Arch. Environ. Contam. Toxicol. 48, 242–250 (2005)
DOI: 10.1007/s00244-003-0262-7

ARCHIVES OF
Environmental
Contamination
and Toxicology

Comparative Sublethal Toxicity of Nine Pesticides on Olfactory Learning Performances of the Honeybee *Apis mellifera*

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Received: 4 January 2004 / Accepted: 23 June 2004

Abstract. Using a conditioned proboscis extension response (PER) assay, honeybees (*Apis mellifera* L.) can be trained to associate an odor stimulus with a sucrose reward. Previous studies have shown that observations of conditioned PER were of interest for assessing the behavioral effects of pesticides on the honeybee. In the present study, the effects of sublethal concentrations of nine pesticides on learning performances of worker bees subjected to the PER assay were estimated and compared. Pesticides were tested at three concentrations. The highest concentration of each pesticide corresponded to the median lethal dose value (48-h oral LD50), received per bee and per day, divided by 20. Reduced learning performances were observed for bees surviving treatment with fipronil, deltamethrin, endosulfan, and prochloraz. A lack of behavioral effects after treatment with λ-cyalothrin, cypermethrin, τ-fluvalinate, triazamate, and dimethoate was recorded. No-observed-effect concentrations (NOECs) for the conditioned PER were derived for the studied pesticides. Our study shows that the PER assay can be used for estimating sublethal effects of pesticides on bees. Furthermore, comparisons of sensitivity as well as the estimation of NOECs, useful for regulatory purposes, are possible.

The hazard assessment of pesticide toxicity to honeybees (*Apis mellifera* L.) is commonly estimated from laboratory studies (median lethal dose: LD50) and from semifield and field experimentations when the pesticides demonstrate a hazard quotient (application rate/LD50) over 50, or when they have a specific mode of action (*e.g.*, insect growth regulators), or when there are indications of indirect effects such as delayed action (EPPO 1992). Because behavioral effects of pesticides in the honeybee have been shown to have the potential to induce a significant impact on the development of colonies (Waller *et al.* 1984; Bendahou *et al.* 1999; Decourtye *et al.* 2004a), such effects also could be used to better estimate the hazard of pesticides to bees. Moreover, it is noteworthy that the EPPO guidelines require recording all abnormal behavioral effects observed during the experiments (EPPO 1992).

Semifield tests, representing more realistic exposure conditions than in laboratory, have been cited as providing good information for the behavioral toxicity assessment of pesticides (Cluzeau 2002). However, the regulatory guidelines give only very limited information on the type of behavioral data that should be collected during the studies or how they should be included and interpreted in the risk assessment scheme (Thompson and Brobyn 2002). Moreover, the semifield tests, even if they are well suited, are technically difficult to maintain and control. Their fluctuating conditions, cost, and the necessity to have trained people to carry them out are bounds limiting the number of facilities able to perform them in practice. Thus, the identification of precise behavioral effects requires additional and specific methods to make appropriate hazard assessment (Pham-Delègue *et al.* 2002). Consequently, the conditioned proboscis extension response (PER) assay should be use to overcome these problems (Decourtye and Pham-Delègue 2002).

The PER assay tentatively reproduces what happens in honeybee–plant interaction: when landing on the flower, the forager extends its proboscis as a reflex when the gustatory receptors set on the tarsae, antennae, or mouth parts are stimulated with nectar. This reflex leads to the uptake of nectar and induces the memorization of the floral odors diffusing concomitantly. Once memorized, the odors play a prominent role in flower recognition during the next trips (Menzel *et al.* 1993). Consequently, an individual associative learning process is important for the effective accomplishment of foraging activities. The associative learning of workers, investigated with the PER assay, may therefore be regarded as having a high ecological significance because it is a prerequisite to the foraging success of the whole colony.

The PER has been successfully reproduced under artificial conditions (Kuwabara 1957; Takeda 1961), and has become a valuable tool in studying various aspects of olfactory learning

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Table 1. Concentrations of agricultural chemicals applied with subchronic exposure before the conditioning procedure

