Available online at www.sciencedirect.com

SCIENCE DIRECT

Pharmacology, Biochemistry and Behavior 82 (2005) 30 – 39

PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR

www.elsevier.com/locate/pharmbiochembeh

# Effects of sublethal doses of fipronil on the behavior of the honeybee (*Apis mellifera*)

Abdessalam Kacimi El Hassani, Matthieu Dacher, Monique Gauthier, Catherine Armengaud *

*Centre de Recherches sur la Cognition Animale, Université Paul Sabatier Toulouse III-CNRS UMR 5169, 118 Route de Narbonne, 31062 Toulouse Cedex 04, France*

Received 16 September 2004; received in revised form 12 July 2005; accepted 13 July 2005
Available online 15 August 2005

**Abstract**

Fipronil is a phenylpyrazole insecticide introduced for pest control, but it can also affect non-target insects such as honeybees. In insects, fipronil is known to block GABA receptors and to inhibit ionotropic glutamate-gated chloride channels, but the behavioral effects of low doses are not yet fully understood. We have studied the effect of sublethal doses of fipronil on the behavior of the honeybee (*Apis mellifera*) under controlled laboratory conditions. The drug was either administered orally or applied topically on the thorax. A significant reduction of sucrose sensitivity was observed for the dose of 1 ng/bee 1 h after a thoracic application. No significant effect on sucrose sensitivity was obtained with acute oral treatment. A lower dose of fipronil (0.5 ng/bee applied topically) impaired the olfactory learning of the honeybees. By contrast, locomotor activity was not affected. Our results suggest a particular vulnerability of the olfactory memory processes and sucrose perception to sublethal doses of fipronil in the honeybee.


*Keywords:* *Apis mellifera*; Fipronil; Memory; Proboscis extension response (PER); Locomotor activity; Sucrose sensitivity; Phenylpyrazole insecticide; Insect

## 1. Introduction

Fipronil is a second-generation phenylpyrazole insecticide widely used in veterinary medicine. It has excellent therapeutic and persistent activity against ticks and fleas when topically administered to domestic animals (Hainzl and Casida, 1996). As fipronil is also effective at low doses against numerous terrestrial insects such as insect pests of crops (Balanca and de Visscher, 1997), it is also used as a pesticide. However, fipronil is highly toxic to non-target insects and the LD 50 on honeybees is very low (Tingle et al., 2003: 4 ng/bee, Decourtye, 2002: 6.2 ng/bee). Besides the well-documented toxicity of fipronil to insects, little is known about the physiological and behavioral effects on honeybees of sublethal doses of fipronil, which bees may encounter during their foraging. This is a major concern as in the South of France there has been a depopulation of hives that could not be accounted for by high mortality, but which occurred after seasons where bees were foraging on sunflowers whose seeds were coated with fipronil. Therefore testing on non-target species is particularly important to determine the suitability of fipronil-based products for registration in different countries or habitats and the potential associated risks to non-target wildlife.

Fipronil is a potent inhibitor of the gamma-aminobutyric acid (GABA)-gated chloride channel (Cole et al., 1993). It triggers hyper-excitation, convulsions and paralysis that cause insect death. Indeed, GABA is an important inhibitory neurotransmitter in invertebrates (Rauh et al., 1990; Sattelle, 1990). GABA-like immunoreactive neurons are widely distributed in the bee brain (Bicker, 1999) and are also clustered within the thorax ganglion of the hymenopthera and orthoptera (Witten and Truman, 1998; Wildman et al., 2002). GABA receptors have been identified in the visceral muscle of the cockroach (Moss and Miller, 1988). In locust

* Corresponding author. Tel.: +33 561 556 436; fax: +33 561 558 444.
*E-mail address:* carmenga@cict.fr (C. Armengaud).

0091-3057/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.pbb.2005.07.008

muscles, GABA receptors resembled those of the motor neuron cell body in their different sensitivity to the vertebrate GABA antagonists picrotoxin and bicuculline (Fraser et al., 1990). Hence, blocking GABA receptors in insects with fipronil could impair locomotor activity.

Several lines of evidence indicate that GABA mediates, in vertebrates, the processing of taste information at each level from the periphery to the cerebral cortex (Yamamoto et al., 1998) and is also involved in olfaction in invertebrates (Bazhenov et al., 2001). In honeybees, picrotoxin disrupts discrimination of molecularly similar odorants but not of dissimilar odorants (Stopfer et al., 1997), whether the treatment occured before conditioning or before testing (Hosler et al., 2000), and similarly fipronil intoxication of the honeybee could impair olfactory perception. We advance the hypothesis that fipronil at sublethal doses can affect gustatory perception, olfactory learning and motor function in the honeybee. These functions are essential to the bees as they are necessary in foraging behavior. Indeed, sucrose sensitivity is important for making foraging decisions (Pankiw and Page, 1999) and organizing the division of labor within the hive (Page and Erber, 2002). Moreover, in the course of foraging, a learning process occurs during which floral features (i.e. odor, color, and shape) are associated to the nectar reward (Menzel, 1999).

Sucrose sensitivity assays, olfactory conditioning of the proboscis extension response (PER) and recording of locomotor activity can be used to assess the sub-lethal effect of pesticides on the honeybee (Lambin et al., 2001; Decourtye et al., 2004a,b). The purpose of this study was to examine under laboratory conditions the effects of acute sublethal doses of orally absorbed or topically applied fipronil on (1) locomotor activity, (2) sucrose sensitivity and (3) olfactory learning.

## 2. Material and methods

### 2.1. Animals

The experiments (locomotor activity, PER induced by antennae sucrose stimulation and olfactory learning) were carried out from September 2003 to February 2005. Worker honeybees were caught through a hole at the top of the hive set in an apiary warmed to 27 °C. Bees were kept for less than 1 h with ad libitum sucrose solution (40% wt/v) in small Plexiglas boxes until being used for a single experiment. For PER to sucrose and the olfactory learning experiments, bees were individually anaesthetized by cooling. Then they were fixed into a small tube with a drop of wax-colophane mixture (9 g and 4 g, respectively) laid on the dorsal part of the thorax and the tube's wall. For locomotor experiments, bees caught from the hive were maintained in the Plexiglas box until they were individually tested for motor activity. Then the test bees received oral or topical application of fipronil and were kept starved in a 5-ml syringe for 1 h until the test for motor activity. The experimental procedures were in compliance with the European laws on the use of animal subjects.

