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Effects of Sublethal Doses of Acetamiprid and Thiamethoxam on the Behavior of the Honeybee (*Apis mellifera*)

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Abstract Acetamiprid and thiamethoxam are insecticides introduced for pest control, but they can also affect non-target insects such as honeybees. In insects, these neonicotinoid insecticides are known to act on acetylcholine nicotinic receptors but the behavioral effects of low doses are not yet fully understood. The effects of acetamiprid and thiamethoxam were studied after acute sublethal treatment on the behavior of the honeybee (*Apis mellifera*) under controlled laboratory conditions. The drugs were either administered orally or applied topically on the thorax. After oral consumption acetamiprid increased sensitivity to antennal stimulation by sucrose solutions at doses of 1 μg/bee and impaired long-term retention of olfactory learning at the dose of 0.1 μg/bee. Acetamiprid thoracic application induced no effect in these behavioral assays but increased locomotor activity (0.1 and 0.5 μg/bee) and water-induced proboscis extension reflex (0.1, 0.5, and 1 μg/bee). Unlike acetamiprid, thiamethoxam had no effect on bees' behavior under the conditions used. Our results suggest a particular vulnerability of honeybee behavior to sublethal doses of acetamiprid.

Introduction

Neonicotinoids are insecticides widely used in agriculture against sucking insects; however, they can also affect useful non-target insects such as honeybees. Nitro-substituted neonicotinoids (imidacloprid and thiamethoxam) applied topically are the most toxic to the honeybee, with contact LD_{50} values in the nanograms per bee range (Iwasa et al. 2004). Cyano-substituted neonicotinoids (acetamiprid and thiacloprid) exhibited a much lower toxicity, with LD_{50} values in the micrograms per bee range (Iwasa et al. 2004; European Commission Acetamiprid, 2004). There is considerable evidence that the targets of the neonicotinoids compounds are the nicotinic acetylcholine receptors (nAChRs), where they act as partial or almost-full agonists (Déglise et al. 2002; Tomizawa and Casida 2003). However, unlike imidacloprid and acetamiprid, thiamethoxam showed no competitive interaction with other neonicotinoids on nAChRs (Tan et al. 2007). Acetylcholine is an important neurotransmitter in the insect brain (Breer 1987; Bicker 1999) and acetylcholine-binding sites are widely present in the honeybee brain (Kreissl and Bicker 1989; Scheidler et al. 1990). In this insect, diverse functions seem to be supported by cholinergic neurotransmission (see Michelsen and Braun 1987; Cano Lozano et al. 1996, 2001; Dacher et al. 2005; Thany and Gauthier 2005). Therefore, even sublethal doses of neonicotinoid can affect honeybees.

Honeybee can be considered an insect particularly vulnerable to pesticides, as its genome has fewer genes encoding xenobiotic detoxifying enzymes compared to other insects' (Claudianos et al. 2006). It has been demonstrated previously that honeybees receiving imidacloprid to a dose >5 ng/bee exhibited an impairment of locomotor activity and sucrose sensitivity (Lambin et al. 2001). Moreover, imidacloprid absorbed orally (12 ng/bee) decreased the retention performances of bees (Decourtye et al. 2004). By contrast, it has been observed that this drug topically applied on the thorax at a low dose (1.25 ng/bee) induced learning and locomotor facilitation (Armengaud

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et al. 2002). Besides the well-documented toxic effect of imidacloprid in insects, little is known of the physiological and behavioral effects of sublethal doses of acetamiprid and thiamethoxam on honeybees. It is thus important to examine the effect of sublethal doses of these neonicotinoid on honeybee functions.

We suggest the hypothesis that acetamiprid and thiamethoxam at nontoxic doses can affect gustatory, motor, and mnemonic functions in the honeybee. It has already been shown that under laboratory conditions, the proboscis extension reflex (PER) elicited by sucrose stimulation of the antennae can be used as an ecotoxicological tool to test different behavioral functions in the honeybee (Mammood and Waller 1990; Pham-Délègue et al. 2002). Sucrose-triggered PER assays and olfactory conditioned PER can be used to assess the sublethal effect of pesticides on sucrose sensitivity and on the olfactory learning abilities of the honeybee, respectively (Lambin et al. 2001; Decourtye et al. 2004; El Hassani et al. 2005). The integrity of these functions is necessary for foraging behavior, for example, the perception of sugar is important to honeybees for making foraging decisions (Pankiw and Page 1999). Furthermore, in the course of foraging behavior, a learning process occurs during which floral parameters (i.e., odor, color, and shape) are associated with a food reward (Erber 1975a, b).

The purpose of this work is to examine under laboratory conditions the effects of acute sublethal doses of orally absorbed or topically applied acetamiprid and thiamethoxam on locomotor activity, water and sucrose sensitivity, and olfactory learning in the honeybee.

