2-h starvation period, each group of 20 bees received 200 µl (10 µl per bee) of the treated or the control sugar solution, in daylight and at 25 (±2) °C. After the consumption of the 200 µl of sugar solution, the bees were put back into an incubator (darkness, 25 (±2) °C, 40 (±10)% RH) and provided with an untreated sugar solution *ad libitum*. Mortality was recorded 48 h after the beginning of the treatment.

2.1.4 Data analysis

In order to calculate median lethal dose (LD_{50}) values, mortality rates of treated groups were corrected, taking into account the mortality of the untreated control group, using Abbott's formula.[29] LD_{50} values were calculated with a probit regression analysis,[30] using the computer program WIN DL (CIRAD-CA/MABIS, Montpellier, France).

2.2 Chemical analysis

To compare required and actual concentrations, samples used for the PER assay were dosed. Frozen samples of contaminated sucrose solution at the concentrations delivered to the bees were sent to the Department of Pharmacology and Toxicology (Limoges, France) for residue analyses. Control untreated samples of sucrose solution were used as a blank matrix to prepare matrix matched standards for calibration. Liquid chromatography-mass spectrometry/mass spectrometry method was used (Lacassie E, unpublished). The limit of detection (LOD) and the limit of quantification (LOQ) obtained for imidacloprid ranged from 1 to 2 µg kg^{-1}, and from 5 to 10 µg kg^{-1} for 5-OH-imidacloprid.

2.3 Proboscis extension response (PER) assay

2.3.1 Insecticides

In Experiment 1, imidacloprid and 5-OH-imidacloprid from Bayer AG were tested at six concentrations, with a geometrical progression of factor 2: 1.5–48 µg kg^{-1} for imidacloprid and 7.5–240 µg kg^{-1} for 5-OH-imidacloprid. The highest concentration corresponded to the LD_{50} value previously determined in the acute oral toxicity tests, divided by 20, to be in the sublethal domain. Moreover, preliminary studies had suggested that imidacloprid at this dose could induce a decrease in learning performances.[31] In Experiment 2, a range of seven concentrations of imidacloprid from Cluzeau Info Labo was used: 1.5–96 µg kg^{-1}, ie the same range of six concentrations as in Experiment 1, complemented by one higher concentration.

All solutions were made up as described previously (500 g $litre^{-1}$ sucrose, 10 ml $litre^{-1}$ acetone). The concentrations were calculated for a consumption of syrup estimated at 33 µl per bee per day (Picard-Nizou AL, pers comm). The control and contaminated sucrose solutions were kept at −20 (±1) °C (during 1–15 days) and defrosted at ambient temperature, in natural daylight, before use.

2.3.2 Honeybees

Experiments were carried out with worker bees of *Apis mellifera ligustica*. Experiment 1 was undertaken on so-called 'winter bees' (December to February). These bees were collected from hives maintained in a heated apiary (25 (±5) °C). Although the foraging activity was reduced as in outdoor hives in the North of France where the laboratory is set, the queens maintained under heated conditions went on laying eggs. Experiment 2 was conducted on summer bees in July, with bees collected from outdoor hives, when the foraging activity of the workers and the queen activity were high. Honeybees of known age were tested. Emerging worker bees were caged in groups of 60 individuals. They were provided with sugar food (mixture of sugar and honey) and water *ad libitum* during the initial 2 days, and with pollen for the following 8 days. After 2 days, the insecticide-treated sugar solutions were provided. The bees were kept in an incubator (33 (±2) °C, 40 (±10)% RH, darkness). The rearing temperature applied is higher than that recommended in the standard acute toxicity method (25 (±2) °C), but corresponds to the hive temperature.[32] Although the toxicity of an insecticide may vary with the temperature, such changes mainly occur with pyrethroids.[33] Therefore, for chronic exposure to imidacloprid and its metabolite, we decided to deliver the insecticide under the temperature conditions encountered in a hive. The exposure lasted until the bees were 14–15 days old, and were used in the PER assay. It has been shown that, on average, worker bees become foragers at that age,[34,35] and give the most consistent performances in the conditioned proboscis extension assay.[36]