### 2.2. Treatment

Fipronil (Cluzeau Info Labo, Sainte-Foy-La-Grande) was dissolved in acetone and diluted either in water for topical application or in sucrose solution for oral treatment. The doses tested were sublethal and inferior or equal to LD 50/5. The final concentration of acetone in sucrose and watery solutions was equal to 0.1% (vol./vol.). The oral treatment consisted in administering (using a 20-μl pipette) 10 μl of sucrose solution (40% wt/v) containing vehicle (0.1% acetone) or fipronil at 0.1, 0.5 or 1 ng/bee to each honeybee. An additional experiment with fipronil at 0.01 ng/bee orally administered was conducted for PER to sucrose and olfactory learning investigations following a pilot experiment suggesting an effect only for the lower doses. The animals did not react to the presence of acetone or fipronil and eagerly drank the sucrose solution. For topical application, 1 μl of vehicle (0.1% acetone) alone or containing fipronil (0.1, 0.5 or 1 ng/bee) was applied to the thorax of the bee with a 2-μl pipette.

### 2.3. Locomotor activity

Locomotor activity was analyzed as previously described (Lambin et al., 2001). The motor activity can be recorded using an open-field like apparatus allowing observation of bee vertical displacements. This test does not reflect the flying ability of the animals, however it is relevant to access motor function of walking bees because pesticides often disturb this function. The effect of fipronil on locomotor activity was studied 60 min after a single topical application or oral dose. Bees were individually introduced into a 5-ml syringe where they were subjected to a starvation period of 60 min. The animals were then restrained by pushing the plunger of the syringe and they received topical or oral administration of fipronil. Honeybees were tested in a PVC open-field-like apparatus (length: 30 cm, height: 30 cm, depth: 4 cm) stood vertically and illuminated from above. The glass front allowed observation and the PVC back area was divided into 30 squares (6 horizontal levels of 5 cm high); with each level divided into squares of $5 \times 5$ cm. The syringe containing the bee was introduced at the bottom right-hand side, a trap door was opened and the bee was allowed to move inside the box for a 3-min observation period. Bees walked from the bottom to the top of the open-field, and some of them tried to fly by remaining in contact with the walls. The position of the animal in a square was recorded every 5 s with a computer. Variables assessed for each animal were the total length walked, the duration of immobility, the number of ascents from one level to a higher one and the

time spent in each of the six levels of the apparatus. These last two parameters were chosen as indicators of geotaxis or phototaxis.

### 2.4. Sucrose sensitivity

The PER can be used to assess sucrose sensitivity (for a review, see Scheiner et al., 2004). Extension of the proboscis is reflexive in response to antennal stimulation with solutions of sucrose. In the current experiments, the PER was used to evaluate the bees' sensitivity to ascending concentrations of sucrose solution (ACSS) and to examine the dose-dependent component of oral and topical applications of fipronil on sucrose responsiveness. For each concentration of sucrose solution, the proportion of animals releasing a PER was calculated. Each animal was tested twice with the ACSS: 60 min prior and 60 min after treatment. Prior to each ACSS, the bees consumed 10 μl of sucrose solution; they were then starved for two periods of 1 h separated by 10 μl of sucrose consumption. This protocol gave the same duration of starvation for topical and oral experiments; in this latter case fipronil was given in the 10 μl sucrose solution. The effect of thirst on sucrose sensitivity was controlled, by allowing bees responding to water to drink water (10 μl) 1 h before the presentation of ACSS. Concentrations of sucrose solution increased in a $\log_{10}$ series of −1.5, −1.0, −0.5, 0.0, 0.5, 1.0, and 1.5 corresponding to sucrose concentrations of 0.03%, 0.1%, 0.3%, 1%, 3%, 10% and 30% (w/v). Solutions were applied to the antennae with a 1-min inter-trial interval. Only bees presenting no response to water 1 min before the lowest sucrose concentration were included in the statistical analysis of the PER to sucrose.

### 2.5. Olfactory learning and memory

This kind of learning can be studied under controlled laboratory conditions by using olfactory Pavlovian conditioning of the PER (Bitterman et al., 1983; Menzel, 1999), which is relevant to the situation bees encounter during their foraging trips (Gerber et al., 1996). Oral or topical treatments were performed 3 h prior to conditioning because 3 h starvation is necessary to enhance the motivational state of the animals.

Classical olfactory conditioning was carried out as previously described by Gerber et al. (1998) and Deglise et al. (2003). The five-trial paradigm with an inter-trial 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% wt/v) applied to the antennae. The CS and the US lasted 3 s, and the US delivery started one second before the end of the CS. The bees were allowed to feed only during the fifth trial of the training phase. In the testing trials, the CS was presented alone 1 h, 24 h and 48 h after the learning session. The proportion of animals releasing a conditioned PER was calculated during learning and retrieval. Daily experiments including bees subjected to fipronil (0.1, 0.5, 1 ng/bee for topical application and 0.01, 0.1, 0.5, 1 ng/bee for oral administration) and control bees were repeated at least 3 times with at least four bees for each condition. Bees were fed with sucrose solution twice a day with at least a delay of 1 h after learning or retrieval.

### 2.6. Data analysis

For the locomotor activity, the nine variables recorded were transformed with natural logarithms or square roots to reach a normal distribution. Analysis of variance (ANOVA) was conducted to analyze the results, using the factor treatment (oral or topical with the four concentrations). As the pairwise Scheffé or Tukey post-hoc tests did not yield any significant result, we used contrast comparison to compare the oral administration group with the topical administration group.