Chemical	Purity	48-h oral LD50 (per bee)	Concentrations in stock solutions	Tested doses in sucrose solutions (per bee per day)	Tested concentrations in sucrose solutions
Deltamethrin	>98%	620 ng (Decourtye 2002)	940 mg · L^{-1}	30 ng	940 μg · L^{-1} [a]
				15 ng	470 μg · L^{-1}
				7.5 ng	235 μg · L^{-1}
λ-Cyalothrin	98.5%	241 ng (Decourtye 2002)	360 mg · L^{-1}	12 ng	360 μg · L^{-1}
				6 ng	180 μg · L^{-1}
				3 ng	90 μg · L^{-1}
Cypermethrin	98.5%	460 ng (Vaughan 1986)	690 mg · L^{-1}	23 ng	690 μg · L^{-1} [b]
				11.5 ng	345 μg · L^{-1}
				5.75 ng	172.5 μg · L^{-1}
τ-Fluvalinate	76%	>200 μg (Barnavon 1987)	3 g · L^{-1}	10 μg	300 mg · L^{-1}
				5 μg	150 mg · L^{-1}
				2.5 μg	75 mg · L^{-1}
Prochloraz	97%	100 μg (Chalvet-Monfrey 1996)	1.5 g · L^{-1}	5 μg	150 mg · L^{-1}
				2.5 μg	75 mg · L^{-1}
				1.25 μg	37.5 mg · L^{-1}
Triazamate	97%	400 ng (Miniggio *et al*. 1990)	600 mg · L^{-1}	20 ng	600 μg · L^{-1}
				10 ng	300 μg · L^{-1}
				5 ng	150 μg · L^{-1}
Endosulfan	97.3% (65.6% α isomer; β isomer)	5 μg (Stevenson 1978)	8 mg · L^{-1}	250 ng	8 mg · L^{-1}
				125 ng	4 mg · L^{-1}
				62.5 ng	2 mg · L^{-1}
Dimethoate	98.5%	350 ng (Gough *et al*. 1994)	580 mg · L^{-1}	17.5 ng	580 μg · L^{-1}
				8.7 ng	290 μg · L^{-1}
				4.3 ng	145 μg · L^{-1}
Fipronil	98.5%	6 ng (Decourtye 2002)	9 mg · L^{-1}	0.3 ng	9 μg · L^{-1}
				0.15 ng	4.5 μg · L^{-1}
				0.075 ng	2.2 μg · L^{-1}

[a] Actual concentrations of deltamethrin equal to 960, 429, and 212 μg · L^{-1}.
[b] Actual concentrations of cypermethrin equal to 782, 388, and 207 μg · L^{-1}.

processes (Bitterman *et al.* 1983; Menzel *et al.* 1993; Sandoz *et al.* 1995). The PER assay with restrained workers has also been used to investigate the behavioral effects of pesticides (Taylor *et al.* 1987; Mamood and Waller 1990; Stone *et al.* 1997; Abramson *et al.* 1999; Abramson and Boyd 2001; Weick and Thorn 2002; Decourtye *et al.* 2003; Abramson *et al.* 2004).

A previous work studying the behavioral toxicity of imidacloprid and deltamethrin to bees indicated that a good relationship was found between effects on olfactory responses in free-flying foragers and in individuals subjected to the PER paradigm (Decourtye *et al.* 2004a). The controlled conditions, the relationship with field conditions, and the ability to quantify the behavior pattern numerically led us to assume that the use of the PER assay, as a method to evaluate the potential effect of pesticides on the honeybees foraging behavior, could help us to assess the toxicity of pesticides in a more comprehensive way than by only considering lethality as currently made in practice (Devillers 2002). However, a survey of the literature showed that only a limited number of chemicals had been tested, and the studies using the PER assay were usually not directly comparable because they were based on different methods for the administration of chemicals, the behavioral response, and so on. Moreover, in these works only one dose, not necessarily sublethal, was generally tested.

To confirm the usefulness of the PER assay as a behavioral toxicity assessment method, the goal of our study was to compare the effects of sublethal exposure of nine pesticides on the olfactory learning performances of worker bees subjected to this assay.

Materials and Methods

Pesticides

The nine studied pesticides (Table 1) were all technical grade. Deltamethrin and prochloraz were obtained from Hoechst Schering AgrEvo S.A. (Aventis CropScience, France). All the other compounds were purchased from Cluzeau Info Labo (Sainte-Foy-La-Grande, France). Their purity was at least 98%, except τ-fluvalinate, which had a purity of 76%.

The pesticides were tested at three different concentrations, with a geometrical progression of factor 2. The highest tested concentration corresponded to the median lethal dose value (LD50 determined 48 h after the oral treatments) divided by 20 (Table 1). From previous results (Decourtye *et al.* 2003), it was assumed that this ratio belonged to a sublethal domain. The 48-h LD50s reported in Table 1 were previously determined from acute oral toxicity tests for deltamethrin, λ-cyalothrin, and fipronil (Decourtye 2002), and from information

gained in the existing literature for the other chemicals. The concentrations were calculated for a consumption of syrup estimated to 33 µl/bee/day (Decourtye *et al.* 2003).

Stock solutions with a given concentration of each chemical were prepared in acetone (Table 1). Acetone was chosen following the EPPO guidelines, because it is a rather generalist solvent (EPPO 1992). Aliquots of the stock solutions were used to make each test solution at a specific concentration. The chemicals were added to a 500 g L^{-1}sucrose solution. The final concentration of acetone in the sucrose solutions was 1% (vol/vol). The effects of insecticide-added solutions were compared with that of an untreated sucrose solution (with 1% acetone vol/vol). Fresh dosing solutions were prepared for each test.

Samples of contaminated sucrose solutions of deltamethrin and cypermethrin delivered to bees were analyzed by gas chromatography/mass spectrometry (K. Le Menach and H. Budzinski, unpublished).

Honeybees

Experiments were carried out with worker bees of *Apis mellifera ligustica* L. They were conducted with bees collected from outdoor hives. 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 2 first days and with pollen for the next 8 days. After 2 days, bees were continuously fed with sucrose solution contaminated or not during 11 consecutive days. The feeders were changed daily with fresh sucrose solutions. The bees were kept in an incubator (33 ± 2°C, 40 ± 10% relative humidity, darkness) until 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 (Sakagami 1953; Seeley 1982) and give the most consistent performances in the conditioned proboscis extension assay (Pham-Delègue *et al.* 1990).