Materials and Methods

Animals

Experiments were conducted from September 2003 to July 2004 at the Paul Sabatier University Campus, France. Worker honeybees were caught at the top of outside hives or were collected from hives maintained in a warmed apiary. Newly emerged workers and drones were excluded from experiments. Honeybees were maintained with ad libitum food in small Plexiglas boxes until the beginning of the individual tests and were maintained in an incubator during the night during the experimental period. The honeybees were tested first for locomotor function and then for sucrose sensitivity and learning capabilities. For locomotor experiments, honeybees were directly and individually introduced into a 5-ml syringe where they received oral treatment or topical application of acetamiprid or thiamethoxam and they were left in the syringe until being tested for motor activity. For PER to sucrose assays and learning experiments, bees were anesthetized by cooling. They were then fixed in a small tube by depositing a drop of wax-colophony mixture onto the dorsal part of the thorax; the head and the forelegs were left free. The experimental procedures were in compliance with the European laws on the use of animal subjects.

Treatment

Acetamiprid (99% purity) and thiamethoxam (97% purity) were purchased from Cluzeau Info Labo, Sainte-Foy-La-Grande, France. Acetamiprid was dissolved in acetone (Sigma Aldrich, France) and thiamethoxam was dissolved in acetonitrile (Sigma Aldrich, France) to obtain the stock solutions. For topical application, the stock solutions were dissolved in water and 1 µl of the final solution was deposited onto the thorax of the honeybee. Control animals received 1 µl of water containing the solvent (10%). For oral treatment, the stock solutions were dissolved in sucrose solution (40%, w/v) that was used to feed honeybees individually with 10 µl. Control animals were fed with 10 µl of sucrose solution containing the solvent (1%). Acetamiprid was used at doses of 0.1, 0.5, or 1 µg/bee and thiamethoxam was used at doses of 0.1, 0.5, or 1 ng/bee.

Locomotor Activity

The effect of acetamiprid and thiamethoxam on locomotor activity was evaluated 60 min after a single topical application or oral dose. Bees were subjected to a starvation period of 60 min before the beginning of the motor test. Locomotor activity was analyzed as previously described (Lambin et al. 2001). Honeybees were tested in an open-field-like apparatus (30 × 30 × 4 cm) standing vertically and illuminated from above. The back area was divided into six horizontal levels 5-cm high; each level was divided into squares of 5 × 5 cm. Honeybees were introduced in the bottom right-hand side and were allowed to move for a 3-min observation period. The position of the animal in a square was recorded every 3 s with a keyboard computer. Variables assessed for each animal were the total distance walked, the duration of immobility, and the number of ascents from one level to a higher one.

Sucrose Sensitivity

Extension of the proboscis is reflexive in response to antennal stimulation with solutions of sucrose. In the

current experiments, the PER is used to sample bees' sensitivity to ascending concentrations of sucrose solution (ACSS) and to examine the dose-dependent relation of orally administered and thoracically applied acetamiprid or thiamethoxam on sucrose responsiveness. Each animal was tested twice with ACSS: 60 min before and 60 min after treatment. The same point of satiety was achieved for orally and topically treated bees by giving animals 10 µl of 40% (w/v) sucrose solution 1 h before each test with ACSS. Allowing bees to drink water ad libitum 1 h before each test with ACSS controlled the effect of thirst on sucrose sensitivity. PER to water was tested 3 min before testing PER to sucrose solution before and after treatment. Concentrations of sucrose solution increased in a $\log_{10}$ series of −1.0, −0.5, 0.0, 0.5, 1.0, and 1.5, corresponding to sucrose concentrations of 0.1%, 0.3%, 1%, 3%, 10%, and 30% (w/v). For each concentration, percentage of PER released by honeybees was recorded. Solutions were applied to antennae with a 3-min intertrial interval. All bees were tested twice with ACSS, but only bees presenting no response to water before the first test with ACSS were included in the statistical analysis of PER to sucrose.

For evaluating PER to water all the honeybees were taken into account. As mentioned before, bees were tested twice: 1 h before and 1 h after treatment, the antennae were touched with a drop of water 3 min before each ACSS. Results for PER to sucrose and PER to water were analyzed separately.

Olfactory Learning

As a 3-h starvation was necessary to enhance the motivational state of the animals, oral or topical treatments were performed 3 h prior to olfactory conditioning. Classical olfactory conditioning was carried out as previously described by Gerber et al. (1998) and El Hassani et al. (2005). A five-trial paradigm with an intertrial interval of 1 min, which leads to long-term memory, was used. In this experiment, honeybees were trained to associate the conditioned stimulus (CS) represented by a coffee odor with an unconditioned stimulus (US) represented by a drop of sucrose (40%, w/v) applied to the antennae. The CS and the US lasted 3 s, and the US was presented 2 s after onset of the CS. No food was allowed to the bee during the training phase until the fifth trial, when a small drop of sucrose (40%, w/v) was presented to the proboscis. In the testing trials, the CS was presented alone 1, 24, and 48 h after the learning session, and the percentage of bees releasing a conditioned PER was recorded for each delay.