2.3.3 Protocol

From 2 to 14–15 days old, the quantity of treated sugar solution provided daily was adjusted to the number of surviving bees (on a basis of 33 µl per bee per day). The mortality and consumption of syrup was recorded daily, and dead bees were discarded. In Experiment 1, every testing day was organised as follows: bees previously exposed to three concentrations of imidacloprid and of its main metabolite were tested, as well as untreated control bees, leading to a total of 60–70 bees tested per day, with 5–6 bees for each treatment. This was done repeatedly, until about 30 bees per treatment were obtained. This procedure minimised possible day effects. As it was not possible to test all six concentrations of the chemicals daily, we chose to test three concentrations in a first set of tests, and the three remaining ones in a second set of tests. Control untreated groups were included in each set. In Experiment 2, the bees subjected to prior exposure to seven concentrations of imidacloprid, and the untreated control bees, were tested daily. This was repeated until the samples of tested bees reached *ca* 30 individuals per treatment.

After treatment, the bees were mounted individually in glass tubes with only their antennae and mouthparts

Table 1. Acute oral toxicity of imidacloprid and 5-OH-imidacloprid in honeybees

Chemical	48-h LD_{50}[a] (ng per bee)	95% CL	Slope
Imidacloprid	30.6	26.7–36.3	2.21
5-OH-imidacloprid	153.5	125.9–196.9	0.88

[a] Tests were performed according to CEB testing guideline No 95.[27] LD_{50} values were calculated using log-probit analysis.[30] The number of bees per group was between 180 and 360.

free. They were starved for 4 h prior to conditioning. They were selected for showing a proboscis extension reflex after stimulation of the antennae with a sucrose solution (300 g litre^{-1}). The number of individuals exhibiting the reflex response was recorded. The ability to produce the reflex response reflects the state of the sensory-motor pathway underlying the PER. Bees were then placed in an airflow (main airflow of 50 ml s^{-1} added to a secondary airflow of 2.5 ml s^{-1}) for 15 s, to be familiarised with the mechanical stimulation and with the experimental background. For the conditioning trials, the conditioned stimulus (10 µl of pure linalool, a standard floral odour,[37] soaked on a filter paper strip inserted in a Pasteur pipette cartridge; Sigma, 95–97% purity) was delivered through the secondary flow (2.5 ml s^{-1}) for 6 s. During odour delivery, the PER was elicited after 3 s by contacting the antennae with a sucrose solution (300 g litre^{-1}) as the unconditioned stimulus, and the same solution was immediately given as a reward, before the odour delivery ended. Three conditioning trials were carried out with 20–30 min inter-trial duration. The individuals were then subjected to one test trial, the conditioned stimulus (pure linalool) being delivered for 6 s. The conditioned PER was recorded as a yes-or-no response when the odour alone was delivered during the 6 s of the test trial.

2.3.4 *Data analysis*

For each chemical, the mean amounts of sugar solution consumed daily over the 11 days of treatment prior to PER testing were compared among the concentrations (including the untreated group) using a one-way analysis of variance ($P < 0.05$). When the F value was significant, a Fisher's least significant difference test (LSD) was applied, with a 5% level of significance. The mortality cumulated over 11 days of treatment with each chemical was compared between every concentration and the control by multiple two-by-two chi-squared tests with 1 *df* (5% level of significance divided by n, n being the number of comparisons where the same control data were used). For each chemical, the number of initial reflex responses and the number of conditioned responses in the test trial, were compared between each concentration of the chemical and the control, by multiple two-by-two chi-squared tests with 1 *df*. When conditions of application of the chi-squared test were not fulfilled according to the Cochran's rule, the Fisher's exact method was applied.[38] The significance threshold was of 5% divided by n, n being the number of comparisons where the same control data were used.

3 RESULTS

3.1 Acute toxicity

Consistent with the guidelines of the 'Commission des Essais Biologiques' method No 95,[27] dimethoate mortality ranged from 50% to 100% and the mortality in the untreated control was less than 10%.

LD_{50} values (and 95% confidence limits) determined 48 h after the oral treatments are presented for the three chemicals (Table 1). The low LD_{50} values (30.6–153.5 ng per bee) revealed high toxicities for imidacloprid and its metabolite. Imidacloprid showed an LD_{50} value of 30.6 ng per bee, which was five times lower than the corresponding LD_{50} calculated for 5-OH-imidacloprid (153.5 ng per bee).

