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Effects of imidacloprid and deltamethrin on associative learning in honeybees under semi-field and laboratory conditions

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Abstract

We have compared the sublethal effects of two insecticides in the honeybee (imidacloprid and deltamethrin) in both semi-field and laboratory conditions. A sugar solution containing 24 μg kg^{-1} of imidacloprid or 500 μg kg^{-1} of deltamethrin was offered to a colony set in an outdoor flight cage. In contrast to imidacloprid, deltamethrin had lethal effect on workers bees. The contamination of syrup with imidacloprid or deltamethrin induced a decrease in both the foraging activity on the food source and activity at the hive entrance. Negative effects of imidacloprid were also observed in an olfactory learnt discrimination task. Free-flying foragers were taken from the contaminated feeder and subjected to a conditioned proboscis extension response (PER) assay under laboratory conditions. As with free-flying bees, no impact of deltamethrin was found on the learning performances of restrained individuals in the PER procedure, whilst significant effects were found with imidacloprid in both semi-field and laboratory conditions.

Keywords: *Apis mellifera*; Imidacloprid; Deltamethrin; Sublethal effect; Associative learning; Olfactory conditioning

1. Introduction

In the course of foraging behavior in the Honeybee (*Apis mellifera* L.), a learning process occurs during which floral parameters such as location, shape, color and odor of flowers are associated to a food reward (nectar or pollen; Menzel and Müller, 1996). Floral odors play a prominent part in the recognition of effective food sites (Menzel et al., 1993). Olfactory processing has been investigated using free-flying foragers visiting artificial flower feeders (Waller, 1972; Greggers and Menzel, 1993; Mauelshagen and Greggers, 1993; Pham-Delègue et al., 1993; Laloi et al., 2000) or using restrained worker bees in the conditioned proboscis extension response (PER) assay (Kuwabara, 1957; Takeda, 1961; Vareschi, 1971; Bitterman et al., 1983; Sandoz et al., 1995). The use of artificial flower feeders allows to simulate a natural foraging situation more closely than does the laboratory tests on restrained worker bees using the conditioned PER procedure. However, although carried out under unnatural conditions, the conditioning of PER has given results that correlated well in terms of time course of memory and olfactory discrimination abilities with the response of free-flying foragers (Mauelshagen and Greggers, 1993; Pham-Delègue et al., 1993; Laloi et al., 2000). Besides the use of this paradigm to study learning and memory processes (Erber, 1981; Menzel, 1990; Smith, 1991), the conditioned PER can be used to assess the sublethal effect of chemicals on the olfactory learning abilities of the honeybee (Taylor et al., 1987; Mamood and Waller, 1990; Stone et al., 1997; Abramson et al., 1999; Decourtye et al., 2003). Evermore, the development of the PER assay as a standardized regulatory method to evaluate the effects of pesticides on the honeybee has been proposed (Decourtye and Pham-Delègue, 2002; Pham-Delègue et al., 2002). However, the transposition of sublethal effects observed in restrained bees, establishing the PER assay under laboratory conditions, to free-flying foraging bees under more natural conditions remains questionable. To address this question, we have

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compared responses of insecticide treated honeybees first allowed to fly freely in an olfactory discrimination task, and then subjected to the PER assay.

The insecticides tested were imidacloprid and deltamethrin. Imidacloprid is a chloronicotinyl insecticide which has a highly specific affinity to the nicotinic acetylcholine receptor of insects (Matsuda et al., 2001). It shows a high insecticidal activity on a wide range of agricultural insect pests, such as aphids, scale insects, whiteflies, leafhoppers, some Coleoptera and some Lepidoptera species (Elbert et al., 1991; Mullins, 1993). This compound has excellent systemic properties and its various applications include granules, pills, foliar spray or seed dressing (Pflüger and Schmuck, 1991). Imidacloprid is the active ingredient of Gaucho® formulation, which is especially used as a sunflower seed coating. As honeybees actively forage on sunflower, and small quantities of imidacloprid were reported in sunflower nectar (Schmuck et al., 2001) several studies were carried out in laboratory, semi-field or field conditions to test sublethal effects of imidacloprid on bees (Kirchner, 1999; Schmuck, 1999; Colin et al., 2000; Curé et al., 2000; Suchail et al., 2000). We have previously showed that subchronic oral treatments with imidacloprid produced a deficit of olfactory learning performances using the PER assay under laboratory conditions (Decourtye et al., 2003).