Data Analysis

Experiments were performed in triplicate and repeated at least three times. For locomotor activity, analysis of variance (ANOVA) was conducted to compare the effects of the different doses of acetamiprid and thiamethoxam. PER rates to the different sucrose solutions, before and after treatment, were compared for each of the control and treated groups using the McNemar test. For PER rates to water, comparison under different treatments was done using Fisher's exact test. When the p-values were significant, we performed pairwise comparisons between all groups. For olfactory learning G tests were used to compare the different doses. All tests were two-tailed and were performed with SPSS12 (SPSS Science, Chicago, IL, USA). A difference was considered to be significant when the obtained p-value was <0.050.

Results

Locomotor Activity

Acetamiprid increased the total length walked in the open-field-like apparatus 1 h after treatment (Fig. 1A; one-way ANOVA; distance covered, $F_{7,100} = 10.78$, $p < 0.001$). To assess the origin of the differences, we compared each treatment with the others, using Tukey or Scheffe pairwise post hoc tests. A significant difference was revealed between controls and treated bees (0.1 and 0.5 µg/bee), topical acetamiprid application inducing an increase in the distance covered (Tukey test; distance covered, $T_{100} = 7.74$, $p = 0.031$, and $T_{100} = 7.74$, $p = 0.035$). Similarly, a significant decrease in the duration of immobility of bees was observed 1 h after topical (ANOVA on data transformed by raising to square, $F_{3,75} = 3.320$, $p = 0.024$) but not oral (ANOVA on data transformed by logarithm, $F_{3,50} = 0.827$, $p = 0.485$) treatment (data not shown). Once again, animals treated with the 0.1 and 0.5 µg/bee doses but not with the 1.0 µg/bee dose differed from the control group. The number of ascents (i.e., flying or climbing from one level to a higher one in the apparatus) was not affected by acetamiprid (data not shown).

By contrast, after oral or topical delivery of thiamethoxan, the locomotor activity of animals was not significantly modified compared to that of control bees (Fig. 1B).

The way pesticides were applied (orally or topically) had an effect on animals' behavior. A significant difference was revealed between orally treated and topically treated animals, whatever the acetamiprid or the thiamethoxan dose (contrasts test: acetamiprid, $T_{100} = 7.74$, $p = 7.95\text{E-}12$; thiamethoxam, $T_{103} = 7.80$, $p = 5.13\text{E-}12$). Topically

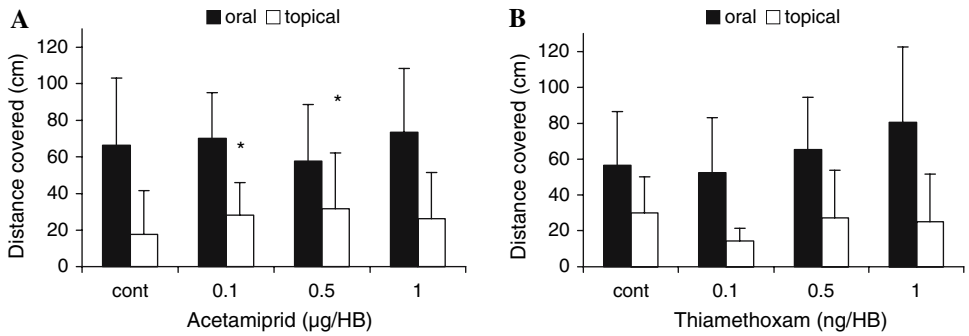


Fig. 1 Mean (+SE) distance covered during 3 min by bees treated with acetamiprid (**A**) or thiamethoxam (**B**) 60 min before the test. *Different from the control group, Tukey test, $p < 0.050$. The numbers of bees used were (A) 15 (oral, control), 12 (oral, 0.1 µg/bee), 14 (oral, 0.5 µg/bee), 13 (oral, 1.0 µg/bee), 14 (topical, control), 14 (topical, 0.1 µg/bee), 14 (topical, 0.5 µg/bee), and 12 (topical, 1 µg/bee) and (B) 12 (oral, control), 14 (oral, 0.1 ng/bee), 14 (oral, 0.5 ng/bee), 12 (oral, 1.0 ng/bee), 14 (topical, control), and 15 (topical, other groups)

treated animals moved less in the box and consequently they covered a shorter distance than orally treated animals.

PER to Sucrose

In acetamiprid experiments, the sucrose sensitivity of the control group was decreased by acetone introduced in an oral solution (Fig. 2A). A significant decrease in the percentage PER was observed for 0.3% and 10% sucrose solutions after oral absorption of solvent (McNemar's test, $p = 0.031$ and $p = 0.031$, respectively). In honeybees treated orally with 0.1 and 0.5 µg acetamiprid, a similar decrease in sucrose responsiveness was observed compared to controls, with significant differences for 1% and 3% sucrose concentrations ($p = 0.008$ and $p = 0.031$, respectively) and for 0.3%, 1%, 3%, and 10% sucrose solutions ($p = 0.006$, $p = 0.016$, $p = 0.016$, and $p = 0.016$, respectively). However, the group of bees treated with 1 µg acetamiprid presented identical response levels to the first and the second test with ACSS (Fig. 2A).

