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SUBCHRONIC EXPOSURE OF HONEYBEES TO SUBLETHAL DOSES OF PESTICIDES: EFFECTS ON BEHAVIOR

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Abstract—Laboratory bioassays were conducted to evaluate the effects on honeybee behavior of sublethal doses of insecticides chronically administered orally or by contact. Emergent honeybees received a daily dose of insecticide ranging from one-fifth to one-five-hundredth of the median lethal dose (LD50) during 11 d. After exposure to fipronil (0.1 and 0.01 ng/bee), acetamiprid (1 and 0.1 μg/bee), or thiamethoxam (1 and 0.1 ng/bee), behavioral functions of honeybees were tested on day 12. Fipronil, used at the dose of 0.1 ng/bee, induced mortality of all honeybees after one week of treatment. As a result of contact treatment at 0.01 ng/bee, honeybees spent significantly more time immobile in an open-field apparatus and ingested significantly more water. In the olfactory conditioning paradigm, fipronil-treated honeybees failed to discriminate between a known and an unknown odorant. Thiamethoxam by contact induced either a significant decrease of olfactory memory 24 h after learning at 0.1 ng/bee or a significant impairment of learning performance with no effect on memory at 1 ng/bee. Responsiveness to antennal sucrose stimulation was significantly decreased for high sucrose concentrations in honeybees treated orally with thiamethoxam (1 ng/bee). The only significant effect of acetamiprid (administered orally, 0.1 μg/bee) was an increase in responsiveness to water. The neonicotinoids acetamiprid and thiamethoxam tested at the highest dose (one-tenth and one-fifth of their oral LD50, respectively) and fipronil at one-five-hundredth of LD50 have limited effects on the motor, sensory, and cognitive functions of the honeybee. Our data on the intrinsic toxicity of the compounds after chronic exposure have to be taken into account for evaluation of risk to honeybees in field conditions.

Keywords—Pesticides Chronic Sublethal Honeybee Behavior

INTRODUCTION

The honeybee *Apis mellifera* is valuable for the economy due to the products of the hive (honey, pollen, royal jelly), which generate considerable income for beekeepers, as well as to its contribution to crop pollination, which is valued at more than $15 billion a year in the United States alone (U.S. Department of Agriculture, http://ars.usda.gov/main/main.htm). Honeybees also contribute to plant biodiversity by pollinating wild plants. Honeybees and their products are potentially exposed to several contaminants present in the environment, such as chemical products released into the hive to fight against diseases and parasites and pesticides used in agriculture against pests.

The continuing need for novel and selective insecticides acting on pests has led to the development of new groups of compounds. The newest major group of insecticides are the neonicotinoids, which include imidacloprid, acetamiprid, and thiamethoxam [1]. Worldwide annual sales of neonicotinoids total $1 billion, and they are used against piercing–sucking pests (aphids, leafhoppers, and whiteflies) of major crops. In France, the use of imidacloprid has been suspended because of concerns that it may have a drastic effect on bee populations, causing loss of honeybees and weakening hives. Acetamiprid and thiamethoxam are presented as potential alternatives to imidacloprid subject to proof that they are harmless to nontarget species. Fipronil belongs to the phenylpyrazole group and is the first product of this group to be introduced for pest control. It is the active molecule of the insecticide Regent Régent TS® (BASF, Ludwigshafen, Germany) with insecticidal properties similar to those of imidacloprid contained in Gaucho® (Bayer AG, Leverkusen, Germany). Fipronil is now a major pesticide for use on crops but also as an antiparasitic, with an estimated world market of $150 million [2]. It is suspected of having harmful effects on honeybees and has been forbidden in France because of its potential involvement in bee declines.

The previously mentioned insecticides are neurotoxic compounds that act on ion channels within the insect nervous system. The neonicotinoids imidacloprid, acetamiprid, and thiamethoxam have the same target at the cellular level, acting mostly as agonists of insect nicotinic acetylcholine receptors (nAChRs) [3]. The α and β subunit compositions of these receptors define different nicotinic receptor subtypes differing in their pharmacological properties. In honeybees, as in insects in general, the subunit composition of nAChRs is unknown. Patch-clamp experiments performed on honeybee brain neurons in culture have shown that imidacloprid is a partial agonist of nAChRs [4–7]. At least two types of nAChRS have been described in the honeybee brain: α-bungarotoxin (α-BGT)-sensitive and α-BGT-insensitive nAChRs [8]. These receptors are involved in tactile and olfactory learning and memory [9–12], which are essential functions for foraging behavior. Fipronil disrupts inhibitory neurotransmission by blocking the γ-amino-butyric-acid (GABA) gated-chloride channels as well as the glutamate gated-chloride channels (GluCl) [4,13]. Since mammals are devoid of this type of chloride channel, the action of fipronil in blocking the glutamate-activated chloride channel is considered to be responsible, at least partially, for its higher selective toxicity to insects over mammals [14]. Gabaergic

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interneurons and neurons bearing GluCl receptors have been found in several honeybee brain neuropiles, where they act as modulators of excitatory synapses [4,15]. In olfactory pathways, gabaergic interneurons contribute to shaping the neural representation of odors [16,17]. Contrary to nAChRs, GABA and GluCl receptors are also found outside the central nervous system on the muscle membrane, where they regulate the excitatory glutamate neurotransmission at the neuromuscular junction [18,19].

We have previously performed experiments on the biological effects of acute sublethal oral or contact exposure of honeybees to acetamiprid and thiamethoxam. Thiamethoxam induced no effect on behavioral functions, whatever the dose and the delivery mode. Acetamiprid had an activating effect on behavior, which appeared as an increase in sucrose responsiveness and in locomotor displacements, but also induced long-term memory impairment after oral absorption [20]. The experiments conducted with fipronil in similar conditions showed a slight decrease in sucrose responsiveness and olfactory memory impairment after topically applied sublethal doses [21].

As neonicotinoids are strongly suggested to be systemic [22], the xylem transport in the plant could result in the presence of tiny quantities of the molecules in nectar and pollen. Uptake of fipronil has also been demonstrated in the root of sunflowers, leading to transport into leaves [23]. Pollen and nectar foraging on plants treated with systemic insecticides can lead to an accumulation of these products in the hive, and young honeybees can be exposed to repeated sublethal doses of pesticides during their early life. The present study examines whether the rather limited behavioral effects we observed after acute exposure to the three pesticides would be amplified by repeated exposure. In the laboratory, we reproduced subchronic intoxication of young honeybees with sublethal doses of acetamiprid, thiamethoxam, and fipronil and are now able to report the effects of oral and contact exposure on sensory perception of water and sugar, locomotor displacements, and olfactory learning and memory capabilities.