Deltamethrin is a type II pyrethroid (Soderlund and Bloomquist, 1989). Its principal molecular mode of action is the modification of the sodium channel kinetics leading to hyperexcitation of the nervous system (Narahashi et al., 1992). Deltamethrin was chosen because it proved highly toxic to honeybees in acute toxicity tests (Atkins et al., 1981; Faucon et al., 1985b) and several studies reported that this insecticide induced sublethal effects during the foraging behavior in the honeybee. More precisely, the decrease of foraging activity was noted on fields treated with Décis® formulation which contains the active ingredient deltamethrin (Bocquet et al., 1980; Faucon et al., 1985a; Florelli et al., 1987). Bos and Masson (1983) demonstrated that this repellent effect could be attributed to additives of Décis® rather than to active ingredient itself. Also, sublethal doses of deltamethrin were found to disrupt the homing-flight of foragers (Vandame et al., 1995).

2. Materials and methods

2.1. Insects

Experiments were conducted with a colony of Italian honeybees (*Apis mellifera ligustica* L.) with about 4000 workers and a fertile 1-year-old queen. Honeybees were confined in a 10-comb Dadant hive with 3 combs (one brood comb, one honeycomb and one empty comb). The combs in each colony were positioned near the middle of the hive body with a division board on each side. Honeybees were purchased from a beekeeping company (Pasini, Italy). These colonies have received sanitary control and had received no chemical treatments (e.g. varroacide) for at least 4 weeks prior to experiments. The experimental colonies were maintained in an outdoor flight cage (2.5 m × 2.5 m, 2 m high) covered with an insect-proof cloth (2 mm × 2 mm mesh) and a ground covered with a double layer of clear polyethylene plastic. Bees were fed on a feeder positioned 1.5 m from the hive entrance, filled with sucrose solution (500 g kg^{-1}, 1% acetone vol./vol.) and pollen. The sucrose solution and pollen were renewed daily except during weekends. The sucrose solution was delivered in a glass bottle set up side down, covered with aluminum paper to avoid exposure to light. The pollen was offered in a sheltered plastic dish. Pollen came from commercial sources. As a control, pollen was analyzed for background contamination with LC-MS/MS technique to detect imidacloprid and its three mains metabolites (hydroxy-imidacloprid, olefin and 6-chloronicotinic acid: limit of detection (LOD) = 5 μg kg^{-1}, limit of quantification (LOQ) = 10 μg kg^{-1}; hydroxy-imidacloprid: LOD = 25 μg kg^{-1}, LOQ = 50 μg kg^{-1}) and with GC/MS method for deltamethrin (LOD = 10 μg kg^{-1}, LOQ = 20 μg kg^{-1}). According to these analyses, the pollen was free of imidacloprid, free of imidacloprid metabolites and free of deltamethrin (Lacassie, unpublished data). The food solution and pollen were removed from the flight cage during each behavioral recording session.

2.2. Chemicals

Technical grade imidacloprid (98%) and deltamethrin (99%) were purchased from Cluzeau Info Labo (France). They were dissolved in acetone and stock solutions of 24 mg kg^{-1} for imidacloprid and 50 mg kg^{-1} for deltamethrin were diluted to final concentrations of 24 and 500 μg kg^{-1}, respectively, in sucrose solution (500 g kg^{-1}). The final concentration of acetone in sucrose solutions was equal to 1% (vol./vol.). Treatment solutions were prepared before each experiment and then stored for up to 2 weeks at −18°C. Each day, food solutions were defrosted at ambient temperature and natural daylight before their use. Treatment with imidacloprid at concentration of 24 μg kg^{-1} was chosen because this concentration corresponds to the lowest observed effect concentration (LOEC) affecting the olfactory learning performances of bees after chronic oral treatment and under laboratory conditions (Decourtye et al., 2003). Deltamethrin administered at a concentration of 500 μg kg^{-1} was chosen because it is the maximum concentration measured in oilseed rape flowers after the spraying of Décis®Micro (6.25%

vol./vol.) with an application rate of 3.75 g a.i. ha^{-1} (Ballanger, unpublished data).

2.3. Flight cage experiments: foraging activity and learning performance

Because the general colony activity and the foraging intensity could vary among colonies, rather than comparing treated and control colonies, we compared responses of honeybees before and after exposure to the insecticide, on the same colony. Thus, three feeding periods were applied: (1) 500 g kg^{-1} sucrose solution (1% acetone vol./vol.) delivered in both the artificial flower feeder and a standard feeder placed in the cage out of the experimental period per se; (2) insecticide-added 500 g kg^{-1} sucrose solution; (3) 500 g kg^{-1} sucrose solution (1% acetone vol./vol.) again. Experiments were conducted in June–July (2000 and 2001).

The foraging activity and the learning performances were evaluated using an artificial flower feeder adapted from the experimental device described by Pham and Masson (1985). This feeder contained six feeding sites distributed on a circular gray tray (50 cm diameter). Each artificial flower was a plastic Petri dish containing glass balls (allowing landing of foragers on the feeding sites) and filled with a sucrose solution contaminated or not by an insecticide. The level of sucrose solution in the Petri dish was maintained as constant. On each side of the feeding sites, an odor could diffuse (pure linalool; 95–97% purity Sigma). To limit the influence of visual or spatial cues, the artificial feeder was rotated slowly $\frac{1}{3}$ rpm. The device was placed 1.5 m from the hive entrance.