After a thoracic application, there was no effect of the solvent on sucrose responsiveness in control animals. The sucrose responsiveness of acetamiprid-treated animals was not modified compared to that of controls (Fig. 2B).

Acetonitril delivered orally or topically in control bees did not induce significant modification of sucrose responsiveness (Fig. 2C and D). Bees treated with thiamethoxam presented identical sucrose responsiveness before and after oral (Fig. 2C) or topical (Fig. 2D) treatment, whatever the dose.

PER to Water

One hour before treatment, the responsiveness to water was tested in control and acetamiprid- or thiamethoxam-treated bees, and it was tested again 1 h after treatment. Data are presented as a water responsiveness index (WRI). This index corresponds to the total number of bees presenting a PER to water before the second test with ACSS minus the total number of bees presenting a PER to water before the first test with ACSS (Fig. 3). Positive index values indicate that treatment induces an increase in responsiveness to water.

Identical water responsiveness was observed in the different groups before the first ACSS and then before treatment (Fisher's exact test, $p>0.05$; data not shown).

No effect of acetamiprid on water responsiveness was found after oral treatment. Topically applied, acetamiprid induced a dose-dependent increase in the PER to water compared to that of controls (Fig. 3B) (Fisher's exact test, 0.1 µg/bee $p = 0.048$, 0.5 µg/bee $p = 0.009$, and 1 µg/bee $p = 0.003$). No significant modification of water responsiveness was observed after treatment with thiamethoxam (Fig. 3C and D).

Olfactory Learning and Memory

Orally absorbed acetamiprid induced no significant impairment of animals' performances during learning (G test with 3 degrees of freedom [df], $p \geq 0.05$) (Fig. 4A). However, percentage PER tested 48 h after learning significantly differed across the groups (G test with 3 df = 8.190, $p = 0.042$). Pairwise comparisons were conducted to assess the origin of this difference; we used Holm's method to adjust the p-values for repeated tests. The performance was significantly lower in the group treated with 0.1 µg/bee than in the control group (G test with 1 df = 7.386, adjusted $p = 0.039$), whereas the performance of animals which received 0.5 and 1.0 µg did not differ significantly from that of any other group (G test with 1 df, adjusted $p \geq 0.05$) (Fig. 4A).