3.2 Chemical analysis

Nominal and dosed concentrations of imidacloprid and of its main metabolite, 5-OH-imidacloprid, in the sucrose solutions delivered to the treated groups are given in Table 2. Residues of both chemicals were analysed and reported for each sample. For imidacloprid-containing solutions (Table 2(A)), greater quantities of the chemical were found after dosage (rate of recovery between nominal and dosed concentrations: from 103% at 48 µg kg^{-1} to 213% at 1.5 µg kg^{-1}). This increase in concentration was probably due to the evaporation of the solvent (10 ml litre^{-1} acetone) during the syrup preparation. Traces of a further metabolite (olefin) were found in the solutions containing imidacloprid at 6 and 24 µg kg^{-1} (<2 µg kg^{-1}), which may be accounted for by metabolisation of imidacloprid into this compound. In syrups treated with 5-OH-imidacloprid (Table 2(B)), nominal and

Table 2. Nominal and dosed concentrations (µg kg^{-1}) of (A) imidacloprid-containing solutions and (B) 5-OH-imidacloprid-containing solutions as used in Experiment 1

	Nominal	Dosed	Nominal	Dosed
A	*Imidacloprid*		*5-OH-imidacloprid*	
	0	<LOD[a]	0	<LOD
	1.5	3.2	0	<LOD
	6	8.8	0	<LOD
	24	32.8	0	<LOD
	48	49.5	0	<LOD
B	*5-OH-imidacloprid*		*Imidacloprid*	
	0	<LOD	0	<LOD
	7.5	<LOQ[b]	0	<LOD
	30	34.1	0	<LOD
	120	83.8	0	<LOD
	240	168.4	0	<LOD

[a] Limit of detection (LOD): 1 µg kg^{-1} for imidacloprid, 5 µg kg^{-1} for 5-OH-imidacloprid.

[b] Limit of quantification (LOQ): 2 µg kg^{-1} for imidacloprid, 10 µg kg^{-1} for 5-OH-imidacloprid.

dosed concentrations were similar (rate of recovery between nominal and dosed concentrations: from 70% at 240 µg kg^{-1} to 113% at 30 µg kg^{-1}). As evaporation of acetone has probably also occurred, increased concentrations were expected in the residue analyses. This was compensated for by metabolisation of 5-OH-imidacloprid into olefin, as shown by the presence of this in the solutions.

3.3 Proboscis extension response (PER) assay

3.3.1 Experiment 1—Effects of imidacloprid and 5-OH-imidacloprid in winter bees

3.3.1.1 Syrup consumption. During the treatment period, ie 11 days, the volume of syrup consumed for control and imidacloprid-treated groups ranged from 28.8 to 33.7 µl per bee and per day, which is in agreement with the consumption initially estimated (33 µl per bee and per day). The consumption was not significantly different in treated and control groups (ANOVA, $F = 0.5$, 6 *df*, $P = 0.78$). This suggests that the tested concentrations of imidacloprid had no antifeedant effect on honeybees. The consumption of 5-OH-imidacloprid-treated syrups was significantly lower than that of syrup control (ANOVA, $F = 2.7$, 6 *df*, $P = 0.015$). Significant differences with the syrup control (volume consumed: 32.4 µl per bee and per day) were found at concentrations of 240, 120 and 30 µg kg^{-1} (23.9, 23.5, 25.8 µl per bee and per day, respectively). Consequently, the potential diet deficiency in bees treated with 5-OH-imidacloprid may have had deleterious effects on mortality or learning performances (see below).

3.3.1.2 Chronic mortality. Cumulative mortality in bees exposed to imidacloprid at concentrations of 1.5–24 µg kg^{-1} did not differ significantly from that of the control group (Table 3(A)). A significant increase in mortality occurred at 48 µg kg^{-1} (20.5% *versus* 11.6% mortality after 11 days, in the treated and control group respectively; $\chi^2 = 7.6$, 1 *df*, $P = 0.006$). The number of dead bees in the group exposed to 240 µg kg^{-1} of 5-OH-imidacloprid (41% mortality) was significantly different ($\chi^2 = 36.3$, 1 *df*, $P < 0.0001$) from that of the control group (17.2% mortality; Table 4).