To initiate the recruitment of foragers about 100 workers were placed on the artificial feeder. Then, the foragers were conditioned to the linalool associated to the food solution (500 g kg^{-1} sucrose solution or insecticide-added 500 g kg^{-1} sucrose solution) in each of the six artificial flowers. Each bee visiting the device was tagged with a color dot on the thorax. The number of tagged bees on the artificial feeder was noted every 5-min as measure of the foraging activity. When the population of marked foragers was stabilized (about 200 individuals under our conditions), they were subjected to the next steps of the experimental procedure. The conditioning (pairing odor/sucrose reward) was conducted over one day from 14:00 to 16:00 hours GMT. Testing was carried out on the following days from 10:00 to 11:00 h or 14:00 to 15:00 h GMT depending on the meteorological conditions. The testing device was set with 3 scented sites alternating with 3 unscented sites, without any food reward. The testing device was presented for 5 min and then replaced by the conditioning device for 15 min, with the odor being again associated with a sucrose solution (contaminated or not). For each observation (every 30 s over the 5 min observation period), the visits on either the scented sites or the unscented ones were noted. After each test, the tray was cleaned with ethanol and the Petri dishes were changed to avoid the deposition of marking scent. The volume of sucrose solution up taken was measured. Climatic conditions inside the cages were recorded during the experiments. Air temperature has fluctuated between 23°C and 35°C for imidacloprid, and between 27°C and 37°C for deltamethrin study. The sky was most of the time cloudy and wind speed was slight throughout the two studies.

Any dead bees found on the ground was counted and discarded daily except during weekends. All anomalies in development and behavior of the honeybee colonies were recorded. The colonies were visited at the end of each period (before, during and after treatment) by a professional beekeeper to assess brood surface, diseases and food quantities (honey and pollen). Brood area was measured using the ellipse (Fresnaye and Lensky, 1961): $S = \pi \times A/2 \times a/2$, where S was the surface of the comb area, A and a the length of the big and small axes of the comb area.

All along the experiment, a bee counter BeeSCAN (Lowlands Electronics bvba, Belgium) set at the hive entrance evaluated the activity of the colony by measuring the number of bees leaving and entering the hive as a function of time (Struye et al., 1994). Bee movement detection is effected via an infrared beam, which takes place in 32 bi-directional channels. A sampled interval time of 15 min was chosen. At the end of the interval, the bee counter delivers the numbers of incoming and outgoing bees during this interval. A computer interface stores this information. At the end of each week, the data were transferred from counter driver to computer.

2.4. Laboratory experiments: olfactory conditioning of PER

At the end of each experimental period in the outdoor flight cage, color-marked foraging bees were collected on the artificial flower feeder immediately after the testing period and were caged in groups of 30–50 individuals. They were maintained in an incubator at 25 ± 2°C, 40 ± 10% relative humidity, and in the dark. They were starved for 4 h prior to odor conditioning in the PER assay.

The classical odor conditioning of the PER is based on the temporal pairing 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 odor (conditioned stimulus) being simultaneously delivered. The proboscis extension is immediately rewarded by the uptake of the sucrose solution. Bees can develop the PER as a conditioned response to

the odor alone even after a single pairing of the odor with a sucrose reward.

Prior to conditioning, honeybees were selected for showing a proboscis extension reflex after stimulation of the antennae with a $300\,g\,kg^{-1}$ sucrose solution. Bees were then placed in an airflow (main airflow of $50\,mL\,s^{-1}$ added with a secondary flow of $2.5\,mL\,s^{-1}$) for 15 s to be familiarized to the mechanical stimulation and to the experimental context. In the conditioning trials, the conditioned stimulus (10 μL pure phenylacetaldehyde deposited on a filter paper strip and inserted in a Pasteur pipette cartridge; 95% purity Sigma) was delivered through the secondary flow ($2.5\,mL\,s^{-1}$) for 6 s, during which the proboscis extension reflex was elicited after 3 s by contacting the antennae with a $300\,g\,kg^{-1}$ sucrose solution as the unconditioned stimulus. Phenylacetaldehyde was chosen as the conditioned stimulus to avoid the possible interaction between the odor used in the flight cage (linalool) and that used in the PER assay. Indeed, it was previously shown that honeybees are able to discriminate these two compounds in an olfactory learning procedure (Sandoz et al., 2001). Three conditioning trials were carried out at 20 min intervals on average (trials C1–C3). The conditioned proboscis extension was recorded as a yes-or-no response when the odor alone was delivered.

2.5. Statistical analysis

In the flight cage experiments, the number of visits to the scented or unscented sites were compared with the hypothesized equal distribution (50% of foragers on either sites) by χ^2 test (1 df, $P<0.05$).