Protocol

For bees from 2 to 14–15 days old, the quantity of the contaminated sugar solution provided daily was adjusted to the number of survivors. The mortality and consumption of syrup were recorded daily, and dead bees were discarded. Every testing day was organized as follows: bees previously exposed to three concentrations of each chemical were tested, as well as untreated control bees, leading to a total of 60–80 bees tested per day, with 16–20 bees for each treatment. Experiments were replicated at least three times, until about 50–60 bees per treatment were obtained.

After treatment, the bees were mounted individually in glass tubes with only their antennae and mouth parts left 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 $\cdot$ L^{-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. The general stimulation conditions as well as the conditioning and testing procedures were adapted from the work of Bitterman *et al.* (1983) and are detailed in Sandoz *et al.* (1995). Bees were then placed in an airflow (main airflow of 50 ml $\cdot$ s^{-1} added to a secondary airflow of 2.5 ml $\cdot$ s^{-1}) for 15 s, to be familiarized with the mechanical stimulation and with the experimental background. For the conditioning trials, the conditioned stimulus (10 µl of pure linalool, a standard floral odor, 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 $\cdot$ s^{-1}) for 6 s. During odor delivery, the PER was elicited after 3 s by contacting the antennae with a sucrose solution (300 g $\cdot$ L^{-1}) as the unconditioned stimulus, and the same solution was immediately given as a reward, before the odor delivery ended. Three successive conditioning trials (Cond1–Cond3) were carried out, followed by five test trials (Test1–Test5). The time interval between trials was 20–30 min. Conveniently, the positive responses at T1 of the individuals are scaled to 100 in order to better characterize the extinction slope. During a test trial, the conditioned stimulus (pure linalool) was delivered for 6 s. The conditioned PER was recorded as a yes-or-no response (*i.e.*, 0 or 1) when the odor alone was delivered during the 6 s of the test trial.

Data Analysis

For each chemical, the mortality accumulated over 11 days of exposure was compared between each concentration and the control by multiple two-by-two χ^2 tests with 1 *df*. To ensure that the experiment error rate was $\alpha = 0.05$, each comparison was carried out according to the Dunn-Sidak method (Sokal and Rohlf 1995) at a critical probability of $\alpha' = 1 - (1 - \alpha)^{1/k}$, where k was the number of intended tests. The significance level was 0.0085 for two-by-two comparisons of the responses to three concentrations of each chemical and one control group.

The number of initial reflex responses and the number of conditioned responses in each trial were compared between the three concentrations of each chemical and the control by multiple two-by-two χ^2 tests with 1 *df*, with a critical probability level of 0.0085, according to the Dunn-Sidak correction of the standard probability level. 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).

Results

Syrup Consumption

During the treatment period (*i.e.,* 11 days) for the nine tested pesticides, the volumes of syrup consumed for control (from 22.0 to 45.2 µl/bee/day) and pesticide-treated groups (from 23.6 to 44.7 µl/bee/day) are not significantly different (ANOVA, 3 *df*, $P > 0.05$, in all cases). The geometrical progression of factor 2 between the different concentrations of chemicals was respected on the whole. These results suggest that the tested concentrations for all pesticides do not have antifeedant effect on honeybees. The volumes of syrup consumed are in agreement with the consumption initially estimated (33 µl/bee/day; Decourtye *et al*. 2003). Consequently, the quantities of chemicals actually ingested by bees are close to wanted quantities.

Chronic Mortality

Cumulative mortality in bees significantly increases from that of the control groups only with dimethoate and fipronil (Table 2). A significant increase in mortality occurs with dimethoate at concentration of 580 µg $\cdot$ L^{-1} (28% *versus* 9.3% mortality after 11 days, in the treated and control groups, respectively; $\chi^2 = 9.3$, 1 *df*, $P = 0.002$). The number of dead

Table 2. Effects of subchronic exposures of nine agricultural chemicals on the survey and reflex responses of the honeybees