Table 4. Chronic oral toxicity of 5-OH-imidacloprid in winter honeybees[a]

Nominal concentrations (µg kg^{-1})	*Mortality (%)*[b]
Control	17.2
7.5	3.3
15	13.3
30	19.4
60	10.5
120	26.6
240	41.0*

[a] The number of bees per group was 180.
[b] In each experiment, the cumulated mortalities in treated groups and in the control group were compared using chi-squared test with 1 *df*.
* $P < 0.0083$.

3.3.1.3 Reflex response. The same level of reflex response in imidacloprid-treated and untreated bees was shown (Table 5(A)). This suggests that the exposure to imidacloprid did not disrupt the sensory and motor components controlling the PER. Reflex response levels obtained after treatment with 5-OH-

Table 3. Chronic oral toxicity of imidacloprid in honeybees

	Experiment	*Nominal concentrations (µg kg^{-1})*	*Mortality (%)*[c]
A	Experiment 1 (winter bees)[a]	Control	11.6
		1.5	12.7
		3	3.0
		6	9.4
		12	11.1
		24	16.1
		48	20.5*
B	Experiment 2 (summer bees)[b]	Control	3.3
		1.5	8.3
		3	8.3
		6	5
		12	7.2
		24	7.7
		48	9.4
		96	17.7†

[a] The number of bees per group was between 180 and 360.
[b] The number of bees per group was 180.
[c] In each experiment, the cumulative mortalities in treated groups and in the control group were compared using chi-squared test with 1 *df*.
* $P < 0.0083$.
† $P < 0.0071$.

Table 5. Effects of imidacloprid on reflex responses in honeybees

Experiment	*Nominal concentrations (µg kg^{-1})*	*Reflex responses (%)*[c]
A Experiment 1 (winter bees)[a]	Control	52.4
	1.5	60.0
	3	44.7
	6	60.0
	12	55.0
	24	42.0
	48	36.6
B Experiment 2 (summer bees)[b]	Control	90.1
	1.5	81.9
	3	85.6
	6	78.6
	12	83.6
	24	80.0
	48	59.0*
	96	69.7*

[a] The number of bees per group was between 68 and 163.
[b] The number of bees per group was between 60 and 66.
[c] In each experiment, the number of reflex responses in treated groups and in the control group were compared using Chi-square test with 1 *df*.
* $P < 0.0071$.

Table 6. Effects of 5-OH-imidacloprid on reflex responses in winter honeybees[a]

Nominal concentrations ($\mu g kg^{-1}$)	*Reflex responses (%)*[b]
Control	61.5
7.5	55.3
15	57.5
30	52.8
60	40.0*
120	29.3*
240	21.4*

[a] The number of bees per group was between 56 and 156.
[b] In each experiment, the number of reflex responses in treated groups and in the control group were compared using chi-squared test with 1 *df*.
* $P < 0.0071$.

imidacloprid solutions at concentrations higher than $30\,\mu g\,kg^{-1}$ were significantly lower than that obtained with the control solution ($60\,\mu g\,kg^{-1}$: $\chi^2 = 9.9$, 1 *df*, $P = 0.0017$; $120\,\mu g\,kg^{-1}$: $\chi^2 = 21.0$, 1 *df*, $P < 0.0001$; $240\,\mu g\,kg^{-1}$: $\chi^2 = 35.2$, 1 *df*, $P < 0.0001$; Table 6).

3.3.1.4 Learning performances. Figure 2 shows the olfactory learning performances represented as the percentage of conditioned PER obtained at the test trial following the training procedure, in bees fed the six concentrations of imidacloprid ($1.5–48\,\mu g\,kg^{-1}$) and in the control bees fed sucrose only. It appears that only bees treated with the highest concentration of imidacloprid ($48\,\mu g\,kg^{-1}$) exhibited significantly lower responses compared to the control group ($\chi^2 = 5.8$, 1 *df*, $P = 0.015$), while the response rate of bees treated with concentrations of imidacloprid below $48\,\mu g\,kg^{-1}$ was equivalent to that of control bees. The high concentrations of 5-OH-imidacloprid induced significantly lower responses compared to that of the control group ($120\,\mu g\,kg^{-1}$: $\chi^2 = 6.4$, 1 *df*, $P = 0.011$; $240\,\mu g\,kg^{-1}$: $\chi^2 = 5.8$, 1 *df*, $P = 0.015$; Fig 3).