In the conditioned proboscis extension experiments, the number of reflex responses and the number of conditioned responses (at each conditioning trials) in treated groups and in the control group were compared using χ^2 test in a contingency table procedure (2 df, $P<0.05$). When a significant difference was found, multiple two-by-two comparisons, using χ^2 test (1 df), were conducted with a significance threshold level which was corrected according to Dunn-Sidák method (Sokal and Rohlf, 1995). The significance level was $\alpha' = 1-(1-\alpha)^{1/k}$, where k was the number of intended tests ($\alpha' = 0.016$). When conditions of application of the χ^2 test were not fulfilled according to the Cochran's rule, the Fisher's exact method was applied (Sokal and Rohlf, 1995).

3. Results

3.1. Flight cage experiments: foraging activity and olfactory learning performance

The treatment period with imidacloprid ($24\,\mu g\,kg^{-1}$) did not lead to additional mortality, whereas the number of dead bees found on the ground of the flight cage during the deltamethrin administration ($500\,\mu g\,kg^{-1}$) was about twice higher than before and after this period (Table 1). Moreover, during deltamethrin treatment, neurotoxic symptoms such as trembling and paralysis were observed in honeybees laying on the ground. Three times over, sample of bees affected by these symptoms were collected to see whether they would recover with time, but all the bees died within 4 h. Therefore, feeding honeybees with sucrose solution added with imidacloprid at the concentration of $24\,\mu g\,kg^{-1}$ or with deltamethrin at the concentration of $500\,\mu g\,kg^{-1}$ might be considered as sublethal and lethal treatments, respectively.

Treatment-related difference was found in the syrup consumption rates (Table 2). Indeed, the addition of imidacloprid or deltamethrin in the food solution

Table 1
Mortality[a] in relation to treatment

Chemicals	Before treatment	Treatment	After treatment
Imidacloprid	70.0±16.4 ($n=5$)[b]	57.7±25.9 ($n=4$)	83.4±31.9 ($n=3$)
Deltamethrin	74.9±22.2 ($n=4$)	156.1±20.9 ($n=4$)	88.0±18.8 ($n=4$)

[a] Data represent mean number of dead workers bees per day (±SEM) which were found on ground of flight cage.
[b] Number of days where mortality was recorded.

Table 2
Consumption[a] of sucrose solution (mL) distributed by the artificial flower feeder

Chemicals	Before treatment	Treatment	After treatment
Imidacloprid	186.0±39.3 ($n=6$)[b]	57.9±9.7 ($n=5$)	38.2±5.3 ($n=5$)
Deltamethrin	93.2±20.2 ($n=5$)	30.7±8.0 ($n=4$)	74.0±14.1 ($n=4$)

[a] Data represent mean volume of syrup consumption per day (±SEM).
[b] Number of days where consumption was recorded.

induced a decrease of consumption by a factor of three compared to the initial period of treatment. This decrease was limited to the treatment period for deltamethrin and lasted after the end of imidacloprid administration.

Considering the brood production, there was a decrease of comb area containing capped brood between the beginning and the end of experiments with the two chemicals studied (Table 3). The reduction of brood size was higher in imidacloprid study. Moreover, the last control visit has revealed irregular capped brood area in both colonies, which may indicate an adverse effect on brood-rearing capabilities of honeybees. The honey and pollen stores in the colony exposed to imidacloprid were strongly reduced at the end of experimental period (absence of uncapped honey and pollen stores), but deltamethrin had no effect on food stores.

Foraging activity on the artificial flower feeder showed that imidacloprid and deltamethrin had a similar repellent effect (Fig. 1A and B). From the beginning of the feeding period with imidacloprid or deltamethrin, a strong decrease was shown in the number of foraging bees in comparison to that observed before the addition of sucrose solution with chemicals. Low foraging activity was prolonged throughout the overall period of imidacloprid or deltamethrin application (i.e. 7 and 8 days, respectively). After the treatment with deltamethrin, return to feeding with control sucrose solution resulted in an increase of foraging bees encountered on the feeder (Fig. 1B). In contrast, low level of foraging was still observed despite the removal of imidacloprid-added solution (Fig. 1A).

Before the treatments, when the foraging bees were offered a choice between sites releasing the odor (linalool) and unscented sites (Fig. 2A and B), the number of visits to the odor (94–96% of landings on scented sites) was significantly higher than hypothesized equal distribution of landings (χ^2, 1 df, $P<0.001$), showing that foragers were conditioned to the odor. When the control solution was replaced by imidacloprid-added solution (Fig. 2A), the percentage of foragers visiting the scented sites was strongly reduced (60% of landings on scented sites). Although there was a decrease of olfactory discrimination performance in