Chemical	% Mortality	% Reflex responses
Deltamethrin		
940 $\mu g \cdot L^{-1}$	12.0	81.6
470 $\mu g \cdot L^{-1}$	10.6	86.7
235 $\mu g \cdot L^{-1}$	9.3	86.9
Control	18.0	90.4
N[a]	*300*	*98–115*
λ-Cyalothrin		
360 $\mu g \cdot L^{-1}$	15.8 (b)[b]	77.7
180 $\mu g \cdot L^{-1}$	8.5 (ab)	84.2
90 $\mu g \cdot L^{-1}$	6.7 (a)	91.6
Control	10.3 (ab)	88.4
N	*165*	*34–38*
Cypermethrin		
690 $\mu g \cdot L^{-1}$	10.5	95.2
345 $\mu g \cdot L^{-1}$	13	96.8
172.5 $\mu g \cdot L^{-1}$	18.5	95.6
Control	15	95.6
N	*200*	*46–63*
τ-Fluvalinate		
300 $mg \cdot L^{-1}$	4.2	70.7
150 $mg \cdot L^{-1}$	9.0	76.3
75 $mg \cdot L^{-1}$	10.3	76.7
Control	8.7	75.0
N	*150*	*28–43*
Prochloraz		
150 $mg \cdot L^{-1}$	7.3	78.4
75 $mg \cdot L^{-1}$	4.3	79.7
37.5 $mg \cdot L^{-1}$	9.6	76.8
Control	8.6	78.2
N	*210*	*33–50*
Triazamate		
600 $\mu g \cdot L^{-1}$	2.0	88.2
300 $\mu g \cdot L^{-1}$	3.3	91.6
150 $\mu g \cdot L^{-1}$	7.3	91.6
Control	4.0	93.9
N	*150*	*33-36*
Endosulfan		
8 $mg \cdot L^{-1}$	5.2 (a)	85.0
4 $mg \cdot L^{-1}$	15.1 (b)	75.0
2 $mg \cdot L^{-1}$	9.0 (ab)	67.5
Control	7.6 (ab)	71.4
N	*150*	*36–42*
Dimethoate		
580 $\mu g \cdot L^{-1}$	28.0 (b)	73.1
290 $\mu g \cdot L^{-1}$	12.6 (a)	68.0
145 $\mu g \cdot L^{-1}$	13.3 (a)	66.0
Control	9.3 (a)	66.0
N	*150*	*39–44*
Fipronil		
9 $\mu g \cdot L^{-1}$	91.1 (c)	—
4.5 $\mu g \cdot L^{-1}$	87.3 (c)	85.0
2.2 $\mu g \cdot L^{-1}$	40.6 (b)	67.5
Control	6.6 (a)	66.6
N	*150*	*11–42*

[a] *N*, number of bees per treatment group.

[b] For each chemical, the number of the cumulated mortality in treated groups and in the control group were compared using χ^2 test or Fisher's exact method with 1 *df* ($P < 0.0083$). Different letters indicate significantly different response levels.

bees in the groups exposed to fipronil at concentrations ranging from 2.2 to 9 $\mu g \cdot L^{-1}$ (40.6–91.1% mortality) are significantly different (χ^2, 1 *df*, $P < 0.0083$, in both cases) from that of the control group (6.6% mortality). Consequently, feeding honeybees with the sucrose solutions with added deltamethrin, prochloraz, endosulfan, λ-cyalothrin, cypermethrin, τ-fluvalinate, or triazamate might be considered as sublethal, contrary to fipronil and dimethoate treatments, which are lethal.

Reflex Response

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 pesticides on the gustatory and motor functions of the PER. At least 66% of bees show a clear PER. For all chemicals, the same level of reflex response in treated and untreated bees is found (χ^2, 1 *df*, $P > 0.0083$, in all cases; Table 2). This suggests that the exposure to pesticides tested does not disrupt the sensory and motor components controlling the PER.

Learning Performances

Table 3 shows the olfactory learning performances represented as the percentage of conditioned PER obtained during the training (Cond1–Cond3) and testing (Test1–Test5) phases, in bees feeding the three concentrations of each pesticide and in the control bees feeding only sucrose. Different letters indicate significantly different response levels (χ^2 test or Fisher's exact method, 1 *df*, $P < 0.0083$). The results for deltamethrin are provided in Figure 1, as an illustrative example of the learning curves that can be drawn in the PER assay.

The percentage of bees treated with the highest dose of dimethoate (580 $\mu g \cdot L^{-1}$) extending their proboscis in response to the first presentation of odor (spontaneous responses observed at Cond1) is significantly higher than is observed with untreated bees (36% *versus* 6%; $\chi^2 = 7.8$, 1 *df*, $P = 0.0052$).

The feeding of workers with sucrose solution contaminated with deltamethrin, prochloraz, endosulfan, or fipronil induces significantly lower responses compared to the untreated bees, considering Cond2–Cond3 for deltamethrin and Test4 for the others pesticides. A reduction of the olfactory learning performances is noted during conditioning trials in bees treated with the highest concentration of deltamethrin (nominal and actual concentrations of 940 and 960 $\mu g \cdot L^{-1}$, respectively): 48% and 60% conditioned responses at Cond2 and Cond3, respectively, *versus* 60% and 84% in the control (χ^2, 1 *df*, $P < 0.0083$, in both cases). At the testing trial Test4, lower levels of responses are obtained with the highest dose of: prochloraz (150 $mg \cdot L^{-1}$), reaching 36% of conditioned responses *versus* 73% in the control group ($\chi^2 = 8.2$, 1 *df*, $P = 0.0048$); endosulfan (8 $mg \cdot L^{-1}$), reaching 6% of conditioned responses, *versus* 45% in the control group ($\chi^2 = 7.8$, 1 *df*, $P = 0.0037$); fipronil (4.5 $\mu g \cdot L^{-1}$), reaching 7% of conditioned responses, *versus* 56% in the control group ($\chi^2 = 12.5$, 1 *df*, $P < 0.001$). Conversely, for the above pesticides, no behavioral effect is observed in the last training trial (Test5) (Table 3).