3.3.2 Experiment 2—Effects of imidacloprid in summer bees

3.3.2.1 Syrup consumption. The consumption of sugar solutions contaminated with all seven concentrations of imidacloprid (consumed volumes ranging

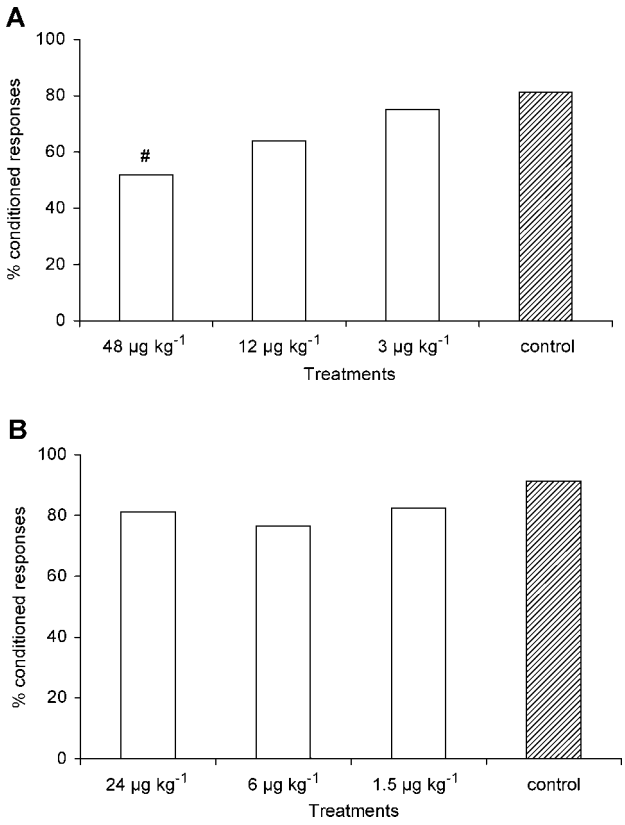

Figure 2. Experiment 1—effects of imidacloprid on learning performances in winter honeybees. (A) Bees exposed to a first set of three concentrations of imidacloprid (white bars; number of bees per group comprised between 27 and 36), and (B) bees exposed to a second set of three concentrations (number of bees per group was between 32 and 35). Control untreated groups (striped bar; number of bees per group was between 32 and 36) were included in each set. The number of conditioned responses at the test trial were compared between each concentration of the chemical and the control using a chi-squared test with 1 *df* (# $P < 0.0166$). When conditions of application of the chi-squared test were not fulfilled, the Fisher's exact method was applied.

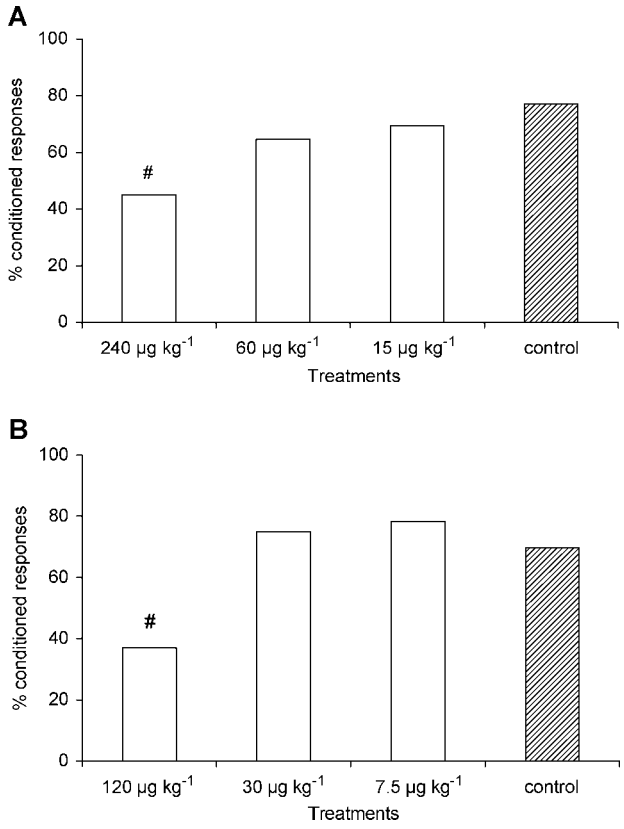

Figure 3. Effects of 5-OH-imidacloprid on learning performances in winter honeybees. (A) Bees exposed to a first set of three concentrations of 5-OH-imidacloprid (white bars; number of bees per group were between 20 and 36), and (B) bees exposed to a second set of three concentrations (number of bees per group comprised between 23 and 36). Control untreated groups (striped bar; number of bees per group were between 33 and 35) were included in each set. The number of conditioned responses at the test trial were compared between each concentration of the chemical and the control using a chi-squared test with 1 *df* (# $P < 0.0166$). When conditions of application of the chi-squared test were not fulfilled, the Fisher's exact method was applied.

