Effect of fipronil on side-specific antennal tactile learning in the honeybee

A. Bernadou, F. Démares, T. Couret-Fauvel, J.C. Sandoz, M. Gauthier *

Centre de Recherches sur la Cognition Animale, UMR CNRS 5169, Université Paul Sabatier, 118 route de Narbonne, 31062 Toulouse Cedex, France

ARTICLE INFO

ABSTRACT

In the honeybee, the conditioning of the proboscis extension response using tactile antennal stimulations is well suited for studying the side-specificity of learning including the possible bilateral transfer of memory traces in the brain, and the role of inhibitory networks. A tactile stimulus was presented to one antenna in association with a sucrose reward to the proboscis. The other antenna was either not stimulated (A+/0 training), stimulated with a non-reinforced tactile stimulus B (A+/B− training) or stimulated with B reinforced with sucrose to the proboscis (A+/B+ training). Memory tests performed 3 and 24 h after training showed in all situations that a tactile stimulus learnt on one side was only retrieved ipsilaterally, indicating no bilateral transfer of information. In all these groups, we investigated the effect of the phenylpyrazole insecticide fipronil by applying a sublethal dose (0.5 ng/ bee) on the thorax 15 min before training. This treatment decreased acquisition success and the subsequent memory performances were lowered but the distribution of responses to the tactile stimuli between sides was not affected. These results underline the role of the inhibitory networks targeted by fipronil on tactile learning and memory processes.

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

Tactile cues play an important role in the life of the honeybee. Within the hive, adult honeybees use mechanical stimuli for the construction of new cells and for intraspecific communication (Kevan, 1987). During foraging, honeybees take into account a set of different stimuli to learn, identify and choose floral food source. Olfactory and visual cues are the most important but tactile stimuli are also used to detect textural characteristics of floral surfaces. Kevan and Lane (1985) demonstrated that tactile perception allows honeybees to discriminate between the epidermal micro-sculptural patterns of different flowers. Due to the orientation of the microsculpturing toward the source of the floral nectar, this pattern would be used as a nectar-guide directing the pollinators to the centre of the blossom.

Honeybees' ability to detect the texture of the petals is linked to the presence of tactile detectors located on various parts of the body. Sensilla trichodea on the tip of the antennae are well suited for texture detection as their size matches the size of the components of the micropatterns on flower petals (Kevan and Lane, 1985). Primary afferents from the tactile and mechanosensory neurons located on the antennae end ipsilaterally in the dorso-lateral portion of the dorsal lobe (Ai et al., 2007; Haupt, 2007; Suzuki, 1975) where they overlap with the dendritic fields of the motor neurons controlling antennal movements (Kloppenburg, 1995). The dorsal lobe in its ventro-medial part also receives terminal afferents from taste hairs located on the antennae (Haupt, 2007) and mouthparts (Barbara et al., 2005a) and may serve for integrating mechanosensory and gustatory inputs coming from the head appendices.

In laboratory conditions, harnessed honeybees can associate a tactile antennal stimulus to a sucrose reinforcement delivered to the proboscis (Erber et al., 1998). Different protocols of tactile learning have been developed. In the operant conditioning procedure, the bee is rewarded when its frequency of antennal contact with an object exceeds a certain threshold (Erber et al., 1997; Haupt, 2007; Kisch and Haupt, 2009). Another protocol consists in rewarding bees after scanning the surface of an object with their antennae in order to learn its texture properties (Erber et al., 1998; Dacher et al., 2005); this form of conditioning follows both operant and classical rules. Lastly, a pure classical procedure has been developed in which mechanosensory stimulation of the base of the antennae is followed by a sucrose reward to the proboscis, regardless of antennal movements (Giurfa and Malun, 2004). These procedures allowed showing that bees can discriminate between tactile patterns of different forms and sizes (Erber et al., 1998; Scheiner et al., 2005) and that the information learned on one side remains confined to the ipsilateral brain (Erber et al., 1997; Giurfa and Malun, 2004; Kisch and Haupt, 2009; Scheiner et al., 2001).

The question of differential, side-specific conditioning has already been addressed using the olfactory conditioning of the

---

* Corresponding author. Tel.: +33 0 561 55 64 71; fax: +33 0 561 55 61 54.
E-mail address: gauthiem@cict.fr (M. Gauthier).

proboscis extension reflex (PER). In this paradigm, the proboscis extension induced by touching the antennae with a sucrose solution is conditioned to the forward presentation of an odorant (Bitterman et al., 1983). Sandoz and Menzel (2001) showed that an unilateral learned information could be retrieved from both sides of the brain. These results indicated either that the learned information was transferred between the two brain hemispheres or that unilateral stored information could be retrieved from both sides of the brain.