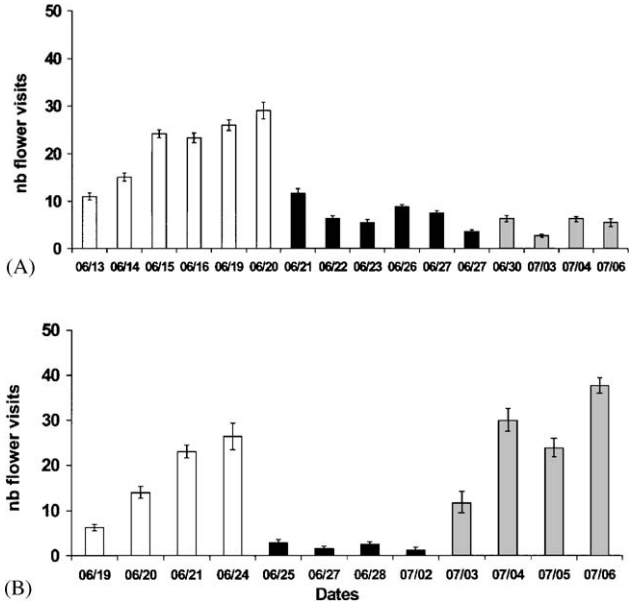

Fig. 1. Foraging activity of honeybees on artificial flower feeder in relation to treatment with imidacloprid (A), and deltamethrin (B). Bars give the mean (± SEM) number of foraging bees which were recorded on the feeding sites. White bars control sucrose solution before treatment period; black bars: imidacloprid-added sucrose solution (24 μg kg^{-1}) or deltamethrin-added sucrose solution (500 μg kg^{-1}); gray bars: control sucrose solution after treatment period.

Table 3
Development of the colony over the time in relation to treatment with imidacloprid (24 μg kg^{-1}) and deltamethrin (500 μg kg^{-1})

Parameters	Imidacloprid			Deltamethrin		
	Before treatment (21/06/00)[a]	Treatment (28/06/00)	After treatment (07/07/00)	Before treatment (18/06/01)	Treatment (02/07/01)	After treatment (09/07/01)
Comb area containing capped brood (cm^2)	850.5[b]	966.8	534.0	1423.9	964.4	919.9
Eggs	+[c]	+	+	+	+	+
Larvae	+	+	+	+	+	+
Uncapped honey	+	+	0	+	+	+
Capped honey	+	+	+	0[d]	0	+
Pollen	+	+	0	+	0	0
Remarks			Irregular brood			Irregular brood

[a] Date where control visit of colony was carried out.
[b] Comb area were calculated with ellipse method (Fresnaye and Lensky, 1961).
[c] Symbol indicates the presence of parameter considered.
[d] Symbol indicates the absence of parameter considered.

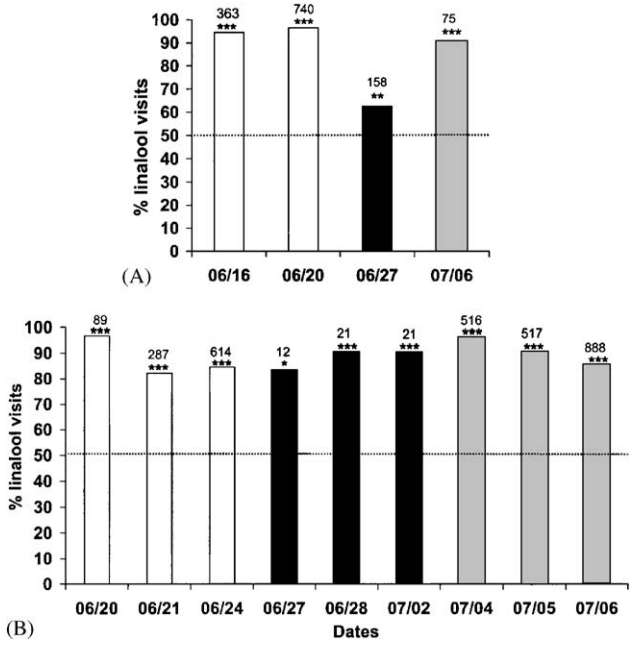

Fig. 2. Olfactory learning performance of free-flying foragers in relation to treatment with imidacloprid (A), and deltamethrin (B). Bars give the percentage of foragers visiting scented sites of the artificial flower feeder. White bars: control sucrose solution before treatment period; black bars: imidacloprid-added sucrose solution ($24\,\mu g\,kg^{-1}$) or deltamethrin-added sucrose solution ($500\,\mu g\,kg^{-1}$); gray bars: control sucrose solution after treatment period. The total number of foragers is indicated above the bars. The observed numbers of visits were compared to hypothesized equal distribution of landings on the scented sites and unscented sites, shown as the 50% line ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).