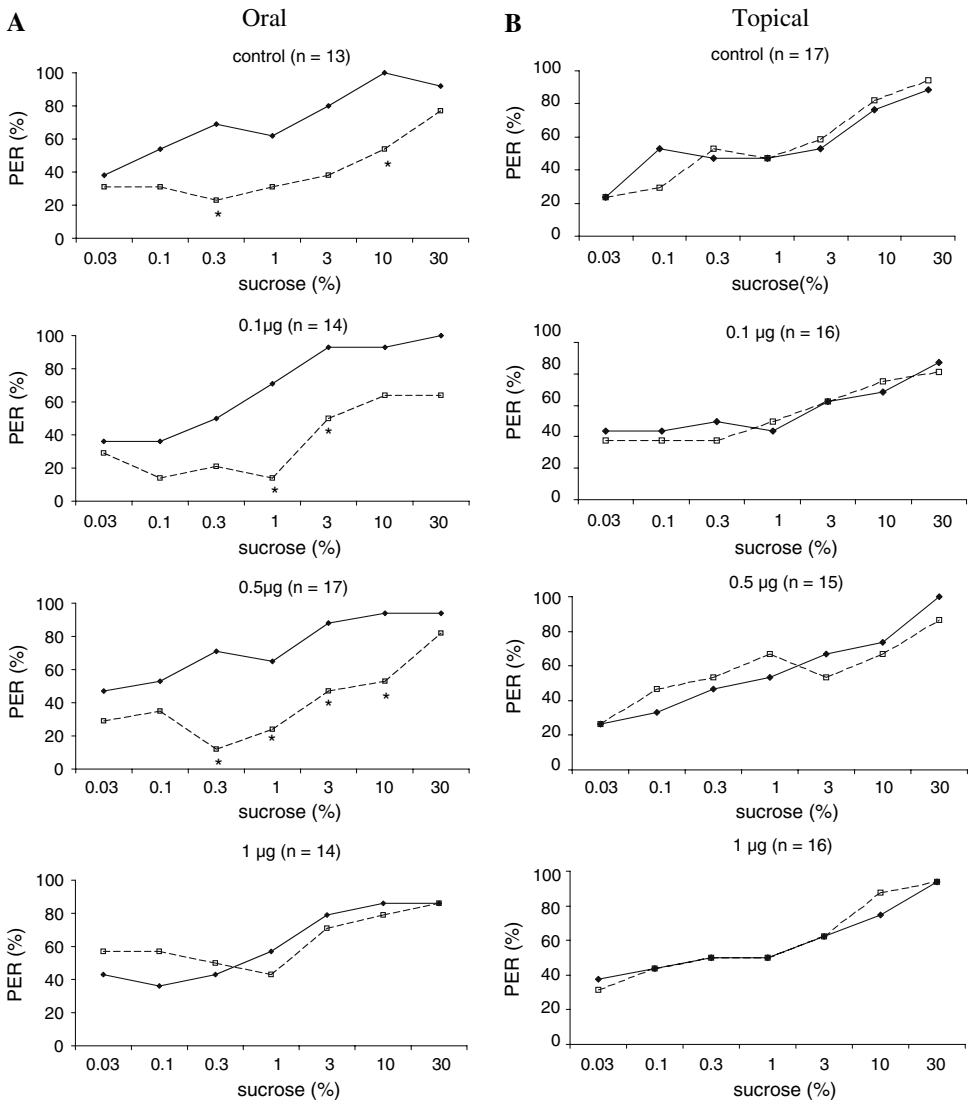


Fig. 2 Percentage PER responses to ACSS when bees were orally (**A**, **C**) or topically (**B**, **D**) treated with acetamiprid (A, B) and thiamethoxam (C, D). Bees were tested twice: 1 h before (solid line) and 1 h after (dashed line) treatment. *The treatment induces a difference (McNemar test, $p < 0.050$)

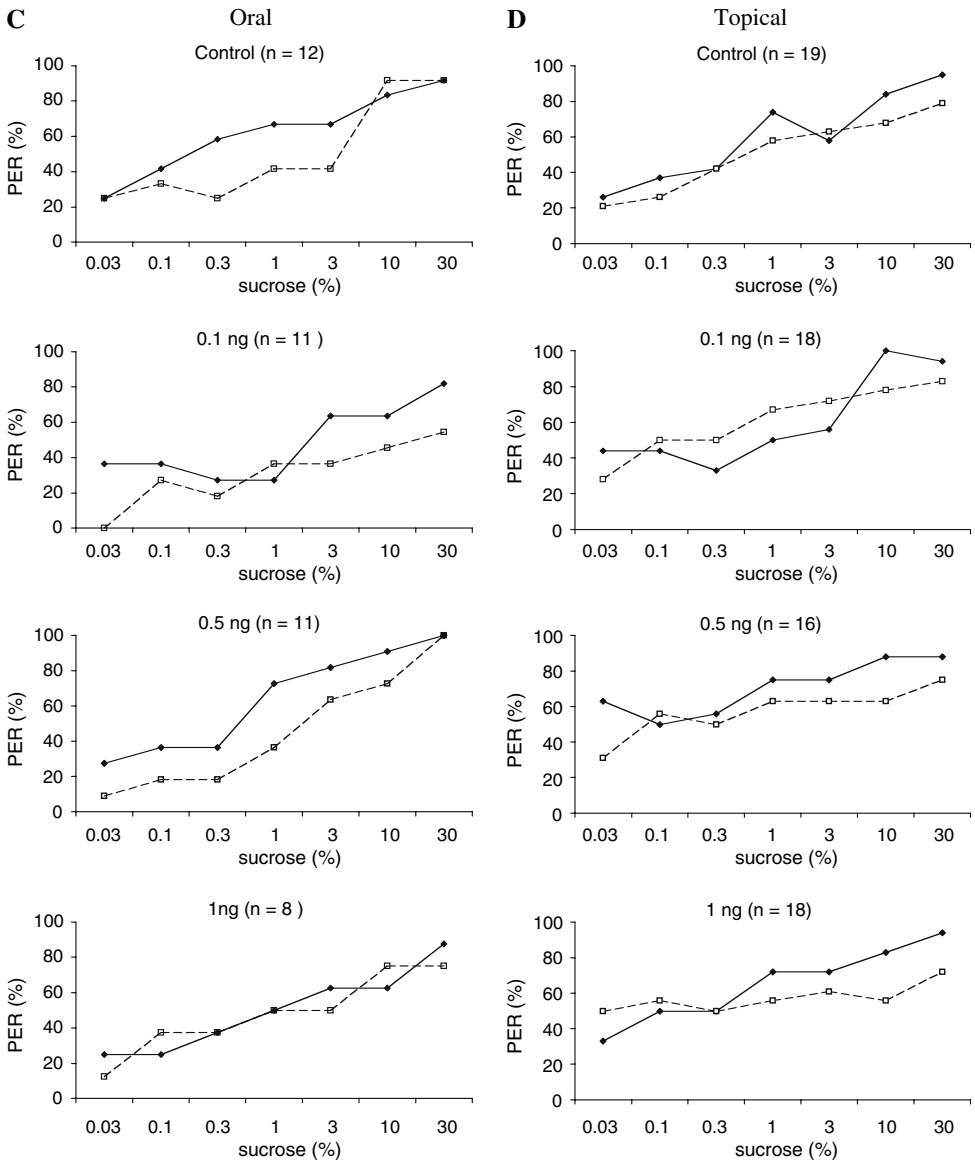
Topical acetamiprid treatment induced no significant effect on learning and retention performances (G test with 3 df, $p \geq 0.05$ for both) (Fig. 4B).