MATERIALS AND METHODS

The following experiments have been performed by two examiners but not in blind tests.

Drugs

Fipronil (98.5% purity), thiamethoxam (97% purity), and acetamiprid (99% purity) all were purchased from Cluzeau Info Labo (Sainte-Foy-La-Grande, France). The three compounds were used at doses of one-fifth to one-five-hundredth of the median lethal dose (LD50) that elicit sublethal effects [20,21,24]. Based on previous studies [25] and our own experiments, the doses used per bee were 0.1 and 0.01 ng (LD50 oral 48 h: 4–6 ng) for fipronil, 1 and 0.1 ng (LD50 oral 48 h: 5 ng; LD50 contact 24 h: 29 ng) for thiamethoxam, and 1 and 0.1 μg (LD50 oral 72 h: 14.5 μg; LD50 contact 24 h: 7 μg) for acetamiprid. Stock solutions of fipronil and acetamiprid were prepared in acetone (Sigma, Saint Quentin Fallavier, France) in accordance with the European and Mediterranean Plant Protection Organism guidelines [26]. Thiamethoxam was dissolved in acetonitrile (Sigma) in accordance with the manufacturer's recommendations. For topical application, the stock solution was diluted with distilled water to obtain the specific concentration. For oral delivery, the specific concentration was obtained after a final dilution in sucrose solution (50% w/v). The proportion of solvent (acetone for acetamiprid and fipronil experiments, acetonitrile for thiamethoxam) was 0.3% (v/v) for oral and 10% (v/v) for topical applications in final solutions.

Animals

The tests were performed all year-round in Toulouse, in the south of France, on emergent honeybees (*A. mellifera*). In September the hives received a single treatment against varroa (Apivar®; Laboratoires Biové, Arques, France), and for the first month after treatment no sampling took place. Since 2004 the pesticides Gaucho and Regent Régent TS have been forbidden in France, thus minimizing exposure of honeybees to these pesticides. Honeybees were collected from hives placed in controlled room temperature (23°C). Working on emergent bees makes it possible to control their age and keep them alive longer in laboratory conditions. The bees were caught on a brood frame when emerging from the cells. They were caged in groups of 40 individuals and maintained in darkness under controlled conditions (40% relative humidity, temperature 33°C). Pollen and sucrose solution (50% w/v) were provided ad libitum for the first week. The bees were then allowed to make a purging flight before returning to their cages, where they were subjected to an 11-d exposure period. During this period, the bees were fed with sucrose solution (50% w/v) and water. The feeders were changed daily with fresh solutions.

Exposure protocols

Two modalities of exposure were used: oral and contact exposure. For oral treatment, the sucrose solution used for feeding the bees contained the test compound or contained the solvent (control). The volume of the test compound sugar solution was adjusted daily to the number of survivors on the basis of a consumption of syrup estimated at 33 μl/bee/d [25]. Control groups ingested a sugar solution containing the appropriate solvent. Individual contact exposure consisted of applying the solution containing the compound under investigation or the solvent alone (control) to the thorax of the honeybee. To do so, each honeybee was caught in the cage daily and maintained with an insect forceps while 1 μl of the solution was applied to the thorax using a micropipette with a tip. After the drop disappeared, which took several seconds, the honeybee was released into a new cage where the treated bees were gradually collected. Throughout the exposure period, the mortality per day was evaluated in control and treated groups and has been presented as cumulative-death curves in the results section.

Behavioral assays

For each pesticide and for each dose, a control group receiving the solvent was tested. The behavioral assays were conducted in parallel in the treated and in the control groups. The numbers of animals for each group and for each experiment are indicated in Table 1. At the end of the exposure period (11 d), honeybees were individually tested for locomotor activity, water and sucrose responsiveness, and learning abilities. Locomotor activity was evaluated in free-moving animals. Experiments to test water and sugar responsiveness and learning were carried out in restrained honeybees. Honeybees were cold anesthetized and individually fixed in plastic tubes and maintained with a drop of wax/resin mixture deposited on the backside of the thorax. The antennae and the mouthparts were left free.

Table 1. The numbers presented in this table represent the number of honeybees used for each experiment. For controls, the values are in italics. Asterisks indicate in the corresponding behavioral assay that at least one value or one parameter shows a significant difference compared to control

	Fipronil				Thiamethoxam				Acetamiprid			
	0.1 ng		0.01 ng		1 ng		0.1 ng		1 μg		0.1 μg	
Size of groups	Oral	Topic	Oral	Topic	Oral	Topic	Oral	Topic	Oral	Topic	Oral	Topic
Mortality												
Treated	17*	16*	36	23	56	32	65	49	58	44	34	29
Controls	*17*	*16*	*37*	*21*	*56*	*33*	*59*	*49*	*55*	*40*	*35*	*31*
Locomotion												
Treated			27	27*	56	32	65	49	30	30	25	22
Controls			*29*	*29*	*56*	*33*	*59*	*49*	*30*	*30*	*27*	*24*
Water experiments												
Treated			34	32*	38	35	40	36	26	26	27*	26
Controls			*33*	*32*	*38*	*35*	*40*	*36*	*29*	*33*	*28*	*28*
Sucrose experiments												
Treated			27*	30	32*	25	28	32	27	30	25	22
Controls			*29*	*30*	*28*	*27*	*24*	*33*	*30*	*30*	*27*	*24*
Learning experiments												
Treated			25*	30*	30	23*	33	29*	29	30	25	25
Controls			*28*	*30*	*28*	*22*	*32*	*29*	*30*	*30*	*27*	*24*

Locomotor activity

The locomotion of naive honeybees was tested in an open-field apparatus consisting of a white Plexiglas box (30 × 30 × 4 cm) with a glass front allowing observation. The box stood vertically and was illuminated from above to induce upward displacements. The back vertical side was divided into squares of 5-cm sides, defining six vertical levels, allowing the bee to be localized into the apparatus. A hole made in the bottom right-hand side allowed individual honeybees to be introduced into the box for a 3-min observation period. The position of the honeybee was recorded every 3 s on the screen of a computer using specially adapted software [27]. Two successive recordings of the honeybee in the same square were reported as a 3-s period of immobility. Relevant parameters were the distance covered in the box, the time spent in each level, and the displacements of the honeybee from lower to upper levels. Observations of the honeybee's behavior such as trembling or abnormal movements of legs or wings were also reported.