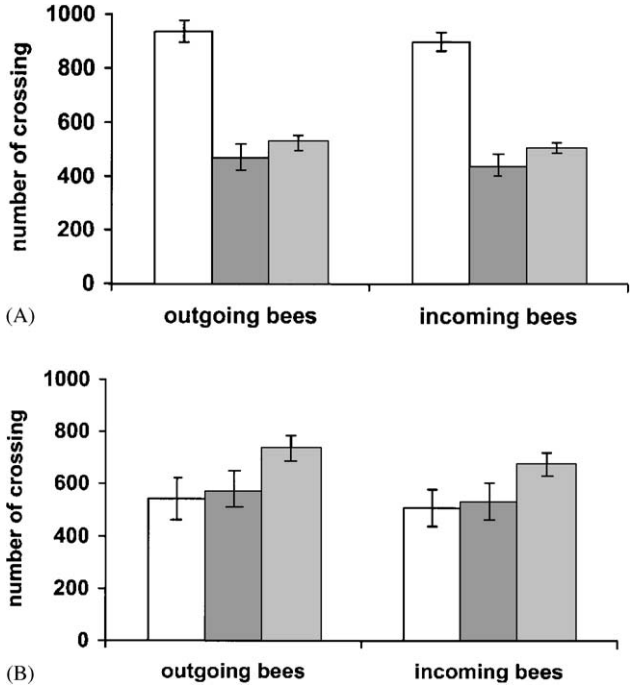

Fig. 3. Flight activity of the colony registered by bee counter in relation to treatment with imidacloprid (A), and deltamethrin (B). Bars give the mean (± SEM) number of crossing which were recorded by the counter (1 h per day for testing period and 2 h per day for conditioning period). White bars: control sucrose solution before treatment period; black bars: imidacloprid-added sucrose solution ($24\,\mu g\,kg^{-1}$) or deltamethrin-added sucrose solution ($500\,\mu g\,kg^{-1}$); gray bars: control sucrose solution after treatment period.

imidacloprid-treated foragers, the number of landings on scented sites was significantly higher than a randomized distribution between scented and unscented sites (χ^2, 1 df, $P<0.01$). Going back to the control solution, the foragers showed again a high level of olfactory discrimination performance (90% of landings on scented sites; χ^2, 1 df, $P<0.001$). In the deltamethrin study (Fig. 2B), the sites scented with linalool were highly discriminated from unscented sites during all experimental periods (82–96% of landings on scented sites; χ^2, 1 df, $P<0.05$).

The activity evaluated by the counter at the hive entrance of the colony treated with imidacloprid showed an evolution similar to that of the foraging activity (Fig. 3A). Thus, the number of outgoing and incoming bees was greater before the treatment with imidacloprid than during treatment period. This result indicates that the colony activity depended on the quality of the food provided on the artificial flower feeder. Imidacloprid-added solution inducing a reduction of activity at the hive entrance. Different colony behavior was observed with deltamethrin (Fig. 3B). Although a clear reduction of forager visits on the artificial flower feeder occurred no changes in the activity at the hive entrance were noted during the delivery of a food solution added with deltamethrin.

3.2. Laboratory experiments: olfactory conditioning of PER

In restrained foraging bees subjected to the PER assay, the number of conditioned responses differed according to the feeding period in the flight cage (Fig. 4A). The feeding of foragers with sucrose solution contaminated with imidacloprid at the concentration of $24\,\mu g\,kg^{-1}$ induced significantly lower responses compared to the foragers collected before the imidacloprid treatment (trials C3: χ^2, 1 df, $P<0.016$). The reduction of olfactory learning performance was also noted in foraging bees collected 9 days after the end of imidacloprid treatment (trials C2 and C3: χ^2, 1 df, $P<0.016$). At the opposite, in all trials the level of responses of foragers fed deltamethrin-added solution was equivalent to that obtained with foragers fed control solution (χ^2, 2 df, $P>0.05$; Fig. 4B). Thus, there was a good concordance between results in free-flying foragers under semi-field conditions and those in restrained foragers under laboratory conditions: de-