Inhibitory networks in the brain of honeybees are essential elements for sensory processing (Stopfer et al., 1997; Sachse and Galizia, 2002), as well as for learning and memory (El Hassani et al., 2008). In insects, nervous inhibitory function is mainly supported by gamma aminobutyric acid (GABA) and glutamate, neurotransmitters that target chloride-gated membrane channels. Gabaergic interneurons have been found in all main neuropile areas. The dorsal lobes, like the antennal lobes show homogeneous GABA-immunoreactivity (Schäfer and Bicker, 1986). The antennal lobes also contain glutamate receptors permeant to chlorure ions (GluCl receptors) (Barbara et al., 2005b). GABA-immunoreactive neurons have also been found in the protocerebrum (Grünewald, 1999). These neurons provide a feedback inhibition from the alpha-lobes to the calyces of mushroom bodies (MBs). These latter structures are multisensory neuropiles strongly involved in the formation of memory (Cano Lozano et al., 2001; Erber et al., 1980; Devaud et al., 2007).

GABA and GluCl receptors are the target of several insecticides. Fipronil is a phenylpyrazol insecticide that is used for pest control in crop cultures and which honeybees can encounter during foraging. Patch-clamp recordings of insect neurons revealed that fipronil blocks both GABA and glutamate-gated-chloride channels (Barbara et al., 2005b; Janssen et al., 2007). Moreover, *in vivo* experiments have shown that sublethal doses of fipronil induce deleterious effect on sucrose responsiveness to low concentrated sucrose solutions, on olfactory memory and on odorant-response specificity (Decourtye et al., 2005; El Hassani et al., 2005, 2008; Aliouane et al., 2009). However, the effects of fipronil on tactile learning have never been investigated. The aim of our work is two-fold: we evaluated the capacity of the honeybee to learn different tactile stimuli applied on each antenna with a focus on the side-specificity of the memory trace and we investigated the effect of topical application of sublethal doses of fipronil on antennal tactile learning and memory. We show that tactile stimulus learnt on one side is only retrieved ipsilaterally and that fipronil decreases learning and memory performance without affecting response distribution between brain sides.

2. Materiels and methods

2.1. Animals

Honeybees (*Apis mellifera*) were housed in a heated hut outside the laboratory. They were captured in a small plastic box at the top of the hive and brought to the laboratory for the experiments. There, they were cold-anaesthetised and fixed in small plastic tubes with a drop of wax/resin mixture (10 and 3 g, respectively) deposited on the backside of the thorax and between the thorax and the back of the head. The forelegs were also immobilized with adhesive strips sticked on the plastic tube. These precautions were taken to precisely control the contacts of the honeybee's antennae with the tactile stimuli. Thus fixed, the bees could only move their antennae and mouthparts (including the proboscis). They were fed with two drops of sugar solution (1.17 M) and left for 2 h without food to enhance their motivation for sucrose in a controlled manner. Fifteen minutes before the end of the starvation period, one half of the animals received a topical application of fipronil (see below) and the other half received the solvent. They were subjected to learning experiments 15 min after treatment.

2.2. Fipronil treatment

Contact toxicity of fipronil was evaluated following the European Plant Protection Organisation (EPPO) guidelines (1992). Fipronil (Cluzeau Info Labo, Sainte-Foy La Grande) was dissolved in acetone and subsequently diluted in water so that the final concentration of fipronil was 1 ng/μl. A volume of 0.5 μl of this solution was applied to the ventral part of the thorax, between the forelegs, using a 1 μl Hamilton syringe. Each honeybee received a sublethal dose of 0.5 ng, which roughly corresponds to $LD_{50}/10$ (contact LD_{50}: 5.9 ng/bee; Durham et al., 2001). Control animals received in the same conditions vehicle solution containing acetone at a final concentration of 0.1% (v/v).

2.3. Conditioning procedure

Honeybees were slightly tipped to the back relative to the experimenter to allow easy presentation of tactile and sucrose stimulations and to limit visual stimulation. The experimenter was still able to perfectly observe the behaviour of bees and possible proboscis extensions. Two metal plates (5-mm wide, 10-mm long, 1-mm thick), one smooth and the other one engraved with a rhomb grid (lines ~200-μm wide, 30-μm deep and spatial wavelength 300 μm) were used as conditioned stimuli (CS).