Water responsiveness

Proboscis extension induced by stimulating the antennae with water is used to test exposed honeybees' responsiveness to water. One hour after being fed with a drop of water and a drop of 50% sucrose solution, restrained honeybees were stimulated on the antennae with a drop of water. The number of animals responding with a proboscis extension was evaluated in control and treated groups. The response to water was tested again, 3 h after the first test. Water consumption was controlled during the exposure period. The volume of consumed water was measured in control and treated groups daily.

Sucrose responsiveness

Stimulating the antennae with a sucrose solution can also induce proboscis extension. The reflex is used to evaluate the sucrose responsiveness in honeybees displaying equal motivation to sugar. One hour after being fed with 50% (w/v) sucrose solution, the honeybees' antennae were stimulated at 3-min intervals with sucrose solutions of ascending concentrations of 0.03, 0.1, 0.3, 1, 3, 10, and 30% (w/v). The percentage of bees responding to sucrose stimulation by a proboscis extension was evaluated in treated and control groups.

Water responsiveness and sucrose responsiveness experiments were conducted in independent groups.

Olfactory learning

We used the olfactory conditioning of the proboscis extension reflex (PER) to assess cognitive functions in honeybees exposed to pesticides. The proboscis extension induced by antennal contact with sucrose can be conditioned to an odorant as long as the odorant precedes the sucrose stimulation. After several paired associations of odor and sugar stimulations (sometimes one association can be sufficient), the odorant becomes a conditioned stimulus (CS), and the honeybee responds to the olfactory stimulation by a proboscis extension (conditioned response). The learned olfactory stimulus can be retained for a short (1-h) or long (24- and 48-h) period, and olfactory memory is evaluated through retrieval tests. After a 3-h fast period, honeybees were trained along five trials with 1-min intertrial intervals. The olfactory stimulation (or CS) was directed toward the antennae for 6 s and diffused through an air puff by means of a 5-ml syringe. The CS was an odor of coffee (5 mg of coffee powder introduced in the syringe) for thiamethoxam experiments. We used one of the two odorants, 1-hexanol and 1-nonanol, (Sigma) as CS for fipronil and acetamiprid experiments. The odorant 1-hexanol or 1-nonanol was diffused from a piece of filter paper placed in the syringe and soaked with 2 μl of the solutions. The odorants were renewed daily. Three seconds after the onset of the olfactory stimulus, the antennae were touched with a drop of sugar water (40% w/v) for 3 s, and this stimulation induced proboscis extension. The honeybee was then allowed to feed on a sucrose solution for 3 s. Memory tests were performed 1, 24, and 48 h after the training phase, presenting the CS alone (coffee flavor for thiamethoxam experiment) or in random presentation with a new odorant (1-hexanol for bees conditioned with 1-nonanol and, vice versa, for acetamiprid and fipronil experiments). A bee that has learned the predictive significance of the odorant for food will extend the proboscis to the olfactory stimulation. When the honeybee did not respond to the sugar stimulation during the training phase, it was excluded from the experiment. A honeybee that did not respond to the odorant during the memory test was tested after 10 min for the sugar-elicited proboscis extension. If no response was observed to

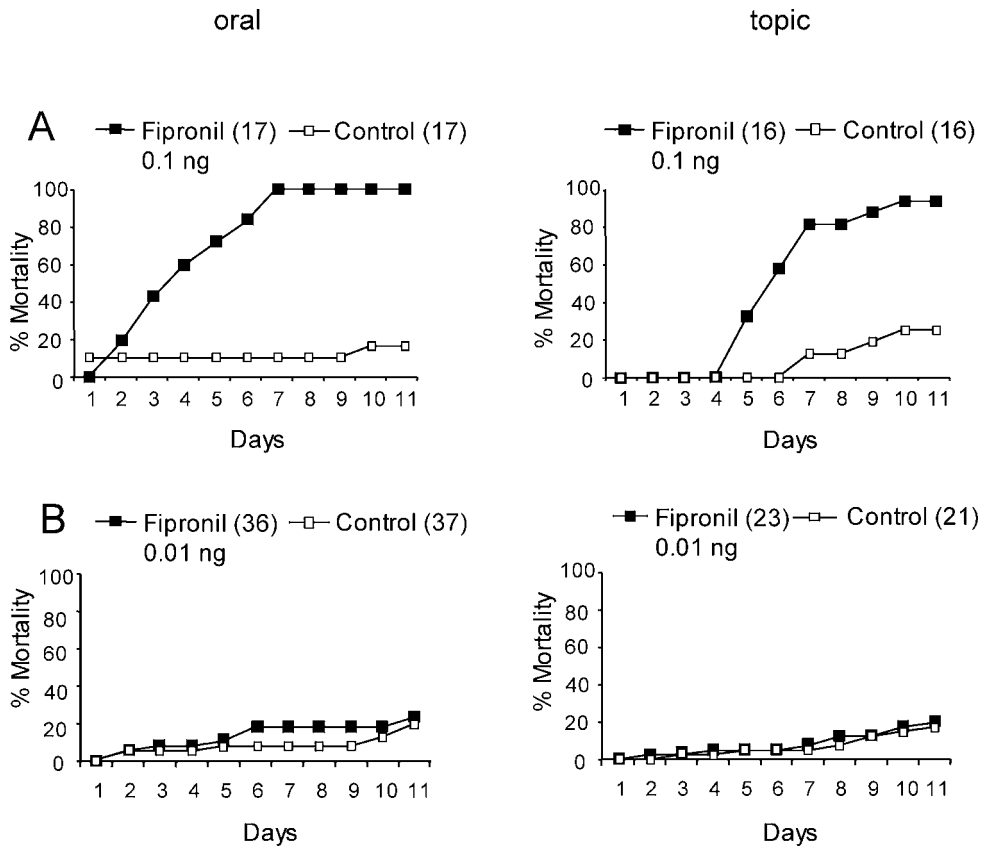

Fig. 1. Percentages of honeybee mortality recorded during 11 d of exposure to fipronil, 0.1 ng (**A**) and 0.01 ng (**B**), after oral and topical treatments. Controls received the solvent. Number of subjects in each group is indicated in parentheses. Statistical comparisons of the curves: For fipronil, 0.1 ng/bee, oral versus control $p = 0.003$; topic versus control $p = 0.011$; for fipronil, 0.01 ng/bee, oral versus control $p = 0.758$, topic versus control $p = 0.893$, as determined by the Kaplan–Meier test.

sugar, the honeybee was discarded. The number of animals that were excluded from the statistical analysis for the previously mentioned reasons was equivalent in control and treated groups and represented less than 5% of the total number.