The PER rates to the different sucrose solutions were compared within the eight treatment groups using a McNemar test (with binomial distribution). For the olfactory learning performances comparison the Fisher exact test was used to compare the different doses. When the $p$-values were significant, we performed pairwise comparisons between all groups. As this involves multiple comparisons (which could artificially decrease the α risk), the $p$-values obtained were corrected using the technique of Holm. All the tests were two-tailed. A difference was considered to be significant when the $p$-value obtained was lower than 0.050. ANOVA and Mc Nemar tests were performed with SPSS12 (SPSS Science, Chicago, USA). Fisher's exact test was performed with R2.0 (R Development Core Team, 2004); R provides algorithms to compare more than two groups with Fisher's exact test.

## 3. Results

### 3.1. Locomotor activity

When introduced in the vertical open-field, honeybees tend to migrate upward against the force of gravity to the light source. This behavior could be defined as negative geotaxis or positive phototaxis. During the 3 min of observation, we evaluated the number of ascents performed (i.e. passing from one level to another higher level; data not shown), the distance covered (Fig. 1A), the duration of immobility (Fig. 1B) and the time spent within each one of the six levels of the apparatus (Fig. 1C).

The first three parameters were significantly different across the different treatments (one-way ANOVA, number of ascents: $F_{7,\ 108}=2.360$, $p=0.028$; immobility duration:

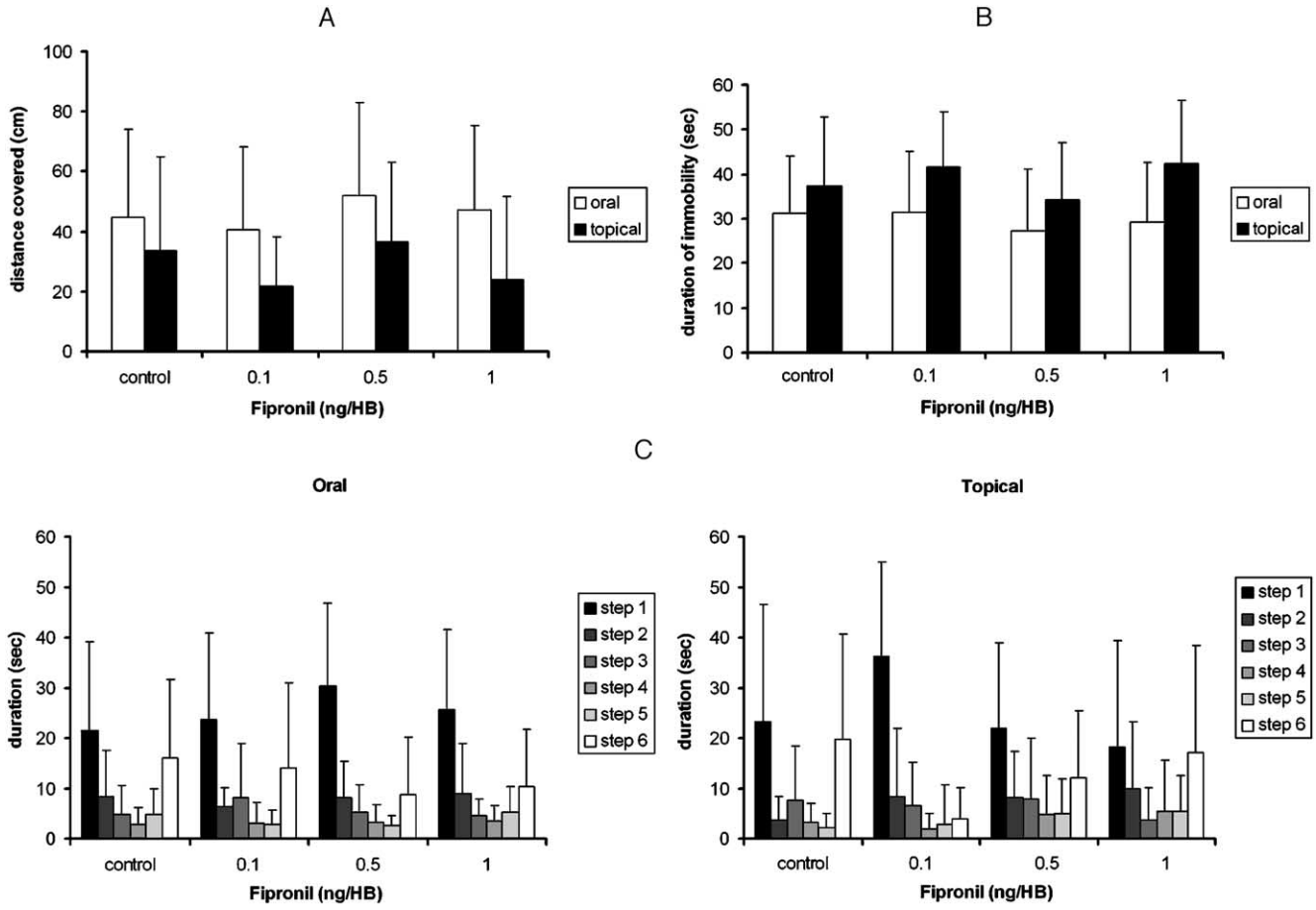


Fig. 1. Locomotor activity of honeybees 1 h after fipronil thoracic application or oral absorption. Results represent the distance covered (A), the duration of immobility (B) and the time spent in each level (C). Columns and vertical bars represent means ± SEM. Number of animals used are: topical, 0.1 ng: 14; topical, 0.5 ng: 13; oral, 0.5 ng: 14; other groups: 15.

$F_{7,\ 108}=2.459$, $p=0.022$; distance covered: $F_{7,\ 108}=2.936$, $p=0.007$). On the other hand, there were no differences between the time spent within each level (one-way ANOVA, level 1: $F_{7,\ 108}=1.573$, $p=0.151$; level 2: $F_{7,\ 108}=1.349$, $p=0.235$; level 3: $F_{7,\ 108}=0.659$, $p=0.706$; level 4: $F_{7,\ 108}=0.544$, $p=0.799$; level 5: $F_{7,\ 108}=1.188$, $p=0.316$; level 6: $F_{7,\ 108}=1.405$, $p=0.211$). To assess the origin of the differences, we compared each treatment with all the others, using Tukey or Scheffe pairwise post-hoc tests, but no differences were seen. Therefore, we performed contrasts comparison, i.e. comparing one group of means versus another group. A significant difference was revealed between orally-treated and topically-treated animals, whatever the fipronil concentration used (contrasts test: number of ascents: $T_{108}=-3.285$, $p=0.0014$; immobility duration: $T_{108}=3.589$, $p=5.0\times10^{-4}$; distance covered: $T_{108}=-3.875$, $p=1.8\times10^{-4}$).