Table 3. Effects of subchronic exposures of nine agricultural chemicals on the learning performances of the honeybee

	% Conditioned responses							
Chemical	Cond1	Cond2	Cond3	Test1	Test2	Test3	Test4	Test 5
Deltamethrin								
940 $\mu g \cdot L^{-1}$	20.7	48.2 (b)[b]	60.3 (b)	100[c]	79.4	87.1	74.3	58.9
470 $\mu g \cdot L^{-1}$	19.3	68.4 (a)	71.9 (ab)	100	91.4	78.7	80.8	74.4
235 $\mu g \cdot L^{-1}$	6.7	55.9 (ab)	74.5 (ab)	100	86.0	79.0	74.4	74.4
Control	19.3	59.6 (a)	84.2 (a)	100	84.3	84.3	82.3	74.5
N[a]		*57–59*				*43–51*		
λ-Cyalothrin								
360 $\mu g \cdot L^{-1}$	7.1	60.7	75.0	100	75.0	50.0	43.8	18.8
180 $\mu g \cdot L^{-1}$	6.2	81.2	75.0	100	68.4	52.6	42.1	36.8
90 $\mu g \cdot L^{-1}$	0.0	81.8	78.7	100	56.0	48.0	32.0	12.0
Control	6.6	66.6	73.3	100	81.0	52.4	23.8	14.3
N		*28–33*				*16–25*		
Cypermethrin								
690 $\mu g \cdot L^{-1}$	12.5	60.0	80.0	100	92.1	92.1	89.4	76.3
345 $\mu g \cdot L^{-1}$	30.0	85.0	92.5	100	88.2	76.4	76.4	70.5
172.5 $\mu g \cdot L^{-1}$	27.5	77.5	75.0	100	96.9	93.9	81.8	87.8
Control	20.0	75.0	82.5	100	97.1	94.2	91.4	82.8
N		*40*				*33–38*		
τ-Fluvalinate								
300 $mg \cdot L^{-1}$	20.6	58.6	65.5	100	75.0	66.7	41.7	33.3
150 $mg \cdot L^{-1}$	10.3	68.9	75.8	100	100	55.6	50.0	33.3
75 $mg \cdot L^{-1}$	12.1	72.7	78.7	100	90.9	72.7	54.5	36.4
Control	9.5	71.4	71.4	100	63.6	45.5	27.3	9.1
N		*21–33*				*11–22*		
Prochloraz								
150 $mg \cdot L^{-1}$	22.8	80.0	88.5	100	87.8	63.6	36.3 (b)	33.3 (b)
75 $mg \cdot L^{-1}$	25.7	85.7	80.0	100	85.7	87.5	78.5 (a)	46.4 (ab)
37.5 $mg \cdot L^{-1}$	23.5	85.2	91.1	100	90.0	80.0	73.3 (a)	63.3 (ab)
Control	25.0	86.1	88.8	100	93.3	86.5	73.3 (a)	70.0 (b)
N		*34–36*				*28–33*		
Triazamate								
600 $\mu g \cdot L^{-1}$	13.3	73.3	73.3	100	77.7	55.5	48.1	40.7
300 $\mu g \cdot L^{-1}$	12.1	75.7	72.7	100	80.7	50.0	46.1	26.9
150 $\mu g \cdot L^{-1}$	39.3	87.8	87.8	100	93.3	66.6	63.3	50.0
Control	9.6	80.6	80.6	100	73.0	53.8	38.4	26.9
N		*30–33*				*26–30*		
Endosulfan								
8 $mg \cdot L^{-1}$	4.0	52.0	68.0	100	64.7	35.3	5.9 (b)	5.9
4 $mg \cdot L^{-1}$	27.7	61.1	77.7	100	83.3	66.7	58.3 (a)	41.7
2 $mg \cdot L^{-1}$	4.7	33.3	47.6	100	83.3	41.7	33.3 (ab)	16.7
Control	8.3	62.5	70.8	100	80.0	55.0	45.0 (a)	10.0
N		*18–25*				*12–20*		
Dimethoate								
580 $\mu g \cdot L^{-1}$	36.6 (a)	66.6	73.3	100	80.0	55.0	30.0	25.0
290 $\mu g \cdot L^{-1}$	34.3 (ab)	68.5	60.0	100	64.7	47.0	35.2	17.6
145 $\mu g \cdot L^{-1}$	31.2 (ab)	71.8	68.7	100	78.2	43.4	13.0	17.3
Control	6.0 (b)	69.7	72.7	100	88.8	62.9	33.3	18.5
N		*26–33*				*17–27*		
Fipronil								
9 $\mu g \cdot L^{-1}$	—	—	—	—	—	—	—	—
4.5 $\mu g \cdot L^{-1}$	18.0	35.2	76.4	100	71.4	21.4	7.1 (b)	14.2
2.2 $\mu g \cdot L^{-1}$	14.8	51.8	66.6	100	68.1	50.0	27.2 (ab)	13.6
Control	10.7	46.4	64.2	100	68.7	56.2	56.2 (a)	31.2
N		*17–28*				*14–22*		

[a] *N,* number of bees per treatment group.
[b] For each chemical, the number of the conditioned responses in treated groups and those in the control group were compared using χ^2 test or Fisher's exact method with 1 *df* ($P < 0.0083$). Different letters indicate significantly different response levels.
[c] Positive responses at T1 are scaled to 100.