The training performance of animals treated orally with thiamethoxam, were not significantly different from that of controls (G test with 3 df, $p \geq 0.05$) (Fig. 4C). Curiously, a significant increase in performance was observed at the third acquisition trial to the dose of 0.5 µg/bee applied topically (Fisher exact test, $p < 0.05$). This was due to an unusual decrease in PER in the control group on the third trial (Fig. 4C). No significant effect was observed on retrieval performance after thiamethoxam either absorbed orally or applied topically (Fig. 4C and D).

Discussion

This report presents a behavioral analysis of the effect of acetamiprid and thiamethoxam on locomotor activity, sucrose gustatory sensitivity, water responsiveness, and olfactory learning and memory in the honeybee. Results described here concern acute oral and contact exposure of adult honeybees to acetamiprid and thiamethoxam. We were interested in the sublethal effect of these pesticides because subtle effects on bees' physiology or behavior may affect the honeybee population. The doses of pesticides used were in the $LD_{50}/100$ to $LD_{50}/10$ range and induced no extra mortality compared to controls. Twenty-four and 48 h after oral or topical contamination, the mortality in the acetamiprid- and thiamethoxam-treated groups was identical to that in the control group. The most significant finding of our study is that bees' physiology and behavior are more affected by acetamiprid than by thiamethoxam, both tested in the $LD_{50}/100$ to $LD_{50}/50$ range. Indeed, contrary to acetamiprid, thiamethoxam induced no significant effect either on locomotor activity or on PER to sucrose and water or on learning and memory. According

Fig. 2 Continued



to available LD_{50} (Iwasa et al. 2004; European Commission Acetamiprid 2004), acetamiprid (0.1 µg/bee = LD_{50}/100) affected locomotor activity, PER to water, and memory performance, whereas thiamethoxam, whatever the dose (0.1 or 1 ng/bee, respectively, LD_{50}/300 and LD_{50}/30), had no effect on the same functions.

In our experiments, the behavior of the bees was tested from 1 to 48 h after acetamiprid treatment. Brunet et al. (2005) indicated that, in the honeybee, more than 50% of acetamiprid was metabolized in <30 min after intoxication, and that only acetamiprid and 6-chloronicotinic acid were present in the head at significant levels over a 72-h period observation. Under our conditions, the observed effect on honeybees' behavior could be essentially attributed to acetamiprid (or maybe 6-chloronicotinic acid), although acetamiprid metabolite effects cannot be excluded, despite their low toxicity (Iwasa et al. 2004). Locomotor activity of the honeybee was stimulated by acetamiprid applied topically at doses of 0.1 and 0.5 µg/bee, whereas locomotion was unaffected by the dose of 1 µg/bee. Under similar experimental conditions the insecticide imidacloprid induced opposite effects on motor activity according to the dose (Lambin et al. 2001). Sixty minutes after topical application of 2.5 ng/bee imidacloprid, honeybees lost their ability to move in the open field, whereas 1.25 ng/bee induced an increase in locomotor activity. Therefore, low, nonlethal doses of nicotinic agonists can affect honeybee displacements, as previously reported by Michelsen and Braun (1987).

The data presented here indicate an effect of acetamiprid on sucrose-elicited PER after oral administration but not after topical application. Doses lower than 1 µg/bee