from 30.3 to 40 µl per bee per day) were equivalent to the consumption of control sugar solution (37 µl per bee per day) (ANOVA, $F = 0.2$, 7 *df*, $P = 0.98$). This result confirms the absence of anti-feeding effect of imidacloprid-treated sugar solutions observed in Experiment 1 on winter bees.

3.3.2.2 Chronic mortality. The cumulative mortality recorded in summer bees (Table 3(B)) was significantly increased in the presence of imidacloprid at a concentration of 96 µg kg^{-1} only, compared with the mortality in the control group ($\chi^2 = 19.9$, 1 *df*, $P < 0.0001$). Compared to winter bees that showed an increased mortality at a concentration of 48 µg kg^{-1}, this suggests that summer bees would be less susceptible to the lethal effects of imidacloprid.

3.3.2.3 Reflex response. Concentrations of 48 and 96 µg kg^{-1} of imidacloprid elicited a significant decrease in the level of reflex responses (Table 5, B) compared with the control (48 µg kg^{-1}: $\chi^2 = 17.3$, 1 *df*, $P < 0.0001$; 96 µg kg^{-1}: $\chi^2 = 9.5$, 1 *df*, $P = 0.002$). A sublethal effect, as measured by the reflex response, appeared in summer bees in contrast to winter bees, which showed no significant difference between treated and untreated bees.

3.3.2.4 Learning performances. Treatment with imidacloprid elicited a general decrease in the olfactory learning performances recorded at the test trial following the training period (Fig 4) compared with the control. This sublethal effect of imidacloprid on the level of conditioned response was significant at 12 µg kg^{-1} ($\chi^2 = 8.7$, 1 *df*, $P = 0.0032$), 24 µg kg^{-1} ($\chi^2 = 8.1$, 1 *df*, $P = 0.0043$), 48 µg kg^{-1} ($\chi^2 = 7.2$, 1 *df*, $P = 0.0063$) and 96 µg kg^{-1} ($\chi^2 = 8.1$, 1 *df*, $P = 0.0043$). The lowest concentration inducing a sublethal effect on the learning performances is lower in summer bees than in winter bees (12 µg kg^{-1} *versus* 48 µg kg^{-1}).

4 DISCUSSION

In France, the standard method for evaluating the toxicity of any insecticide likely to be encountered by honeybees is the calculation of the acute toxicity value (LD_{50}) following the 'Commission des Essais Biologiques' method No 95.[27] The acute oral tests undergone on summer bees revealed that the 48-h LD_{50} value of imidacloprid (30 ng per bee) was five times lower than that of 5-OH-imidacloprid (153 ng per bee). This slightly differs from other data reported by Suchail *et al*,[39] who quote an LD_{50} of 60 ng per bee for imidacloprid, this value being close to that of the metabolite. However, our data fit with those of Nauen *et al*,[40] which indicated that the 48-h oral LD_{50} of 5-OH-imidacloprid was 159 ng per bee. More generally, the oral LD_{50} of imidacloprid reported in the present paper lies in the range of published data: 3.7 to >81 ng per bee.[5,7,39–41] Thus, although the LD_{50} value of imidacloprid may vary widely (up to a factor of >100 whereas most insecticide LD_{50} values vary by a factor of 2),[39] our data confirmed previous results demonstrating the high acute toxicity of imidacloprid and 5-OH-imidacloprid to honeybees. This also indicates that the insecticide sensitivity of our biological material is not different from that reported in other papers.