Preliminary experiment: a preliminary experiment was carried out on non-treated honeybees to ensure that the smooth and the grid-engraved plates were equally well-detected and corresponded to equally salient stimuli for the bees when used as CS. Two groups of animals were subjected to a differential conditioning procedure in which one stimulus (A) was presented to both antennae and was reinforced by touching the proboscis with the sugar solution (Unconditioned Stimulus, US-1.17 M) and allowing the bee to suck the sucrose solution for 4 s. The other stimulus (B) was presented to both antennae but was non-reinforced. CS presentation lasted 5 s, and the US was presented after 3 s. The two metal plates were balanced as A and B in two parallel groups. The training phase comprised 6 presentations of stimulus A and 6 presentations of stimulus B in a pseudorandom order (ABBABAABABBA) with 5-min inter-trial intervals (ITI). Bees were then subjected to memory tests after 3 and 24 h, by stimulating the antennae in a random order with A and B for 5 s without reinforcement (5 min ITI). Both metal plates gave similar acquisition, as well as similar memory performance after 3 and 24 h (supplementary materials; see also Fig. 4A and B). They were thus considered as similarly salient.

Principle of side-specific conditioning protocol: one of the tactile stimuli was presented laterally to the bee during 5 s so that only one antenna could scan the plate. Three seconds after the onset of the plate presentation, the proboscis was directly stimulated with the US. Each animal whose antenna did not scan the plate, spontaneously responded to the plate before training or which did not show a PER to sucrose was discarded. The high concentrated sucrose solution used in our experiment (1.17 M) was chosen to exclude a fipronil effect on sucrose responsiveness (El Hassani et al., 2005). Different conditioning procedures were carried out in each experiment (see below for details). After each trial the metal plate was cleaned in pure ethanol and dried with absorbing paper, making sure that no ethanol was left. Two retrieval tests were performed 3 and 24 h after training in order to test mid-term and long-term memory (Menzel, 1999). The tests consisted in presentation in a random order of both stimuli to each antenna. For both conditioning trials and retrieval tests, a delay of 5 min was respected between right and left antennal tactile stimulations; the same antenna was stimulated with a 10 min ITI. The two sides and the two stimuli were balanced between animals so that as many animals received a given stimulation pattern on the left side and on the right side. If no conditioned PER was observed during a

retrieval test, a sucrose stimulation of the proboscis was applied 5 min after the end of the four tests. If no response occurred, the animal was discarded from the analysis (5.7% and 6.4% in control and fipronil-treated animals, respectively). After the 3 h retrieval test, bees were fed *ad libitum* with sucrose (1.17 M) and were left to rest until the 24 h test.

Three different protocols were used, in which two tactile stimuli, A and B, were either rewarded or unrewarded, and presented either on the left or on the right antenna. The first experiment examined if the unilateral tactile conditioning allows transfer between sides. For that, the CS was presented to one antenna and the other antenna was not stimulated (A+/0). The second experiment was designed as side-specific differential conditioning in which a tactile stimulus was presented to one antenna and was reinforced and the other tactile stimulus was presented to the other antenna without reinforcement (A+/B−). This experiment verifies whether bees responded to the reinforced CS due to its association with the US rather than due to simple exposure to the training stimulus independently of reinforcement (in which case, bees would respond to B on the B− side). The third experiment was side-specific conditioning (A+/B+) in which each antenna was stimulated with a different tactile stimulus reinforced with sucrose. The purpose was to test whether honeybees are able to learn one specific tactile stimulus on each side.

2.4. Statistical treatment

A proboscis extension beyond a virtual line linking the opened mandibles was recorded as a PER. The graphs plot the proportion of honeybees releasing a PER during acquisition or test trials. To analyze performances during the conditioning phase, we used analyses of variance (ANOVAs) for repeated measurements. For the first experiment (A+/0), we used *conditioning trials* as the first (repeated) factor and *treatment* (control and fipronil) as the second fixed factor. For the second (A+/B−) and third experiments (A+/B+), *conditioning trials* and *conditioned stimuli* (CS) were used as the two repeated factors and *treatment* (control and fipronil) as the third fixed factor. Monte Carlo studies have shown that it is permissible to use ANOVA on dichotomous data only under controlled conditions (Lunney, 1970; for an example see Vergoz et al., 2007), which are met by our experiments (equal cell frequencies and at least 40 degrees of freedom of the error term). Responses during the test phase were compared using Cochran's Q test. It was completed, when significant, with pairwise comparisons following the Marascuilo and McSweeney method (1967, in Zar (1999)). Comparisons between groups (control group and fipronil group) during the test phase were made using χ^2 tests. For the three experiments, we verified that bees learnt the CS in the same way between their left and right sides (ANOVA for repeated measurements, *side effect*: A+/0, $F_{1,112} = 0.001$, $p = 0.981$; A+/B−, $F_{1,85} = 0.983$, $p = 0.324$; A+/B+, $F_{1,234} = 0.203$, $p = 0.652$).