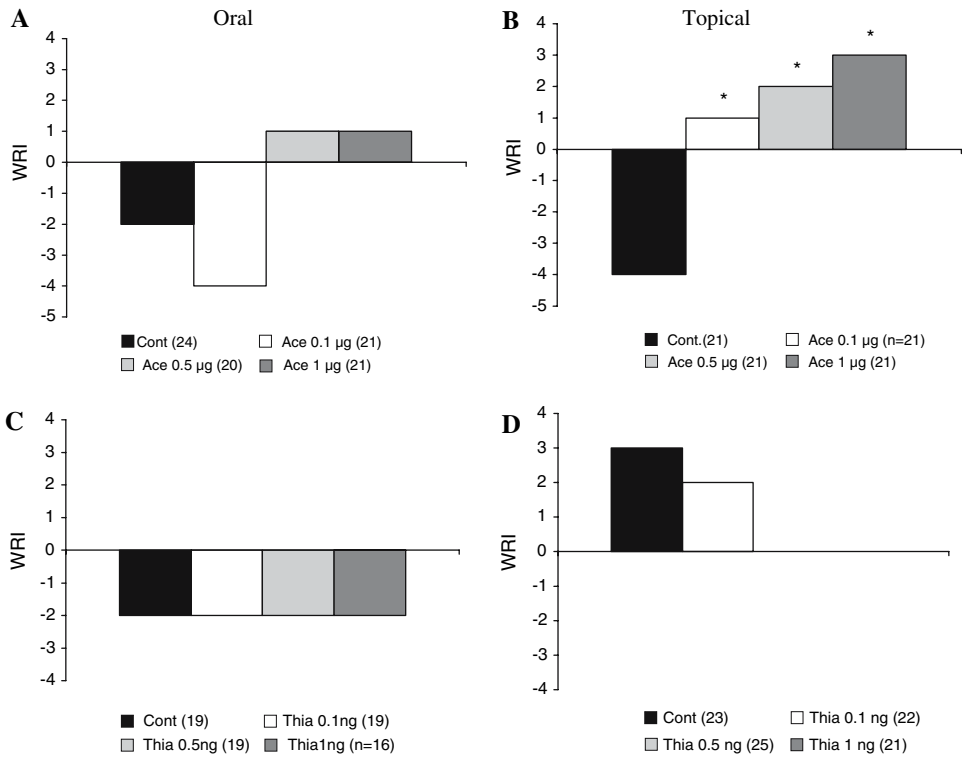


Fig. 3 Water responsiveness index (WRI) of bees treated orally and topically with acetamiprid (**A**, **B**) or thiamethoxam (**C**, **D**). The WRI is the total number of bees that presented a PER to water before the second test with ACSS minus the total number of bees that presented a PER to water before the first test with ACSS. Cont, control; Ace, acetamiprid; Thia, thiamethixam. *Different from the control group (Fisher exact test, $p < 0.050$)

induced a decrease in sucrose-triggered PER comparable to that observed after acetone absorption. However, animals that consumed 1 µg acetamiprid presented no modification of PER to sucrose. Thus, it seemed that the dose of 1 µg/bee reversed the effect of solvent by increasing the sucrose sensitivity. This effect of acetamiprid on sucrose responsiveness is consistent with the previously described effect of nicotinic drugs on sucrose-triggered PER (Braun and Bicker 1992) and on sucrose sensitivity (Thany and Gauthier 2005). Water-triggered PER was increased in a dose-dependent manner after thoracic application of acetamiprid. It can be hypothesized that acetamiprid has an effect on thirst but not when it is orally administered. Before oral or topical treatment, the groups presented identical responsiveness to water and sucrose. This suggests that the tested honeybees were not different in caste, age, genotype, or foraging experience (Pankiw and Page 1999; Pankiw et al. 2001). So the differences observed between groups can be attributed to the effect of the pesticide.

Results of learning experiments indicated that oral treatment with a sublethal dose of acetamiprid (0.1 µg/bee = LD_{50}/100) lowered bees' memory performance only for the long-term retention delay (48 h after acquisition), leaving short-term memory intact. Therefore, acetamiprid seems specifically harmful to the long-term memory of the honeybee. We must also consider the possibility that a lower sensitivity in the PER assay (induced by acetone) can decrease the PER rate. The fact that the performance level of controls in oral experiments was weaker than in topical experiments reinforces this hypothesis.

Acetamiprid and imidacloprid are neonicotinoid compounds acting agonistically on nAChRs (Tan et al. 2007) and they share the same binding site (Kayser et al. 2004). Their behavioral effect on honeybees does not totally match the effect of nicotinic agonists. Oral treatment of honeybees with imidacloprid (12 ng/bee = LD_{50}/2.5) impaired medium-term olfactory memory and lower doses had no effect on learning and memory processes (Decourtye et al. 2004). A facilitation of olfactory memory was observed after nicotine injection into the brain (Thany and Gauthier 2005). A facilitation of nonassociative memory tested through the habituation of the PER was also induced by imidacloprid (Lambin et al. 2001; Guez et al. 2003). Interestingly, we previously reported differential effects of nicotinic antagonists on memory processes. One class of nicotinic antagonists specifically affected long-term memory, whereas another class affected short-term memory (Cano Lozano et al. 1996; Cano Lozano et al. 2001; Dacher et al. 2005; Gauthier et al. 2006). All together, these results point to the existence of different nicotinic receptor subtypes, which could be differently affected by neonicotinoids. Genome sequencing has revealed a greater diversity of nAChRs in honeybees compared to *Drosophila* or mosquitoes (Jones et al. 2006).

An interesting aspect of the present results is the non-linear effect on behavior of increasing concentrations of acetamiprid, a result already observed with imidacloprid