STATISTICAL METHODS

Comparison of the mortality curves between the control and treated groups was performed with the Kaplan–Meier test. Locomotor activity was evaluated through analysis of three relevant parameters: the path length during the 3-min observation period, the duration of immobility, and the time spent in each level of the box. Student's *t* tests were performed for mean comparison between treated and control group values after variance comparison with Levene's test [28]. The mean water consumption per day per bee was calculated as the total amount of water consumed each day in each group divided by the number of surviving honeybees, and this calculation does not allow error bars to be represented on the figure. The daily values of consumed water were compared between the treated and the control groups with a Student's *t* test. Figures representing the proportion of bees releasing a PER in response to water, sugar solution, or olfactory stimulation do not include error bars, which is the general practice for this type of data, as standard errors or standard deviations do not accurately reflect the variability for proportions. Responsiveness to water was compared between control and treated groups at 1 and 3 h with a chi-square test. The comparisons between the groups for sucrose responsiveness were conducted using Fisher's exact test, which directly yields a *p* value. For olfactory learning, the values were compared between control and treated groups for acquisition (from the second to the fifth trial) and for each retention test (at 1, 24, and 48 h) using Fisher's exact test. Within-group comparison for level response to conditioned odorant versus new odorant was performed using McNemar's test [29]. For each of these tests, a *p* value of less than 0.050 was considered significant. All the statistical tests were performed with SPSS®12 software (SPSS Science, Chicago, IL, USA).

RESULTS

All the honeybees in the treated and control groups consumed the syrup distributed daily. The consumption of the total amount ensures that honeybees were exposed orally to pesticides. Attacks between animals were visible in fipronil-treated honeybees at the dose of 0.1 ng/bee and at the end of the 11 d of exposure at the dose of 0.01 ng/bee. These attacks were associated with frequent behavior of wing fluttering accompanied by the emission of alarm pheromone.

Mortality

Cumulative mortality curves were established for each pesticide, for each dose and for each modality for the 11-d exposure period. Fipronil (0.1 ng/bee, oral and contact) induced the death of all the animals one week after the beginning of treatment. Mortality increased significantly compared to control, from day 3 of fipronil oral exposure (Fig. 1A, $p = 0.003$), and from day 5 when fipronil was topically applied (Fig 1A, $p = 0.011$). At the dose of 0.01 ng/bee, fipronil induced 25

and 20% mortality, respectively, in orally and topically treated animals at the end of the treatment (Fig. 1B). These percentages were not statistically different from mortality in control individuals (21.6 and 17.5%, $p = 0.758$ and 0.893, respectively). Because fipronil at the dose of 0.1 ng induced complete mortality, the dose of 0.01 ng/bee was retained to study the behavioral effects of chronic intoxication. At the end of the exposure period with thiamethoxam (11th day), the percentage of mortality in bees treated orally and topically with 1 ng was 10% (data not shown). The mortality in these groups was not different from that of controls (4 and 10% respectively). A maximum of 20% of dead bees was observed in the groups exposed orally and topically to 0.1 ng thiamethoxam. The same mortality level was observed in control animals (15% for oral and 20% for topic exposure). Acetamiprid (1 μg/bee) orally and topically administered induced, respectively, 29.3 and 31.8% mortality after 11 d, but these values were not different from the control (21.8 and 22.5%, respectively). At the dose of 0.1 μg/bee, mortality of orally and topically treated animals was equivalent to their respective controls. Mortality ranged from 20 to 26% for all the groups (data not shown).

Locomotor activity

Topical treatment of fipronil at the dose of 0.01 ng/bee produced only the effect of spending more time in immobility (Student's test, $t = -2.631$, $p = 0.011$, $df = 58$). The path length and vertical displacements in the box decreased in treated animals, but the difference between the treated animals and the controls was not significant in either case. No effect of fipronil was observed at the same dose after oral treatment. Thiamethoxam and acetamiprid had no significant effect on the three parameters of locomotor activity compared to controls, regardless of dose (0.1 and 1 ng/bee for thiamethoxam and 0.1 and 1 μg/bee for acetamiprid) or exposure route (oral delivery or topical application). In all cases, honeybees seemed less active than controls in the box and spent less time in the sixth upper level of the box and more time in levels 1 and 2. The time spent in immobility was increased, and the distance covered was reduced in treated honeybees. None of these differences were significant. We never observed honeybees trembling on the floor, fallen backward, or displaying abnormal movements of wings, legs, or body.

Water consumption and water responsiveness

A significant increase of the volume of water consumed by honeybees topically treated with fipronil was observed (Student's test, $p = 0.0001$), and this response was obvious during the first week of treatment (Fig. 2A). No effect was observed on water responsiveness after fipronil oral treatment. Thiamethoxam induced no effect on water consumption and responsiveness. Acetamiprid given orally induced an increase in water responsiveness (0.1 μg/bee) at 1 h (χ^2 test, $p = 0.0002$) and at 3 h (χ^2 test, $p = 0.0006$) (Fig. 2B) but had no effect on water consumption.

Sucrose responsiveness

Oral exposure to fipronil (0.01 ng/bee) induced a decrease in sucrose responsiveness with a significant difference for the 0.3 % sucrose concentration (Fig. 3A; Fisher's exact test, $p = 0.042$). Topically applied fipronil had no significant effect (Fig. 3A). Oral thiamethoxam (1 ng/bee) induced a decrease of honeybees' sucrose responsiveness to 3 and 10% sucrose concentrations (Fig. 3B; Fisher's exact test, $p = 0.001$ and 0.008, respectively). Oral exposure to a dose of 0.1 ng/bee and contact exposure to thiamethoxam (0.1 and 1 ng/bee) had no effect on sucrose responsiveness (see Fig. 3B for contact 1 ng/bee). Acetamiprid 0.1 μg had no effect on the response rates of honeybees to the ascending concentrations of sucrose solutions for oral and topical treatment (data not shown). At the dose of 1 μg/bee, topical treatment induced a nonsignificant increase of responses to sucrose concentrations (Fig. 3C). We note that this is the only case where the response curve of treated animals exceeded that of control bees.