Beside acute toxicity tests, in the case of systemic compounds such as imidacloprid, longer term effects are not excluded, since the product is potentially present at low concentrations in the nectar of plants whose seed was dressed with imidacloprid,[7,42] and could be collected along the flowering. Therefore, the effects of long-term exposure were considered. Laboratory chronic feeding tests showed that imidacloprid and 5-OH-imidacloprid at high concentrations (48–96 µg kg^{-1} and 240 µg kg^{-1} respectively) were lethal to caged worker bees. Interestingly, the concentration of imidacloprid inducing a chronic toxicity in winter bees (48 µg kg^{-1}) was lower than that producing the same effect in summer bees (96 µg kg^{-1}). This may account for differences in the physiological state of the two types of bees, especially in the amount of adipose tissues which are known to be more abundant in winter bees,[43] and to be the site of pesticide bioaccumulation.[44] Therefore, winter bees

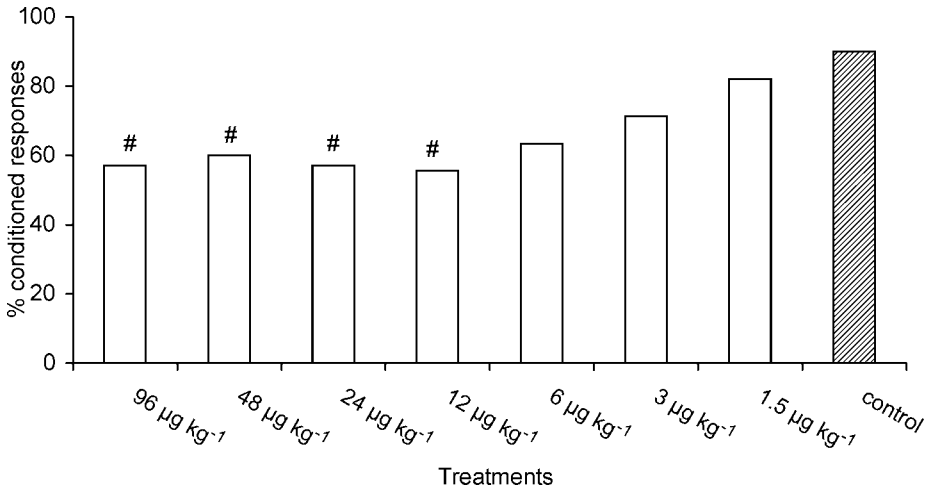

Figure 4. Experiment 2—effects of imidacloprid on learning performances in summer honeybees. The number of bees per group was between 27 and 30. The number of conditioned responses at the test trial were compared between each concentration of the chemical (white bars) and the control (striped bar) using chi-squared test with 1 *df* (# $P < 0.0071$). When conditions of application of the chi-squared test were not fulfilled, the Fisher's exact method was applied.

should be more sensitive to lower doses of pesticides over time. Chronic toxicity was found even at the lower concentrations of imidacloprid, as shown in a laboratory 10-day chronic oral test with bees fed treated syrup (0.1–10 μg kg^{-1}).[39]

Bees surviving chronic exposure to imidacloprid have reduced learning performances in the PER assay. Interestingly, it appears that the initial reflex response and the conditioned response obtained after training are differentially affected by the treatment. Thus, the lowest observed effect concentration (LOEC) is always lower for the conditioned response than for the reflex response (Table 7), suggesting that gustatory and motor functions involved in the PER would be less sensitive to the treatment than the integrative processes underlying memory acquisition and recall of learned information. This is consistent with the work of Mamood and Waller[22] reporting that prior administration of permethrin induced deleterious effects on the conditioned responses but not on the reflex response. Effects of imidacloprid were also found on a non-associative learning task.[45] The neurotoxic action of imidacloprid would affect both non-associative and associative learning, since nicotinic cholinergic receptors, the major site target of imidacloprid and of its metabolites,[4] are present in cerebral structures of the honeybee which are implicated in memory processes (ie antennal lobes and mushroom bodies).[46] Moreover, application of imidacloprid to the honeybee brain surface resulted in modification of cytochrome oxidase staining, used as an endogenous marker of neuronal metabolism, in antennal lobes and mushroom bodies.[47] Thus, it is more likely that the performance deficit caused by imidacloprid is due to a memory disruption, rather than to sensory, motor or motivational changes in treated bees.