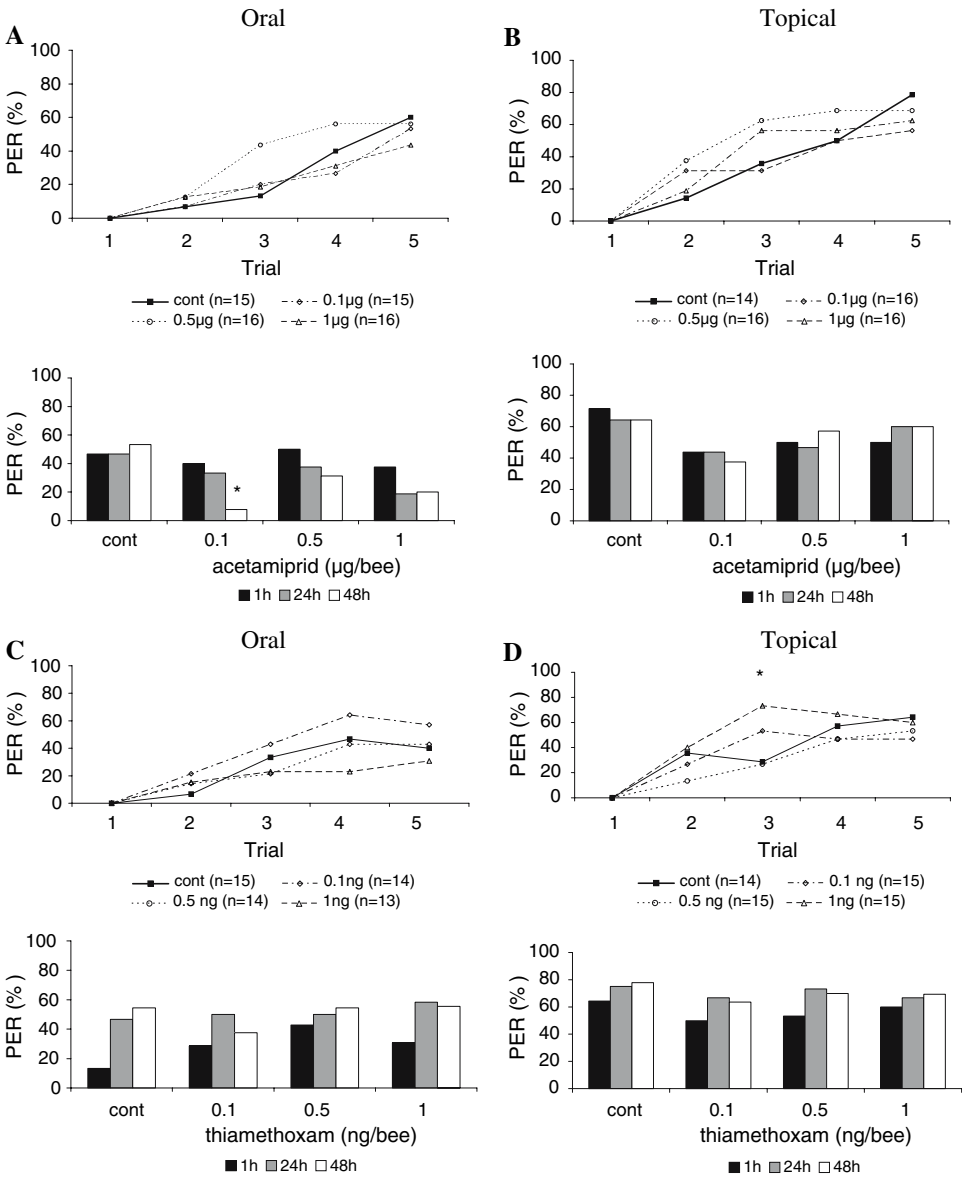


Fig. 4 Olfactory learning and retention performances of bees 3 h after an oral (**A**, **C**) or topical (**B**, **D**) acetamiprid (A, B) or thiamethoxam (C, D) treatment. The same animal was tested 1, 24, and 48 h after learning. *Different from the control group (*G* test, $p < 0.050$)

(Lambin et al. 2001) and with fipronil, a phenylpyrazole insecticide (El Hassani et al. 2005). Indeed, intermediate doses of acetamiprid (0.1 or 0.5 µg/bee) modified learning performance and locomotor activity, whereas the highest dose (1 µg/bee) did not. One explanation of this phenomenon could be the existence of two receptors for acetamiprid, as has been suggested for imidacloprid (Nauen et al. 2001; Guez et al. 2003).

In the present study, thiamethoxam failed to cause any effect on honeybees' behavioral functions. These results are in agreement with patch-clamp recording performed on cockroach neurons (Tan et al. 2007). In this study, all neonicotinoids tested (including imidacloprid and acetamiprid), except thiamethoxam, caused inward currents that were blocked reversibly by methyllycaconitine, a nAChR antagonist. Thiamethoxam, even at 100 mM, failed to cause an inward current and showed no competitive interaction with other neonicotinoids on nAChRs (Tan et al. 2007). Similar results were reported for noctuid neurons (Nauen et al. 2003). These results indicate that thiamethoxam is not a direct-acting agonist or antagonist. The conversion of thiamethoxam into the toxic metabolite clothianidin has been proposed as the cause of its biological effect (Nauen et al. 2003). Laboratory bioassays have demonstrated the toxicity of thiamethoxam and clothianidin to honeybees, with contact LD_{50} values of 30 and 22 ng, respectively, comparable to the LD_{50} value for imidacloprid (18 ng) (Iwasa et al. 2004). Thus we cannot exclude the hypothesis that, in honeybees as in noctuids, thiamethoxam is a neonicotinoid precursor for clothianidin, which exhibits high activity as an agonist on isolated neurons. At present, we have no hypothesis to explain the

absence of behavioral effects caused by sublethal doses of thiamethoxam delivered to honeybees.

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