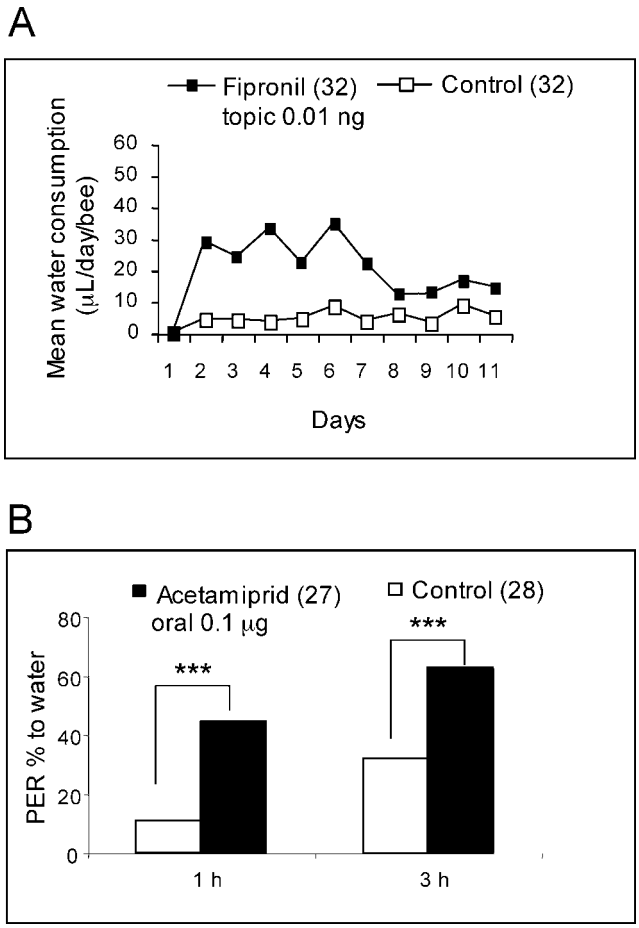

Fig. 2. Honeybees' behavioral response to water after fipronil exposure (**A**) or acetamiprid exposure (**B**). (**A**) Water consumption measured daily in fipronil-treated and control groups. The total water volume per day was divided by the number of survivors in each group, giving the mean water consumption per day per bee. See text for statistical analysis. (**B**) Water responsiveness evaluated after chronic oral exposure with acetamiprid (0.1 μg/bee). *** $p < 0.001$ (χ^2 test). PER = proboscis extension reflex.

Olfactory learning

Fipronil (0.01 ng/bee) absorbed orally or applied topically for 11 d had no effect on learning performance. An example of the result for oral intoxication is given in Figure 4A. The conditioned PER level was approximately 60% in each group. Comparison of performance in the control group between the 1-, 24-, and 48-h memory tests revealed a decay of memory with time. This observation was valid for the control groups of thiamethoxam and acetamiprid experiments as well (see Fig. 4B and C). No effect of fipronil on the response to CS compared to controls was observed, regardless of the time of the test. This indicated no memory impairment induced by fipronil