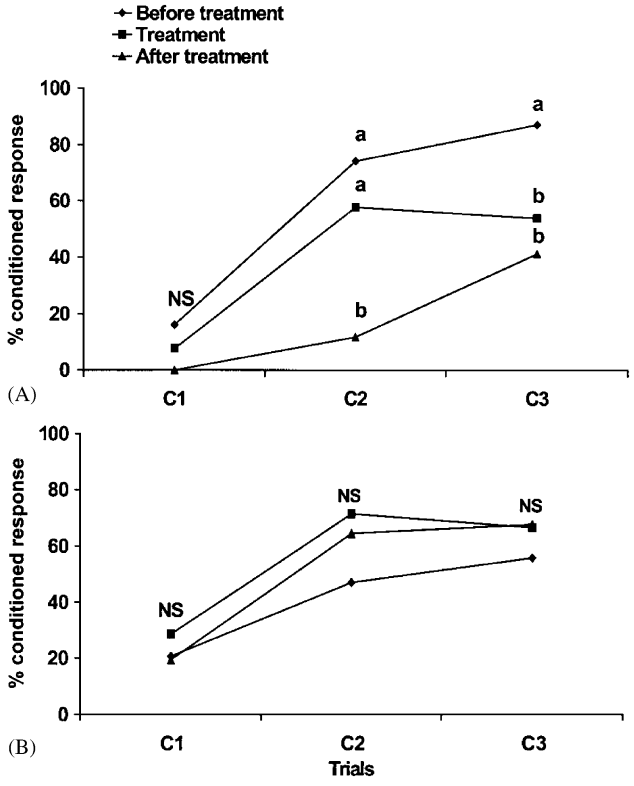

Fig. 4. Olfactory learning performance of restrained foragers bees during olfactory conditioning of PER in relation to treatment with imidacloprid (A), and deltamethrin (B). Number of bees per treatment group: 17–31 in imidacloprid study, 21–34 in deltamethrin study. The number of conditioned responses obtained during the conditioning trials (C2–C3, C1 showing the level of spontaneous response) was compared among experimental period using χ^2 test (2 df, $P<0.05$; NS: non-significant), followed by multiple two-by-two comparisons using χ^2 test or Fisher's exact method (1 df). Different letters indicate significant differences in this test (corrected significance threshold $\alpha' = 0.016$).

crease of learning performances with imidacloprid, but not with deltamethrin.

The comparison of the number of reflex responses obtained when the antennae were contacted with a sucrose solution, in treated and control bees, was used to evaluate the effects of the tested insecticides on the gustatory and motor functions of the PER. Only the foraging bees collected after removal of the solution containing imidacloprid showed significant decrease in PER rates (52–58% of reflex responses) in comparison with responses recorded before and during treatment (90–100% of reflex responses; from trial C1 to C3: χ^2, 1 df, $P<0.016$). A disruption of sensory-motor activity underlying PER in foragers tested after the end of imidacloprid treatment might be the basis of the decrease observed in conditioned responses level (Fig. 4A). For deltamethrin, the foraging bees collected during the three experimental periods (before, during and after treatment) presented similar PER rates (76–95% of reflex responses; from trial C1 to C3: χ^2, 2 df, $P>0.05$).

4. Discussion

To compare the sublethal effects observed in laboratory conditions to free-flying foraging bees under more natural conditions, we have evaluated responses of insecticide-treated honeybees first allowed to fly freely in an olfactory discrimination task, and then subjected to the conditioning of PER assay. For the two insecticides tested, imidacloprid and deltamethrin, a good concordance was demonstrated between the assessment of toxicity on the olfactory learning performances under semi-field conditions, in free-flying foragers, and those obtained in laboratory, in restrained foragers. After feeding with sucrose solution contaminated with imidacloprid at the concentration of $24\,\mu g\,kg^{-1}$, negative effects on the learning performances were observed in foraging bees subjected to both olfactory conditioning procedures. The behavioral effects of imidacloprid are in accordance with previous works reporting the negative effect of imidacloprid on the learning during a conditioning of PER (Decourtye et al., 2003).

In contrast to imidacloprid, both behavioral assays indicated no difference between the performance of foragers collected during exposure to deltamethrin at the concentration of $500\,\mu g\,kg^{-1}$ and those collected before or after the treatment. Thus, the hypothesis that using the observation of free-flying foragers under semi-field conditions in complement to the recording of conditioned PER in laboratory conditions would reveal similar toxicological profile for imidacloprid and deltamethrin was clearly demonstrated. Such correlation between responses observed in restrained bees subjected to the PER procedure and free-flying bees foraging on an artificial flower feeder was previously shown on the time course of memory and olfactory discrimination abilities (Mauelshagen and Greggers, 1993; Pham-Delègue et al., 1993; Laloi et al., 2000).

We should take notice that the deltamethrin treatment has a lethal character. Indeed, the number of dead bees counted on the ground of the flight cage increased under deltamethrin treatment, but not under imidacloprid treatment. Thus, the exposure to deltamethrin can result in a selection of worker bees staying alive because they are less sensitive to this chemical. Such resistant bees may give responses in the sublethal toxicity assessment not representative from that on the whole of population. To correct this fact, further experiments following the same experimental setup as the one described in this paper could be conducted with lower concentrations of deltamethrin.