These results indicated that fipronil had no effect on locomotor activity whatever the route of its administration. Fipronil did not affect the honeybees' ability to move inside the apparatus. However, the method of pesticide application employed (oral or topical) had an effect on the animals' behavior. Putting a drop of solution a bees' thorax induced a reduction in mobility compared with the locomotor activity of the animals that drank the same volume of solution. A mechanical constraint has been evoked to explain this observation.

### 3.2. Sucrose sensitivity

One hour before treatment, the responsiveness to water was tested in control and fipronil-treated bees; animals that presented a PER to water before the first ACSS were not taken into account for sucrose sensitivity analysis. In the control groups, the sucrose sensitivity was not modified by acetone 1 h after an oral administration or a topical application (McNemar tests, $p>0.050$, Fig. 2A–B). Orally absorbed fipronil had no significant effect on sucrose responsiveness to the 4 doses tested (McNemar tests, $p>0.050$, Fig. 2A). However, a nearly significant decrease was observed for the 1% sucrose solution after treatment with 0.01 ng of fipronil (McNemar tests, $p=0.070$) and for the 0.3% sucrose solution after treatment with 0.5 ng of fipronil (McNemar tests, $p=0.063$). By contrast, 1 h after 1 ng of fipronil was topically applied, a significant decrease of the PER to sucrose solutions was observed for the 0.1% and

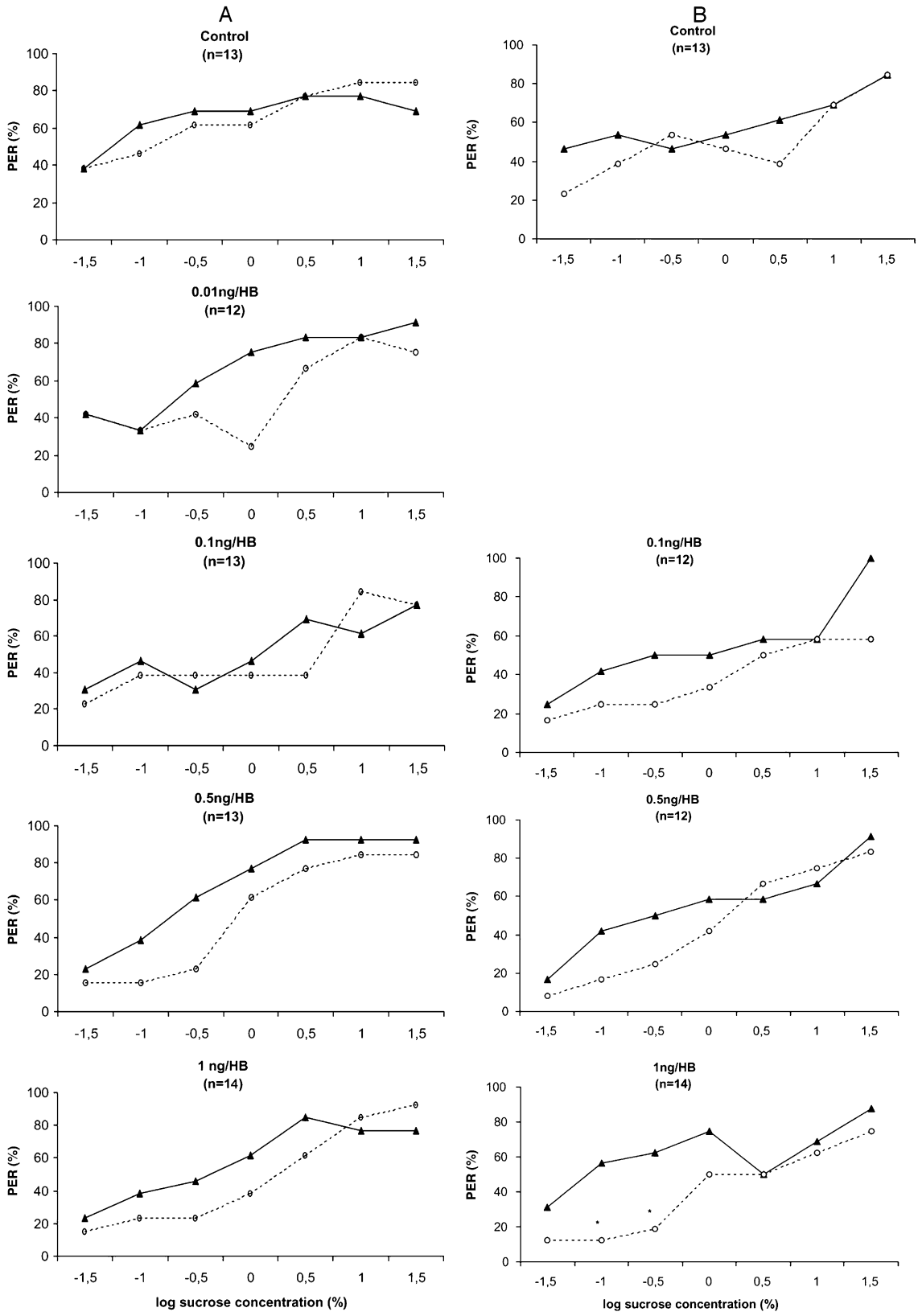


Fig. 2. Sucrose–concentration response curves of oral (A) and topical (B) fipronil treated bees. Bees were tested twice: 1 h before treatment (black line) and 1 h after treatment (dotted line). Abscissa indicates sucrose concentrations (0.03%, 0.1%, 0.3%, 1%, 3%, 10%, 30%) in logarithmic scale.*$p<0.05$ McNemar test, compared to before treatment.