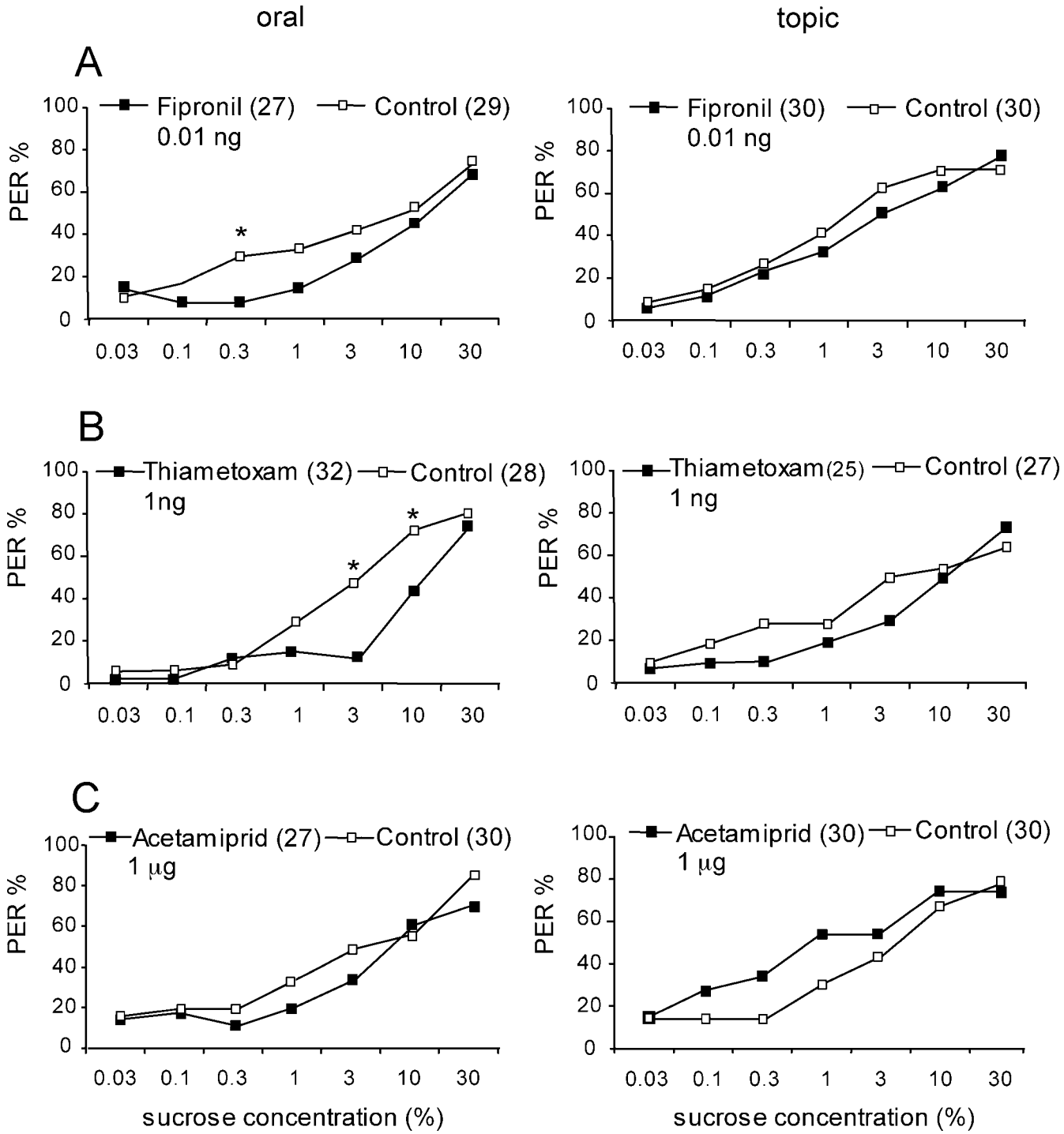

Fig. 3. Sucrose responsiveness of honeybees after oral or contact exposure to insecticide compared to controls. The nature and the dose of the insecticide are indicated in the figure. The performance is expressed as the percentage of proboscis extension reflex (PER) to antennal stimulation with sucrose solutions of increasing concentrations recorded in each group. Number of subjects in each group is indicated in parentheses. * $p < 0.050$ (Fisher's exact test).

1, 24, and 48 h after learning. At these times the honeybees were also tested for a new odorant. Control honeybees responded significantly more to the CS than to the new odorant (McNemar's tests, $p = 0.002$ and 0.031 for tests performed at 1 and 24 h, respectively). This was also the case for treated honeybees at the 1-h test (McNemar's test, $p = 0.031$). For later retrieval tests (at 24 and 48 h), the response levels to the CS and the new odorant were not different in fipronil-treated animals, indicating a problem of generalization of the conditioned response to a new odorant for long periods. The same effect was observed with fipronil 0.01 ng/bee after topical exposure (data not shown). Oral treatment of thiamethoxam (0.1 and 1 ng) induced a slight and nonsignificant decrease of performance during learning and in retrieval tests (data not shown). Only with topical application did we observe a significant decrease of learning performance or retention level. For a dose of 0.1 ng/bee, the learning curve of topically treated animals was not different from the control curve, and the

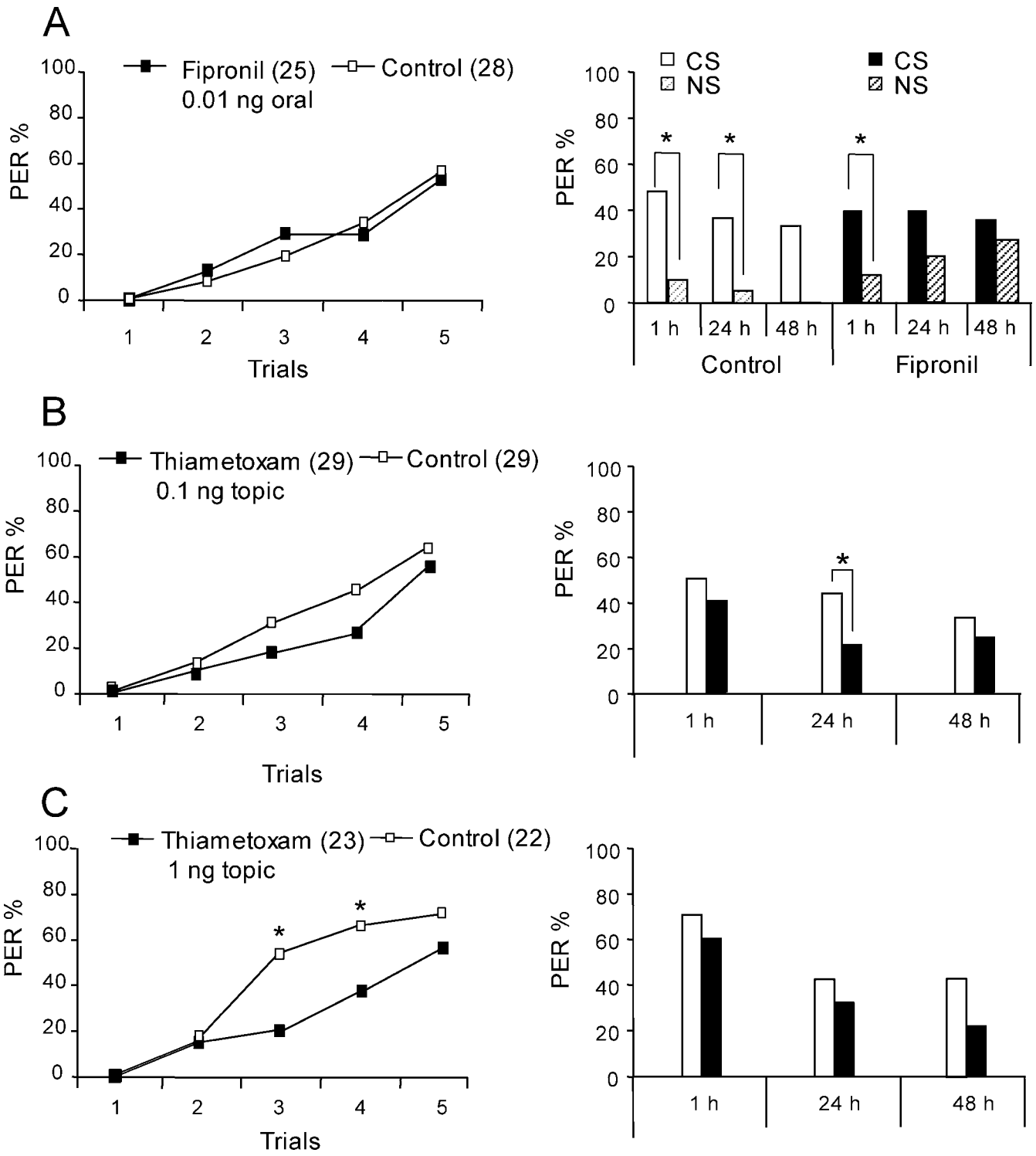

Fig. 4. Olfactory learning and memory performances of honeybees exposed to insecticides compared to controls. Performances during training (left) and memory tests (right) are expressed as percentage of proboscis extension reflex (PER) to conditioned stimulus (CS) recorded in each group. (**A**) Honeybees treated orally with fipronil and control honeybees were conditioned to one odorant (CS) during training and were tested for CS and a new olfactory stimulus (NS) at 1, 24, and 48 h. * $p < 0.050$ (MacNemar's test for within-group comparison of response to CS and NS). (**B** and **C**) Honeybees exposed topically to thiamethoxam (0.1 and 1 ng) and control honeybees were conditioned to respond by a PER to a coffee flavor during training and memory tests. Number of subjects in each group is indicated in parentheses. * $p < 0.050$ (Fisher's exact test for between-group comparison).