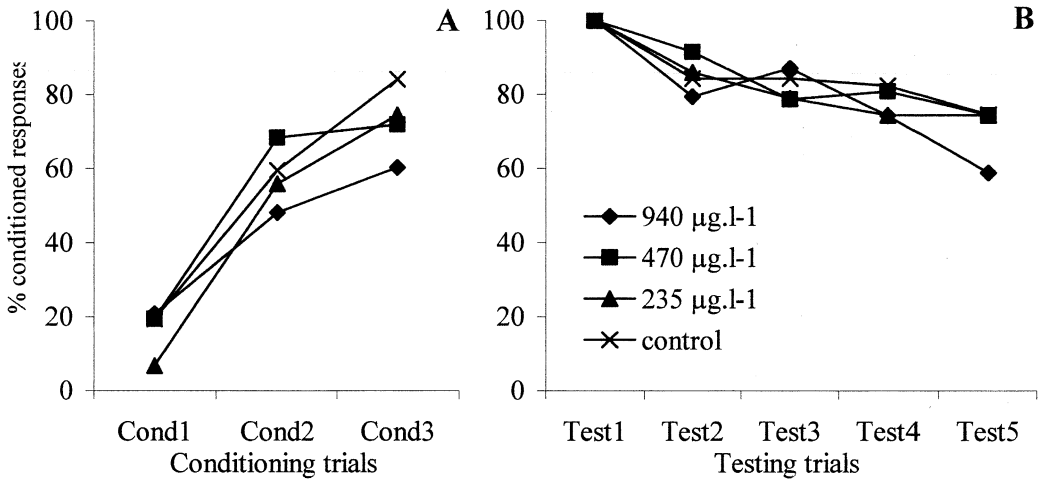

Fig. 1. Learning performances of deltamethrin-treated bees during conditioning (A) and testing (B) procedures of PER assay

In all trials, the level of responses of bees exposed to λ-cyalothrin, cypermethrin, τ-fluvalinate, and triazamate is equivalent to that obtained with control bees (χ^2, 1 *df*, $P >$ 0.0083, in all cases). For these four chemicals, 66–93% of conditioned responses are obtained in treated bees at the last conditioning trial (Cond3) and 71–83% in the untreated bees.

Discussion

It is of interest to characterize honeybee behaviors that can be routinely used as indicators of sublethal exposure to pesticides. The possible long-term exposure to a toxic agent by contamination of stored food has been established by studying the transfer into the colony of pesticides sprayed on a crop (Fries and Wibran 1987; Koch and Weisser 1997; Russel *et al.* 1998; Villa *et al.* 2000). Thus, it is necessary to evaluate the viability of worker bees newly involved in foraging duties based on their learning ability, after being fed with a contaminated food within the hive. The preconditioning treatment applied in the present study leads to determining whether or not a pesticide exposure applied prior to a learning task may affect the bees' performances. Among the nine pesticides tested, only fipronil, deltamethrin, endosulfan, and prochloraz yielded behavioral effects during the PER assay. This is consistent with previous works reporting that the PER assay was adapted to the screening of the adverse effects of various pesticides to bees (Taylor *et al.* 1987; Mamood and Waller 1990; Stone *et al.* 1997; Abramson *et al.* 1999; Abramson and Boyd 2001; Weick and Thorn 2002; Decourtye *et al.* 2003; Abramson *et al.* 2004). Conversely, our results clearly indicate that the range of tested concentrations of λ-cyalothrin, cypermethrin, τ-fluvalinate, and triazamate does not affect the learning performances of bees. However, cypermethrin and τ-fluvalinate are less toxic to honeybees than λ-cyalothrin and triazamate. These results corroborate those of Taylor *et al.* (1987) showing that among a set of six pyrethroids, cypermethrin and τ-fluvalinate yielded the least impact on the honeybee learning.

The originality of our approach consists in taking into account different concentrations in the PER assays. The determination of the threshold toxicity concentrations is also possible. Thus, the no-observed-effect concentration (NOEC) for the conditioned PER is set to 2.2 $\mu g \cdot L^{-1}$, 470 $\mu g \cdot L^{-1}$ (actual concentration equals 429 $\mu g \cdot L^{-1}$), 4 $mg \cdot L^{-1}$, and 75 $mg \cdot L^{-1}$ for fipronil, deltamethrin, endosulfan, and prochloraz, respectively. Considering the consumption of contaminated syrup and the number of bees, we can estimate that the no-observed-effect dose of pesticide received per bee and per day is 0.07 ng for fipronil (LD50 divided by 80), 15 ng for deltamethrin (LD50 divided by 40), 125 ng for endosulfan (LD50 divided by 40), and 2.5 µg for prochloraz (LD50 divided by 40). Thus, fipronil is the most effective of the above pesticides tested to induce learning performances impairment. Under similar experimental conditions, the NOECs for imidacloprid and hydroxy-imidacloprid were estimated to 6 and 60 $\mu g \cdot L^{-1}$, corresponding to the DL50 value divided by 160 and 80, respectively (Decourtye *et al.* 2003). As regards λ-cyalothrin, cypermethrin, τ-fluvalinate, and triazamate, we can only say that the NOECs of these pesticides are superior to 360 $\mu g \cdot L^{-1}$, 690 $\mu g \cdot L^{-1}$ (actual concentration of 782 $\mu g \cdot L^{-1}$), 300 $mg \cdot L^{-1}$, and 600 $\mu g \cdot L^{-1}$, respectively.