0.3% sucrose solutions (McNemar tests, $p=0.039$ and $p=0.039$, respectively). For sucrose concentrations higher than 0.3% no more modification of sucrose responsiveness was observed (McNemar test, $p>0.050$, Fig. 2B). For lower doses of fipronil, no significant decrease of sucrose sensitivity was observed (McNemar test, $p>0.050$), though 0.1 ng of fipronil induced a nearly significant decrease of the PER for the 30% sucrose solution (McNemar test, $p=0.063$, Fig. 2B).

### 3.3. Olfactory learning and memory

Orally absorbed fipronil induced no significant impairment of learning and retention performances (Fig. 3A). A moderate but not significant decrease of performance was observed at the fourth acquisition trial with the fourth dose tested (Fisher exact test: $p>0.05$). One hour after training, the 0.01 ng group exhibited a non-significant decrease in retention. Retention tested 24 h and 48 h after learning was

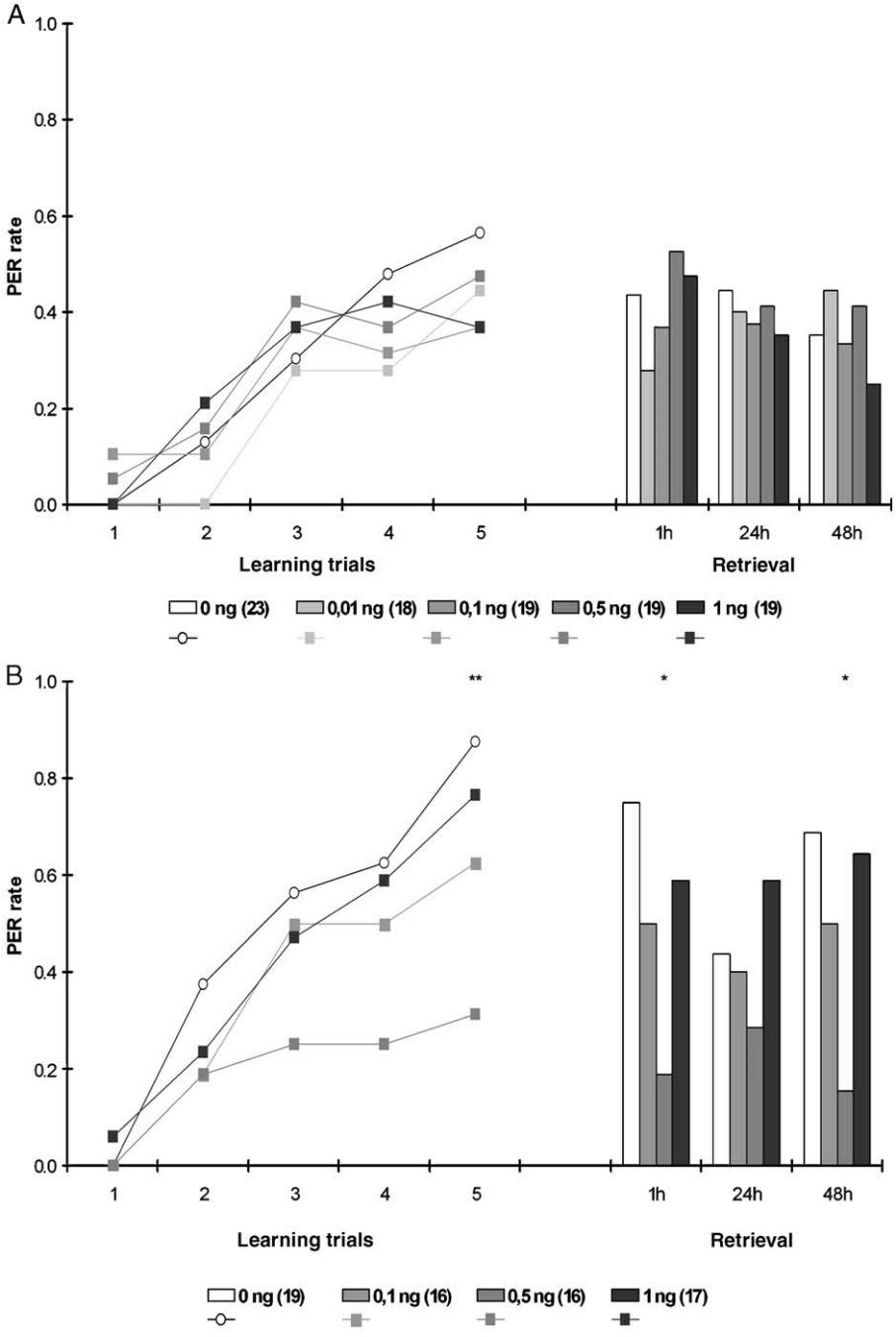


Fig. 3. Olfactory learning performances of bees 3 h after an oral (A) or topical (B) fipronil treatment. Retention performances of the same animals were tested 1 h, 24 h and 48 h after learning. The control and fipronil treated groups were run in parallel. *: The four groups are different ($p<0.050$, Fisher exact test); **: The four groups are different ($p<0.010$, Fisher exact test).

not significantly impaired in the treated groups (Fisher exact test: $p > 0.05$).

Topical treatment induced an effect on learning according to the dose used. Indeed, as can be seen in Fig. 3B, the 0.5 ng dose impaired the animal's performance whereas 0.1 ng or 1 ng did not differ from control group. This is particularly clear in the 5th learning trial, where the four groups (control, 0.1 ng, 0.5 ng and 1 ng) are different (Fisher's exact test, $p = 0.006$). The 0.5 ng dose differed from control group (Fisher's exact test, $p = 0.019$, adjusted by Holm's method) whereas the other doses did not significantly differ when compared to each other or to the control (Fisher's exact test, $p > 0.050$, adjusted by Holm's method). The same holds true during retrieval tests performed 1 h after learning, i.e. 4 h after treatment (Fisher's exact test, $p = 0.011$; 0.5 vs. control, $p = 0.023$, adjusted by Holm's method; other pairwise comparisons, p>0.050, adjusted by Holm's method) or 48 h after learning; i.e. 51 h after treatment (Fisher's exact test, $p = 0.021$; 0.5 vs. control, $p = 0.047$, adjusted by Holm's method; other pairwise comparisons, $p > 0.050$, adjusted by Holm's method). Though performance was similar among the four groups, 24 h after retrieval or during acquisition (Fisher's exact test, $p > 0.050$) the same pattern of performance was observed. Thus, topical application of 0.5 ng fipronil impaired the formation of the memory trace, but lower or higher doses did not significantly affect learning processes.