1-h retention level was equivalent in the two groups, with a performance approximately of 50%. The memory test performed 24 h after learning showed a significant decrease in performance in the treated group compared to controls (Fisher's exact test, $p = 0.020$), but at 48 h, there were no more differences between the two groups (Fig. 4B). At the dose of 1 ng/bee, topical application of thiamethoxam induced a significant decrease in learning performance for the third and fourth trials (Fisher's exact tests, $p = 0.025$ and 0.033, respectively). At the end of the learning session, control bees

reached 70% response rate, whereas thiamethoxam-treated bees reached only 50% response rate. Consequently, memory performance of the latter was lower than that of controls at 1, 24, and 48 h, but the difference was not significant (Fig. 4C). Acetamiprid induced no effect on learning and memory (data not shown). At the end of the learning session (fifth conditioning trial), all the animals gained a conditioning level of 60%. During the retention tests, the response level to the CS was lower in treated honeybees than in controls at each point in time, but the difference was not significant. In control and treated bees, the response rate to the new odorant was low and significantly different from the response rate to the conditioned odor.

DISCUSSION

Repeated exposure of honeybees to fipronil at the dose of 0.1 ng/bee (LD50/50), defined as sublethal in our previous work [21], induced complete mortality in individuals exposed for one week. This effect on mortality was not observed after neonicotinoid exposure. Sustained exposure to fipronil (LD50/500) or to neonicotinoids ($1/5 < LD50 < 1/100$) induced limited behavioral modifications. Chronic sublethal doses of acetamiprid induced no greater effect than at acute doses [20] on water responsiveness and induced less effect than at acute doses on locomotor activity, sucrose responsiveness, and olfactory memory. The experiments with thiamethoxam show that repeated exposure to a dose that has no behavioral effect when applied in acute conditions [20] results in the appearance of some behavioral deficits. We may conclude from these observations that acetamiprid seems to be the least toxic of the three molecules for honeybees after repeated exposure to sublethal doses.

We reported a mean mortality level of 21% for acetone and 12% for acetonitrile in control animals orally or topically treated with the solvent. Comparison to the mortality level (10%) reported in nontreated animals for a 10-d observation period [30] indicates that acetone enhances mortality of individuals in our experiments and can be considered partly responsible for the mortality of fipronil-treated (0.01 ng/bee) and acetamiprid-treated (1 μg/bee and 0.1 μg/bee) animals.

Oral thiamethoxam delivered at the highest dose (one-fifth of the LD50 corresponding to 30 μg/L) had no significant effect on mortality. Similarly, chronic oral exposure of honeybees to either imidacloprid or its plant metabolites induced no lethal effect at concentrations of 20 and 40 μg/L [26]. Acetamiprid 1 μg/bee (one-tenth of the LD50) induced the highest observed mortality level (30%), but this level was not statistically different from that of the control group. Oral fipronil at a dose of one-fiftieth of the LD50 (0.1 ng /bee, 3 μg/L) induced complete mortality after one week of treatment. This result is in agreement with the 40% mortality obtained with 2.2 μg/L fipronil in the subchronic exposure study conducted by Decourtye et al. [25]. By contrast, acute contact with the dose of 0.1 ng/bee induced no additional mortality compared to controls in the 24 h following the treatment [21], indicating that fipronil at a sublethal dose becomes lethal on repeated exposure. It is noteworthy that no significant mortality was obtained in conditions of chronic exposure when the dose was decreased to 0.01 ng /bee, which corresponds to a dose of one-five-hundredth of the LD50.

We report limited effects of the three pesticides on the motor, sensory, and cognitive functions of the honeybee. The behavioral functions we have taken into account are linked to the foraging profile of the honeybee. Inside the honeybee colony, division of foraging labor correlates directly with sucrose responsiveness. Pollen foragers respond to lower concentrations of sucrose than do nectar foragers [31]; they perform better on associative learning assays [32], and they display increased walking activity compared to nectar foragers [33]. Therefore, induced modifications of one (or all) of these functions by pesticide intoxication may have repercussions on honeybee foraging, leading to a perturbation of foragers' activity and, as a consequence, a disruption of nectar and pollen hoarding. Contrary to what was observed after acute treatment [21], fipronil chronically applied to the thorax affected locomotor activity. The exposed honeybees stayed in the lower part of the apparatus, and the time spent in immobility was significantly increased. None of the relevant parameters (time spent in each level, duration of immobility, and number of displacements) were different from their counterpart control values for other treatments regardless of dose and delivery method. We previously observed an activating effect of neonicotinoids at low doses on locomotor activity. Acetamiprid in acute topical treatment at doses of 0.1 and 0.5 ng/bee increased locomotor activity [20], but in the present experiment this effect was not observed. Imidacloprid (LD50: 10–20 ng/bee) in acute topical treatment at doses of 2.5, 5, 10, and 20 ng/bee induced an inability of the honeybees to move in the apparatus. However, an increase of locomotor activity was induced by the lowest dose of 1.25 ng/bee [27]. These results are in agreement with the fact that at low doses these compounds act as agonists of the cholinergic system and induce excitation, whereas at higher doses they evoke a toxic effect. However, repeated exposure to low doses of acetamiprid did not transform motor excitation into significant immobility and decreased displacements. Thiamethoxam, which, along with imidacloprid, belongs to the nitroguanidine neonicotinoid group, did not induce this activating effect after acute [20] or chronic exposure (present study).