The most striking result is the great difference in sensitivity to learning impairment between winter and summer bees. Indeed, a significant learning impairment was found from 12 μg kg^{-1} in summer bees, whilst such an effect appeared only at a concentration of 48 μg kg^{-1} in winter bees (Table 5). This difference in the LOEC may reflect a general greater sensitivity of honeybee behaviour in summer, compared to winter, although these bees were kept in a heated apiary. Both types of bee received similar laboratory rearing conditions after emergence, they were tested at the same age, and they were checked to avoid pathologies. Therefore, differences in sensitivity of bees to imidacloprid relied on other parameters. Thus, the quality and amount of pollen available during the larval stage may have differed. Indeed, it has been shown that the degree of sensitivity of the worker bee to pesticides may depend on its pollen diet, at larval and early adult stages.[48] It has been suggested that the increase in sensitivity of bees fed poor pollen is caused by a decrease in enzymatic detoxification of the pesticide. In our study, it is conceivable that winter bees emerging from colonies maintained in a heated room, artificially fed a rich pollen supply (40 plant species; Loublier Y, pers comm), are less sensitive than summer bees emerging from outdoor colonies, and fed stored pollen with a narrow range of floral origin (eg the work by Louveaux reported that the pollen gathered by foragers in July consisted mainly of that from chestnut).[49]

Table 7. Nominal concentration thresholds of imidacloprid on lethal and sublethal parameters in winter and summer honeybees (Experiments 1 and 2)

Parameters	*Thresholds*	*Winter bees (Experiment 1) (μg kg^{-1})*	*Summer bees (Experiment 2) (μg kg^{-1})*
Chronic toxicity	NOEC[a]	24	48
	LOEC[b]	48	96
Reflex responses	NOEC	>48	24
	LOEC	—	48
Conditioned responses	NOEC	24	6
	LOEC	48	12

[a] NOEC: no observed effect concentration.
[b] LOEC: lowest observed effect concentration.

We mentioned above physiological differences between winter and summer bees, leading to a greater chronic mortality in winter bees. However, when considering the learning performance, summer bees appeared much more sensitive to imidacloprid. These contradictory data may be accounted for by the fact that long-term exposure to a toxicant would induce higher mortality in winter bees, but a good tolerance to the toxicant in the surviving individuals. Summer bees would survive better a chronic exposure, but in a state which significantly affected their performance in a learning task. These results emphasise the need to combine the recording of lethal and sublethal effects in the interpretation of the toxicity of a chemical. When the lethal effect of a compound is not obvious, additional testing could give information on the mechanisms that it may possibly disrupt.

In the case of imidacloprid, as the compound is more likely to be found by the bees on summer crops, attention would be drawn mainly on the effects found in summer bees. It appears that in these bees, mortality appears only for high and unrealistic concentrations of the product. However, a behavioural effect can be found at a concentration potentially encountered in plant tissues. In a greenhouse assay, the imidacloprid residues were examined in pollen and nectar of sunflowers which seeds were dressed with radiolabelled imidacloprid. Imidacloprid was detected in nectar and pollen at concentrations of 1.9 and 3.3 μg kg^{-1} respectively.[6] Moreover, Wallner *et al.*[42] showed that nectar sampled in honey gut of foragers visiting *Phacelia* plants treated with imidacloprid contained between 3 μg kg^{-1} and 10 μg kg^{-1} of imidacloprid residues. Considering all these studies, the highest concentration of imidacloprid potentially found in nectar of plant treated is established at 10 μg kg^{-1}. Our study shows that an effect can be found on the learning abilities of summer bees in the

range of 6 to 12 $\mu g\,kg^{-1}$. Therefore we cannot exclude that foragers visiting imidacloprid seed-dressed sunflowers may encounter an amount of insecticide residue that can affect their learning abilities, although such a possibility remains questionable (eg Schmuck *et al*[7] consider that a sunflower seed dressing with imidacloprid poses no risk to honeybees).

It remains to be determined whether a decrease in the olfactory learning ability as detected in the PER assay would significantly affect the foraging behaviour in such a way that bee populations would suffer severely. Preliminary studies indicate that the decrease in learning performance induced by imidacloprid observed at the individual level in the PER assay is confirmed at the colony level in an olfactory discrimination task.[31] Moreover the sublethal effects of imidacloprid on the PER can be related to a reduction in the foraging activity and to changes in the dancing behaviour, when sucrose solution containing imidacloprid at a concentration higher than 20 $\mu g\,kg^{-1}$ is delivered to foraging bees.[50] Further work is still needed to establish a better correlation between the behavioural responses observed under laboratory conditions and those observed in field studies.

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