At the end of the experiment, all the bees were tested for the PER induced by sucrose stimulation of the antennae. No significant impairment of the PER to sucrose (40%) was observed over a 48-h period. This indicated that topical and oral fipronil treatments neither affected motor response nor sucrose (40%) perception during the 48-h period observation.

### 3.4. Mortality

During the three experiments, the number of dead bees was counted. The significant effects of fipronil on the PER to sucrose 1 h after a thoracic application and on olfactory memory tested up to 27 h after treatment were not associated with differential mortality among the different groups. Indeed, the treatment with fipronil topically applied or orally administered did not lead to additional mortality during this observation period. However, 48 h after topical treatment, the mortality was significantly different across the three groups (Fisher exact test: $p < 0.05$). Moreover, no difference was found between the different fipronil treated groups on the mortality rate (Fisher exact test: $p > 0.05$) whereas no mortality was observed in acetone control group. Hence, fipronil treated groups exhibited higher mortality than the control group 48 h after a thoracic application.

Forty-eight hours after oral treatment fipronil induced an increase of mortality compared to control group (58% vs. 21%, Fisher exact test: $p = 0.04$). The dose of 0.5 ng leads to a non-significant (Fisher exact test: $p > 0.05$) increase of mortality (19%) 48 h after oral absorption.

The LD 50 proposed by Decourtye (2002) was obtained by feeding groups of 20 bees with 200 µl of glucose syrup containing different doses of fipronil. By comparison with this oral contamination of group of bees, individual contamination seems to be more toxic. Although our experiments are not designed to calculate a LD 50 but a lethal time for a given dose, they indicate that the LD 50 for fipronil absorbed by harnessed bees is lower than 5 ng/bee.

## 4. Discussion

This report presents a behavioral analysis of the effect of fipronil on locomotor activity, sucrose gustatory sensitivity and on olfactory learning and memory in the honeybee. Results described here concerned acute oral and contact exposure of adult honeybees to fipronil. We were interested in the sublethal effect of the pesticide because subtle effects on bees' physiology or behavior may affect the honeybee population. The fipronil effects observed in our study were complex because they depended on the function studied, the dose tested and the way the pesticide was administered (oral vs. topical). Oral administration, which implicates digestive metabolism, induced less or lighter effects. Also, there was no clear dose–effect relationship on learning and memory functions.

### 4.1. Locomotor activity

Locomotor activity of the honeybee was not affected by fipronil at the doses given and for the short periods of time tested. We have previously observed with the same open-field test that the insecticide imidacloprid induced opposite effects on motor activity depending on the dose (Lambin et al., 2001). Sixty minutes after a 2.5 ng imidacloprid topical application, honeybees lost their ability to move in the open-field whereas 1.25 ng induced an increase of locomotor activity.

The absence of an effect of fipronil on locomotor activity was surprising because GABAergic signaling networks within the central nervous system play an important role in modulating locomotor activity in insects. Indeed, GABA is present at the peripheral inhibitory neuromuscular junction of locust extensor-tibiae muscle fibers (Usherwood and Grundfest, 1965). In addition, feeding adult female flies with a yeast-sucrose medium containing GABA transport inhibitors, resulted in diminished locomotor activity, deficits in geotaxis, and the induction of convulsive behavior with a secondary loss of the righting reflex (Leal and Neckameyer, 2002). As fipronil's main target is GABAergic signaling, we first hypothesized that fipronil would have an effect on locomotion. It might be supposed that a longer delay between treatment and test would lead to a significant impairment of locomotor activity. Another explanation

could be that the dose necessary to achieve this impairment is too close to the lethal dose. In this case, it would not be possible to observe any effect on motor function without affecting the survival of the animal (which was the purpose of the present study and is relevant to the situation encountered in the South of the France).

### 4.2. PER to sucrose

The data presented indicated that the fipronil effects on the sucrose-elicited PER were greater after topical application than after oral administration. The significant decrease of the PER for animals that received a topical application of 1 ng fipronil was observed for low sucrose concentrations. Hence the perception of a sugar solution of low concentrations was reduced by this treatment.

The responsiveness of bees to sucrose is an important indicator of honeybee foraging decisions. By offering increasing concentrations of sucrose and determining the concentration that elicits proboscis extension, one can determine the response threshold of an individual bee (Scheiner et al., 2004). Using this technique Page et al. (1998) determined that pollen and nectar foragers perceive the concentration of sucrose differently. Pollen foragers have lower sucrose response thresholds than nectar foragers (Pankiw and Page, 1999). Workers with the lowest response threshold became water foragers, followed with increasing response thresholds by pollen foragers, nectar foragers, bees collecting both pollen and nectar, and finally those returning to the colony empty (Pankiw et al., 2001). Following our results, it can be suggested that pollen foragers would be more affected by fipronil than nectar foragers, because they have a low sucrose response. As sucrose sensitivity is a critical parameter in organizing the division of labor (Page and Erber, 2002), fipronil could be harmful for hives as sublethal doses would prevent a proper organization of hive work.

In addition, we cannot exclude an effect of fipronil on amino acid gustatory perception of the bees. Indeed, some insects (i.e. beetles) possess sensory receptors that respond to GABA (Mullin et al., 1994). These receptors could be a target for fipronil.