To evaluate the usefulness of PER as a measure for toxicity assessment, it is necessary to compare these responses to standard toxicity endpoints such as mortality. Learning performances after treatment with the highest concentration of deltamethrin, endosulfan, or prochloraz are decreased, in contrast to survival, which is not affected. The NOEC of hydroxy-imidacloprid for the mortality was estimated to be 120 $\mu g \cdot L^{-1}$, whilst the NOEC for the conditioned responses was established at 60 $\mu g \cdot L^{-1}$ (Decourtye *et al.* 2003). From this study, it appears that most often the impairments in olfactory learning abilities are shown for chemical concentrations at which no additional mortality occurred.

The choice of sublethal concentrations of pesticides is a crucial problem when an attempt is made to estimate the effects of pesticides on bee behaviors. In this study, for each chemical, the highest tested dose was the 48-h oral LD50 value

divided by 20. Considering the low mortality observed for most of the tested pesticides, it appears that this choice was acceptable.

In case of lethal treatment, the exposure to insecticide can result in a selection of worker bees staying alive because they are less sensitive to this pesticide than the other congeners. Such tolerant bees can give an intact conditioned response level in the PER assay. For example, bees treated with Decis® (0.5% a.i. deltamethrin) at a high dose exhibited similar pattern of learning performances than control bees (Abramson *et al.* 1999). In the current study, an adverse effect of dimethoate at its highest concentration (580 $\mu g \cdot L^{-1}$) is shown on survival of honeybees, but not on their learning performances. Previous studies have assessed the effect of chlorpyrifos ethyl, an organophosphorus insecticide like dimethoate, on the behavior of parasitoids (*Leptopilina heterotoma*). Females of parasitoids were conditioned to associate an odor with the oviposition in host larvae of *Drosophila* (Rafalimanana *et al.* 2002). Parasitoids exposed to the LD20 value of chlorpyrifos oviposited the host larvae more quickly than controls did. In our experiment, higher levels of spontaneous responses were obtained in bees treated with the highest concentration of dimethoate (580 $\mu g \cdot L^{-1}$). Thus, current results and those found in the literature suggest that the high behavioral response levels in organophosphorus-treated insects were probably linked to pharmacological action. These chemicals act by inhibiting acetylcholinesterase and consequently by prolonging activity of synapses (Padilla 1995). We assume that the increase in spontaneous responses in dimethoate-treated bees may be due to amplification of stimulus perception or of response motricity. To confirm this hypothesis, further experiments should be necessary. Works by Abramson *et al.* (1999) should provide some insight to perform them.

The adverse effects are observed during a conditioning or testing procedure according to the chemical tested. Ingestion of deltamethrin significantly reduces the level of conditioned responses in the conditioning procedure. This result suggests an adverse effect of deltamethrin on the ability of treated animals to learn the temporal relation between the unconditioned stimulus and the conditioned one. In addition to conditioning procedure, the testing procedure points out the resistance of bees to extinguish the response to a conditioned stimulus no longer associated with a reward. Abramson *et al.* (1999) reported that endosulfan tested at the concentration recommended to control the cotton boll weevil influenced extinction of the conditioned response. The authors suggested that motor system disruption was responsible for this event rather than an effect on the learning process. Our results clearly indicate that endosulfan, as well as fipronil and prochloraz, do not affect either the reflex response or the conditioned response level in the conditioning procedure, but the decrease of response level in the testing procedure occurs more rapidly compared to the control group.

The conditioning and testing phases are two independent processes that could be differentially affected by a toxic exposure. This may rely on the fact that different steps of the memorization are involved. If we refer to the model of memory temporal schedule in the honeybee as described by Menzel (1999), the conditioning covers the information storage in the short-term memory, whilst long-term memory is already established when the testing phase occurs. Deltamethrin would affect the first step of information storage, whereas endosulfan, fipronil, and prochloraz would interfere with the retrieval process resulting in the capacity to restore the conditioned response. However, further work is still needed to investigate more precisely the effects of these chemicals on the different parameters of the memory (acquisition, retrieval, short-, medium- and long-term memory) during an olfactory conditioning of the PER, as investigated with imidacloprid (Decourtye *et al.* 2004b).