We observed modifications of behavioral response to water after treatment with pesticides. Fipronil induced an increase in water consumption during the exposure period. Oral acetamiprid treatment (0.1 μg/bee) induced the enhancement of water responsiveness, and a nonsignificant increase of sucrose responsiveness was induced by topical acetamiprid (1 μg/bee). These modifications were previously observed after acute exposure to acetamiprid [20]. Acetamiprid exposure of honeybees could modify the hive equilibrium, shifting nectar foragers with a high sucrose threshold to pollen foragers with a low sucrose threshold. Only oral exposure with thiamethoxam at the highest repeated dose (1 ng/bee) induced a partial decrease of sucrose responsiveness. The same dose in acute treatment had no significant effect [20]. Fipronil had no obvious effect on sucrose responsiveness, only a tendency to a decrease after oral exposure, an observation reminiscent of the one observed after acute treatment [21]. We observed that sucrose responsiveness was decreased in emergent control bees compared to foraging adult bees [20,21], a difference that can be related to age and foraging experience [34]. The difference is clear for weak sugar concentrations up to 3% and can explain the fact that sucrose responsiveness of young bees was less affected by acetamiprid and fipronil than in foragers.

Contact thiamethoxam (0.1 ng/bee) induced a decrease of memory 24 h after learning followed by a recovery at 48 h that rules out long-term memory impairment. Learning performance was decreased in bees treated with thiamethoxam at

the dose of 1 ng/bee, but there were no significant repercussions on olfactory memory. No effect of fipronil was observed on learning performance and olfactory memory, a result already found in honeybees chronically consuming a higher dose (0.075 ng/bee) [25]. However, fipronil induced an impairment of odorant response specificity; this is shown by the fact that, after long postlearning periods, treated honeybees responded indiscriminately to the learned odorant and the new one, a phenomenon known as odor generalization. Data in the literature have shown that each of the odorants we used as CS (1-hexanol or 1-nonanol) induces a low response level to the other one [35]; thus, in our experiments, the two odorants should be perceived as dissimilar by bees. The lack of odorant-specific memory suggests that fipronil favors increased odor generalization. This effect was not observed after acetamiprid treatment, as honeybees still discriminated between known and unknown odorants. These results suggest that cholinergic and gabaergic pathways do not support the same role in olfactory processes. Anatomical and physiological evidence indicates that the olfactory message is conveyed from the antennae to higher brain centers through activation of cholinergic neurons. Modifying the excitatory level of the cholinergic pathways with acetamiprid treatment seems to have no major effects on olfactory perception. Inhibitory local circuits within the antennal lobe are necessary for building up odorant-specific signals. It has been suggested that GABA and GluCl receptors play this role [4,16]. Fipronil blocking of these receptors can be responsible for the olfactory generalization.

The effects of the low doses of fipronil after subchronic exposure can be linked to sensitization of receptors following prolonged stimulation. In cockroach thoracic ganglion neurons, the repetitive activation of GluCl receptors by the major metabolite of fipronil, fipronil sulfone, decreases the 50% inhibitory concentration values, indicating that the receptors are more sensitive to the inhibitor [36]. Receptor sensitization can be responsible for the death of animals that have undergone repeated exposure at the dose of 0.1 ng/bee and for the behavioral effects we reported at the dose of 0.01 ng/bee (increased water consumption, decreased mobility, odor generalization). Although all neonicotinoid insecticides act selectively on insect nAChRs, their agonist actions vary from partial to full efficacy. A recent study conducted on cockroaches has shown that the agonist efficacy, defined as the maximum inward current induced by neonicotinoid insecticides on isolated neurons, is positively correlated with insecticidal activity [37]. Injected at toxic doses, low-efficacy compounds like imidacloprid cause excitatory symptoms, whereas high-efficacy compounds (acetamiprid) cause depressive/paralytic symptoms. Using acute intoxication with sublethal doses, we failed to find such a distinction between imidacloprid [27] and acetamiprid [20], the two compounds showing an activating effect on honeybees' biological functions. In the vertebrate, a 3-d exposure to imidacloprid, thiacloprid, or nicotine up-regulates the neuronal $\alpha4\beta2$ nAChRs that are insensitive to α-BGT [38. Patch-clamp recordings performed on cultured rat cortical neurons show $\alpha4\beta2$ nAChR desensitization induced by 30-min exposure to nicotine [39]. It was originally proposed that nicotine-induced up-regulation is related to desensitization of the receptor, but the relations between the two phenomena are not clearly defined [38]. Such up-regulation of nAChRs has not been described in insects and cannot be counted for the behavioral modifications observed in honeybees after neonicotinoid exposure. In the study of Tan et al. [37] on *Periplaneta americana* neurons, thiamethoxam failed to activate the neuronal nAChRs, a result already found by Nauen et al. [40] on *Heliotis virescens* neurons. Both studies report as a certainty that thiamethoxam does not interact with the nicotinic receptors. Nevertheless, the biological effect of thiamethoxam is in most cases comparable to other neonicotinoid insecticides [41]. The toxic action of thiamethoxam is then related to its rapid conversion to clothianidin, a metabolite compound that binds to insect nAChRs with high affinity and is considered a full agonist of these receptors [40]. However, we failed to find any relevant biological effect of thiamethoxam on the honeybee after acute sublethal treatment [20], and we observed only a limited impairment of sucrose sensitivity and olfactory learning after chronic treatment (present study). No explanations can be put forward for these results, as bioassays in honeybees have shown comparable toxic effects of imidacloprid, thiamethoxam, and clothianidin, with LD50 values induced by contact treatment ranging from 18 to 30 ng/bee [41].

?2

In our evaluation of the sublethal toxicity, we cannot estimate the no-observed-effect-concentration for fipronil. At a concentration of 0.3 μg/L (corresponding to the oral dose of 0.01 ng/bee), at least one behavioral parameter was statistically different from the control value (Table 1). By contrast, acetamiprid and thiamethoxam have no or limited effect when applied chronically at sublethal doses. Therefore, the no-observed-effect-concentration for the behavioral assays are 3 mg/L and 3 μg/L for acetamiprid and thiamethoxam, respectively. Hence, fipronil appears to be the most toxic of the three molecules tested. The evaluation of the pesticide risk to honeybees (the hazard ratio) will be a combination of the intrinsic toxicity of the molecules as we have attempted to define it and the exposure of bees to the compounds in natural conditions, which will depend on their status (larvae, workers, foragers) in the hive.

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