### 4.3. Effect of fipronil on learning and memory

Our behavioral results indicated that fipronil at a sublethal doses (topical 0.5 ng per animal) modified the acquisition and retention performances tested in the conditioned PER paradigm. The impairment of retention observed 4 h after the treatment (1 h after acquisition) was most likely due to the impairment of memory formation rather than retrieval. This effect on retention performance was neither associated to a decrease of sucrose sensitivity nor to a toxic effect of fipronil. This effect could not be due to a lower sensitivity to sucrose; in the opposite case the unconditioned stimulus would be powerless. In fact, we have shown that the topical dose of 0.5 ng per animal was without effect on the PER to sucrose and no significant mortality was observed with this dose over 24 h. Furthermore, one can exclude an impairment on olfactory perception, as this would have been observed consistently for all learning trials and retrieval tests, which was not the case for any of the doses.

Insect ionotropic GABA receptors are the target of numerous insecticides especially fipronil. In crickets, GABA levels increased in the haemolymph during learning (Jaffe et al., 1992). This suggests an involvement of this neurotransmitter in the learning processes. Moreover, electrophysiological recordings indicated that GABA inhibition of the Kenyon cells could play a role on the odor-evoked oscillation patterns observed in the mushroom bodies (MacLeod and Laurent, 1996; Stopfer et al., 1997). It can be postulated that the impairment of learning and memory performance after fipronil was associated to an inhibition of ligand-gated chloride channels involved in GABA transmission. Blocking the actions of fipronil on glutamate-induced $Cl^-$ currents has been studied in cockroaches (Raymond et al., 2000; Ikeda et al., 2003; Zhao et al., 2004) and honeybees (Barbara et al., 2003). In honeybees the glutamatergic transmission seemed also to be involved in memory processes (Maleszka et al., 2000). The vertebrate NMDA receptor antagonist: MK-801, and the glutamate transporter inhibitor: L-trans-2,4-PDC, used both with pretraining and pretesting injections lead to an impairment of long-term (24 h) memory. However these treatments had no effect on short-term (1 h) memory of an olfactory task (Si et al., 2004). Thus the effects of fipronil could be attributed to the multiple cellular targets of this pesticide, including non-desensitizing glutamate-gated chloride channels (Zhao et al., 2004). In addition, fipronil sulfone is rapidly formed from fipronil in biological systems and plays a major role in its toxicity. Fipronil can also be converted into the photoproduct desulfinyl fipronil which is generally more toxic and more potent at the chloride channel than the sulfone (Hainzl et al., 1998). Fipronil photoconversion is enhanced with topical application and limited with oral consumption. This process could explain the differences in the results observed between these two kinds of administration.

### 4.4. Conclusion

Fipronil blocks glutamate-gated chloride channels in the cockroach (Ikeda et al., 2003) and the honeybee (Barbara et al., 2003); our results indicated that ligand-gated chloride channels are involved in learning and memory in the honeybee. An interesting aspect of these results is the non-linear effect on behavior of increasing concentrations of fipronil, a result already observed with imidaclopride, a neonicotinic insecticide (Lambin et al., 2001). It could be suggested that fipronil affects different receptors with a different affinity for each of them. The lowest concentration

of fipronil could block a first receptor, triggering the behavioral effects; then a higher concentration would block another receptor which would antagonize the effects of the first one. Glutamate and GABA receptors could be potential candidates. Alternatively, this non-linear effect could also be triggered by different metabolites of fipronil.

Testing fipronil on honeybees under laboratory conditions was particularly important to determine the suitability of fipronil-based products for registration, and to evaluate the potential associated risks to non-target wildlife. However, the transposition of acute effects observed on restrained bees to free-flying bees that encountered fipronil on crops needs two other kind of experiments: (1) chronic fipronil treatment under laboratory condition, and (2) field experiments. Our results provide a framework for these experiments.

## Acknowledgments

This study was supported by a grant from the French Ministry of Agriculture (no. 407, ONIFLHOR). We thank Sandrine Paute and Vincent Garry for technical assistance and Michel Lambin for his advice on the locomotion experiments.

## References

Balanca G, de Visscher M. Impacts on non-target insects of a new insecticide compound used against the desert locust [Schistocerca gregaria (Forskal 1775)]. Arch Environ Contam Toxicol 1997;32:58–62.

Barbara G, Zube C, Rybak J, Gauthier M, Grünewald B. Ionotropic receptors of cultured honey bee antennal lobe neurons. In: Eisner N, Zimmermann H, editors. Proceedings of the 5th meeting of the German neuroscience society. New York: George Thyme Verlag Stuttgart; 2003. p. 856–7.

Bazhenov M, Stopfer M, Rabinovich M, Abarbanel HD, Sejnowski TJ, Laurent G. Model of cellular and network mechanisms for odor-evoked temporal patterning in the locust antennal lobe. Neuron 2001;30:569–81.

Bicker G. Histochemistry of classical neurotransmitters in antennal lobes and mushroom bodies of the honeybee. Microsc Res Technol 1999;45:174–83.

Bitterman ME, Menzel R, Fietz A, Schäfer S. Classical conditioning proboscis extension in honeybees (*Apis mellifera*). J Comp Psychol 1983;97:107–19.

Cole LM, Nicholso RA, Casida JE. Action of phenylpyrazole insecticides at the GABA-gated chloride channel. Pestic Biochem Physiol 1993; 46:47–54.

Decourtye A. Etude de l'impact des produits phytopharmaceutiques sur la survie et l'apprentissage associatif chez l'abeille domestique (*Apis mellifera* L.). PhD Thesis University Paris, X.I., d'Orsay 2002.

Decourtye A, Armengaud C, Renou M, Devillers J, Cluzeau S, Gauthier M, et al. Imidacloprid impairs memory and brain metabolism in the honeybee (*Apis mellifera* L). Pestic Biochem Physiol 2004a;78:83–92.

Decourtye A, Devillers J, Cluzeau S, Charreton M, Pham-Delegue MH. Effects of imidacloprid and deltamethrin on associative learning in honeybees under semi-field and laboratory conditions. Ecotoxicol Environ Saf 2004b;573:410–9.

Deglise P, Dacher M, Dion E, Gauthier M, Armengaud C. Regional brain variations of cytochrome oxidase staining during olfactory learning in the honeybee (*Apis mellifera*). Behav Neurosci 2003;117:540–7.