Besides behavioral effects of fipronil, an increase in the mortality after 11 days appears in bees treated with this pesticide. The lowest lethal dose of fipronil (0.1 ng per bee per day corresponding to a concentration of 2.2 $\mu g \cdot L^{-1}$) is 60 times lower than the LD50 value. At the same time, a lethal effect is significantly observed for bees exposed to the highest dose of dimethoate (20 ng per bee per day corresponding to a concentration of 580 $\mu g \cdot L^{-1}$). Although the long-term lethal effect of dimethoate was previously demonstrated (Waller *et al.* 1984), we have determined for the first time the chronic toxicity of fipronil to the honeybee. Using a similar laboratory chronic oral test with bees fed with contaminated syrup, chronic toxicity can be found even at low concentrations of imidacloprid (Suchail *et al.* 2000; Decourtye *et al.* 2003; Dechaume-Moncharmont *et al.* 2003). In chronic toxicity studies, imidacloprid reacts at doses 60 to 6000 times lower than those required to produce the same effect in acute intoxication studies (Suchail *et al.* 2001). Thus, the acute toxicity tests, performed according to the EPPO guidelines (EPPO 1993), appear to give only a partial measure of the lethal effects because of the short duration of these tests (1 to 3 days in most cases). Now, when the acute lethal effect is not obvious, additional testing could give information on the long-term lethal effects possibly induced by the toxic, as that was proved with dimethoate and fipronil.

Although spraying of dimethoate-based formulations is prohibited on flowering crops, the field application of formulations containing deltamethrin, fipronil, or imidacloprid is allowed. In the current study, concentrations of 455 and 227.5 $\mu g \cdot L^{-1}$ of deltamethrin (actual concentrations of 429 and 212 $\mu g \cdot L^{-1}$) were tested. They are realistic because 500 $\mu g \cdot L^{-1}$ corresponds to the maximum concentration measured in oilseed rape flowers after spraying of Decis®Micro (CETIOM unpublished data). We noted the absence of lethal and behavioral effects after administration of these concentrations. In an outdoor flight cage, representing more realistic exposure conditions than those performed in a laboratory, a sugar solution containing 500 $\mu g \cdot L^{-1}$ of deltamethrin offered to a colony had no effects on an olfactory learning discrimination task in free-flying foragers and in the PER procedure of restrained individuals (Decourtye *et al.* 2004a). Thus, the intact learning performances in treated bees at a realistic concentration of deltamethrin during a PER assay are in agreement with those obtained in semifield conditions, at the colony level. This suggests that in field conditions, foraging bees could not suffer from behavioral effects of deltamethrin after visiting of flowering crops treated with Decis®Micro. Contrary to the current study, the impact of deltamethrin has been shown on survival of worker bees in the flight cage. This discrepancy might result in differences between the dose received per bee and per day. In the flight cage

study, we can estimate that the highest dose of deltamethrin received per bee and per day was about equal to the LD50 value, while the value of 30 ng obtained in the current laboratory study corresponds to the LD50 divided by 20.

Fipronil and imidacloprid, being the active ingredient of the Regent® and Gaucho® formulations, respectively, are authorized as a sunflower seed coating. In France, imidacloprid and fipronil were accused of being a cause for the decline of sunflower honey production. It is suspected that these products or theirs metabolites could migrate into nectar or pollen of treated sunflowers and induce deleterious effects in foraging bees after ingestion of contaminated food. Despite the fact that several semifield and field tests indicated that seed dressing with imidacloprid posed no risk during sunflower flowering (Curé *et al.* 2000; Schmuck *et al.* 2001), a behavioral effect in the laboratory can be found at a concentration potentially encountered in plant tissues (Decourtye *et al.* 2003). From 1999 onwards, the French Ministry of Agriculture decided to suspend the registration of the seed treatment product Gaucho® in sunflowers according to the precautionary principle. As with imidacloprid, our study shows that an effect of fipronil can be observed on the learning performances of bees in the range of 2.2 to 4.5 $\mu g \cdot L^{-1}$. Additional experiments are needed to establish the threshold concentration of fipronil or of its metabolites from which the forager bees could be exposed and possibly induces drastic bee population losses, as observed by French beekeepers in colonies foraging on sunflowers treated with Regent®.

Our application of the PER assay for pesticide toxicity assessment led to characterization of effects on a behavioral endpoint related to ability to associate an odor stimulus with sucrose reward. In general, a detailed basic knowledge on behavior relevant for honeybee assays in ecotoxicology is still relatively scarce, and this is especially true for the influence of principal general test variables on foraging behavior. The crucial problem in behavioral toxicology is the lack of standardization for the tests. Therefore, the PER assay could be a useful tool in the studies on behavioral effects of pesticides, especially on foraging behavior, since it guarantees a good control of bee-rearing conditions and of exposure to chemicals. During bee-rearing under laboratory conditions, the quality of the food and the olfactory environment of the individuals must be strictly controlled because these factors can later influence pesticide sensitivity (Wahl and Ulm 1983) and learning performances in the PER assay (Sandoz *et al.* 2000), respectively.

Our study shows that the observations on conditioned PER can be of interest in the assessment of behavioral effects of pesticides to the honeybee, because this endpoint can be used to compare different pesticides by accounting for various concentrations for increasing the accuracy of the results and for deriving NOECs. In a previous study, we showed that the behavioral toxicity of imidacloprid observed in laboratory conditions at the individual level (conditioned PER assay) was consistent with results obtained in semifield experiments at the colony level (Decourtye *et al.* 2004a). For the pesticides tested in the current study, further studies are still needed to establish similar relationships.

Acknowledgments. We wish to thank M. Charreton and B. Roger for technical assistance in bee rearing. This work benefited from a grant by the French Ministry of the Environment (MATE-01133-Evaluation et réduction des risques lies à l'utilisation des pesticides).

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