Considering the consumption of syrup contaminated and the number of foragers visiting the artificial flower feeder, we can estimate the dose of chemical received per forager and per day: about 10 ng for imidacloprid, which correspond to LD50 (Decourtye et al., 2003) divided by three, and about 700 ng for deltamethrin,

which correspond to LD50 (Decourtye, 2002). Thus, the assessment of the dose of chemical received per forager is in accordance with the sublethal characteristic of imidacloprid treatment and the lethal characteristic of deltamethrin treatment. Moreover, neurotoxic symptoms such as trembling and paralysis ("knock-down" effect) were observed during deltamethrin treatment. Similar behavior after deltamethrin application has been classically described in laboratory and field experiments (Faucon et al., 1985a, b). The significantly increased mortality and the "knock-down" effect in the deltamethrin-exposed foragers might lead to a reduction of the foraging activity, since it could reduce the proportion of effective foragers. Unlike deltamethrin, the avoidance behavior observed with imidacloprid would mainly result from an antifeedant/repellent effect but not to a lethal or "knock-down" effect. A similar negative effect on the feeding behavior was reported for sucrose solutions contaminated with imidacloprid in aphids (Nauen and Elbert, 1997) and in honeybees (Nauen et al., 2001). In semi-field or field conditions, several authors reported that foraging bees reduced their visits to a syrup feeder when it was contaminated with, respectively, $2\,mg\,kg^{-1}$ (Mayer and Lunden, 1997), $100\,\mu g\,kg^{-1}$ (Kirchner, 1999) and $50\,\mu g\,kg^{-1}$ of imidacloprid (Colin et al., 2000). We found that the lowest adverse-effect concentration of imidacloprid on the foraging behavior on an artificial flower feeder was $24\,\mu g\,kg^{-1}$. Interestingly, a recovery of the foraging activity occurred after deltamethrin treatment, whilst the activity on the feeder remained low after imidacloprid treatment. This might rely on the fact that the lethal effect induced by deltamethrin killed a part of the foragers visiting the contaminated source but did not affect the renewal of naive foragers visiting the feeder after treatment. In the case of imidacloprid delivery, foragers got a negative experience during treatment, but were not killed. This population associated on a long-term basis the feeder to a negative reward and avoided it even when noncontaminated food was again delivered. They survived on stored food, as could be seen by the reduction of food surface into the hive.

The activity of the colony registered by the bee counter at the hive entrance decreased when the feeder provided food contaminated with imidacloprid, whereas similar activity was obtained before and during the delivery of deltamethrin-added food. Thus, the number of incoming and outgoing bees was not always directly correlated with the foraging activity on the feeder device, which was previously reported under field conditions (Struye et al., 1994). Therefore, it might be assumed that deltamethrin acted only at the foragers level via its paralyzing and lethal action, whereas imidacloprid acted at both the level of foragers behavior (avoidance behavior, learning deficit) and of the colony (reduction of activity). The impact of imidacloprid on the behavior of workers inside the colony has already been tested. Under field conditions, Kirchner (1999) investigated the effect of sucrose solutions containing imidacloprid at concentrations ranging from 20 to $100\,\mu g\,kg^{-1}$ on the recruitment dances. The author described modifications of dance frequencies with imidacloprid concentration of $20\,\mu g\,kg^{-1}$ and above. With regard to the short distance between the hive and the feeder in our flight cage experiment (1.5 m), the presence of food resource close to the hive is communicated to the other bees by round dances which are not directional (von Frisch, 1967). Thus, the slowing down of the activity at the hive entrance and of the recruitment activity which were noted during imidacloprid treatment might be due to decrease of effectiveness of the round dances produced to stimulate bees to search for a food source.

Our experimental results can tentatively be related to the field situation of bees exposed to deltamethrin and imidacloprid. For deltamethrin, a realistic concentration (maximum concentration measured in oilseed rape flowers after the spraying of Décis®Micro) was used suggesting that in treated fields, foraging bees could suffer from nervous symptoms and finally die. We may assume that the nervous symptoms occurring immediately after an exposure of the foragers to deltamethrin could limit the chemical transfer inside the hive, since we have noted no alteration in the development of colony during and after the treatment period. For imidacloprid, the experimental concentration is 2–10 times higher than the maximal concentration of imidacloprid potentially found in the nectar of plants after seed coating with Gaucho® insecticide (Wallner et al., 1999; Schmuck et al., 2001). Although a significant decrease in the attractiveness of the food source was found at this concentration, no evidence of such repellent effect has been reported experimentally for lower realistic concentrations. Additional experiments are needed to establish the threshold concentration from which the foraging behavior could be affected and possibly induce drastic bee population losses as observed by French beekeepers in colonies foraging on sunflower treated with Gaucho® (Belzunces and Tasei, 1997).

5. Conclusions

Although correlation of data gained in laboratory and field studies remains difficult to obtain (Smirle et al., 1984), we showed that the behavioral toxicity of imidacloprid observed in laboratory conditions at individual level (conditioned PER assay) was consistent with results obtained in semi-field experiments at colony level. Therefore, we assume that the PER assay should be a useful tool to investigate the behavioral effects of toxicants preferentially to more natural approaches,

such as studies in semi-field conditions, because it allows a better control of treatment and conditioning parameters. It remains to establish whether the use of the conditioned PER as a measure of the sublethal effects of pesticides on honeybees can be a reliable indicator of the hazards associated with the exposure to sublethal doses of toxic in field conditions.

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