Fraser SP, Djamgoz MBA, Usherwood PNR, O'Brien J, Darlison MG, Barnard EA. Amino acid receptors from insect muscle: electrophysiological characterization in *Xenopus* oocytes following expression by injection of mRNA. Mol Brain Res 1990;8:331–41.

Gerber B, Geberzahn N, Hellstern F, Klein J, Kowalksy O, Wüstenberg D, et al. Honeybees transfer olfactory memories established during flower visits to a proboscis extension paradigm in the laboratory. Anim Behav 1996;52:1079–85.

Gerber B, Wustenberg D, Schutz A, Menzel R. Temporal determinants of olfactory long-term retention in honeybee classical conditioning: non-monotonous effects of the training trial interval. Neurobiol Learn Mem 1998;69:71–8.

Hainzl D, Casida JE. Fipronil insecticide: novel photochemical desulfinylation with retention of neurotoxicity. Proc Natl Acad Sci U S A 1996;93:12764–7.

Hainzl D, Cole LM, Casida JE. Mechanisms for selective toxicity of fipronil insecticide and its sulfone metabolite and desulfinyl photoproduct. Chem Res Toxicol 1998;11:1529–35.

Hosler JS, Buxton KL, Smith BH. Impairment of olfactory discrimination by blockage of GABA and nitric oxide in the honey bee antennal lobes. Behav Neurosci 2000;114:514–25.

Ikeda T, Zhao X, Kono Y, Yeh JZ, Narahashi T. Fipronil modulation of glutamate-induced chloride currents in cockroach thoracic ganglion neurons. Neurotoxicology 2003;24:807–15.

Jaffe K, Blau S, Zabala N. Amino acid levels during learning and memory consolidation of an aversive conditioning task in crickets. Pharmacol Biochem Behav 1992;43:205–14.

Lambin M, Armengaud C, Raymond S, Gauthier M. Imidacloprid-induced facilitation of the proboscis extension reflex habituation in the honeybee. Arch Insect Biochem Physiol 2001;48:129–34.

Leal SM, Neckameyer WS. Pharmacological evidence for GABAergic regulation of specific behaviors in Drosophila melanogaster. J Neurobiol 2002;50:245–61.

MacLeod K, Laurent G. Distinct mechanisms for synchronization and temporal patterning of odor-encoding neural assemblies. Science 1996;8:976–9.

Maleszka R, Helliwell P, Kucharski R. Pharmacological interference with glutamate re-uptake impairs long-term memory in the honeybee, *Apis mellifera*. Behav Brain Res 2000;115:49–53.

Menzel R. Memory dynamics in the honeybee. J Comp Physiol A 1999;185:323–40.

Moss JI, Miller TA. A pharmacological study of the hyperneural muscle of *Periplaneta americana*. J Insect Physiol 1988;34:997–1003.

Mullin CA, Chyb S, Eichenseer H, Hollister B, Frazier JL. Neuroreceptor mechanisms in insect gustation: a pharmacological approach. J Insect Physiol 1994;40:913–31.

Page R, Erber J. Levels of behavioural organisation and the evolution of division of labour. Naturwissenchatfen 2002;89:91–106.

Page RE, Erber J, Fondrk MK. The effect of genotype on response thresholds to sucrose and foraging behavior of honey bees (*Apis mellifera* L). J Comp Physiol A 1998;182:489–500.

Pankiw T, Page RE. The effect of genotype, age, sex, and caste on response thresholds to sucrose and foraging behavior of honey bees (*Apis mellifera* L). J Comp Physiol A 1999;185:207–13.

Pankiw T, Waddington KD, Page RE. Modulation of sucrose response thresholds in honey bees (*Apis mellifera* L): influence of genotype, feeding, and for aging experience. J Comp Physiol A 2001; 187:293–301.

Rauh JJ, Lummis SC, Sattelle DB. Pharmacological and biochemical properties of insect GABA receptors. Trends Pharmacol Sci 1990;8:325–9.

Raymond V, Sattelle DB, Lapied B. Co-existence in DUM neurones of two GluCl channels that differ in their picrotoxin sensitivity. NeuroReport 2000;11:2695–701.

R Development Core Team. R: A language and environnment for statistical computing. R Foundation for statistical computing, 2004, http://www.R-project.org.

Sattelle DB. GABA receptors of insects. Adv Insect Physiol 1990; 22:1–113.

Scheiner R, Page RE, Erber J. Sucrose responsiveness and behavioral plasticity in honey bees (*Apis mellifera*). Apidology 2004;35:133–42.

Si A, Helliwell P, Maleszka R. Effects of NMDA receptor antagonists on olfactory learning and memory in the honeybee (*Apis mellifera*). Pharmacol Biochem Behav 2004;77:191–7.

Stopfer M, Bhagavan S, Smith BH, Laurent G. Impaired odour discrimination on desynchronization of odour-encoding neural assemblies. Nature 1997;390:70–4.

Tingle CC, Rother JA, Dewhust CF, Lauer S, King WJ. Fipronil: environmental fate, ecotoxicology, and human health concerns. Rev Environ Contam Toxicol 2003;176:1–66.

Usherwood PN, Grundfest H. Peripheral inhibition in skeletal muscle of insects. J Neurophysiol 1965;28:497–518.

Wildman M, Ott SR, Burrows M. GABA-like immunoreactivity in nonspiking interneurons of the locust metathoracic ganglion. J Exp Biol 2002;205:3651–9.

Witten JL, Truman JW. Distribution of GABA-like immunoreactive neurons in insects suggests lineage homology. J Comp Neurol 1998;398:515–28.

Yamamoto T, Nagai T, Shimura T, Yasoshima Y. Roles of chemical mediators in the taste system. Jpn J Pharmacol 1998;76:325–48.

Zhao X, Yeh JZ, Salgado VL, Narahashi T. Fipronil is a potent open channel blocker of glutamate-activated chloride channels in cockroach neurons. J Pharmacol Exp Ther 2004;310:192–201.