3. Results

3.1. Mortality

Although fipronil was used at a sublethal dose, we checked for the mortality rate induced by the insecticide at the end of the 2 experimental days. We found 37.0% mortality in control animals ($N = 61$) and 34.6% in fipronil-treated animals ($N = 55$) and the
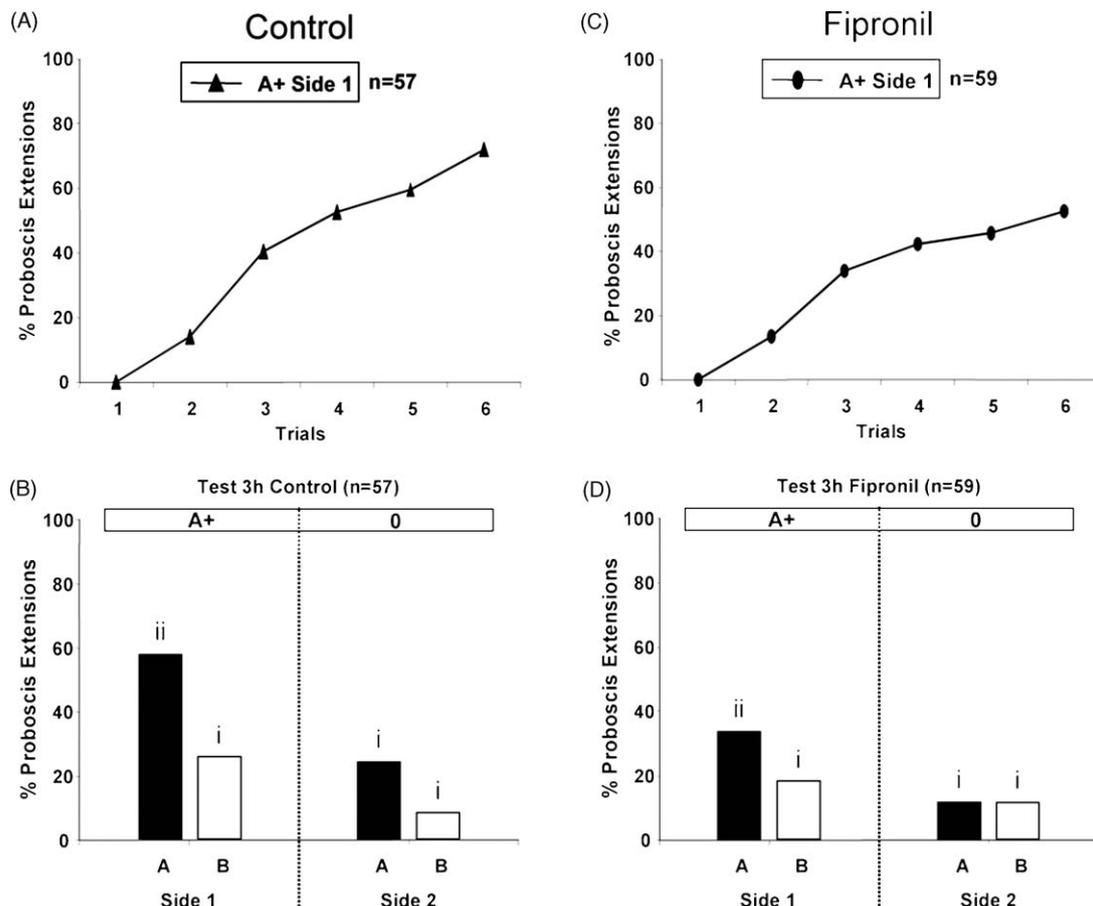
Fig. 1. Acquisition curves for control (A) and fipronil-treated (C) honeybees in a unilateral tactile conditioning procedure (A+/0); (B) and (D) performance in the test phase after 3 h retention for control and fipronil-treated animals respectively. Control honeybees responded more strongly to stimulus A on the trained side than on the untrained side, and less to stimulus B (new stimulus) on both sides. Different letters indicate significantly different levels of responses (pairwise comparisons after Cochran's Q test).

difference was not significant (Chi-square test, $\chi^2 = 0.014$, $p = 0.90$). These mortality rates are relatively high but when compared to the 32% mortality rate of the animals used for the preliminary experiment ($N = 38$), no difference was observed between the three groups (Chi-square test, $\chi^2 = 4.475$, $p = 0.10$). This observation rules out the possibility that the solvent itself may have an effect on mortality that could mask the effect of fipronil.

Experiment 1 (A+/0): in this experiment, we tested whether bees that learn a unilateral tactile stimulus can retrieve this information from the contralateral side. For each animal, the CS was presented to one antenna (and the US to the proboscis) and the other antenna was not stimulated during training. Training comprised 6 reinforced trials with 10 min ITI. The nature of the tactile stimulus and the stimulated antenna (right or left) were balanced within control and fipronil-treated groups. Retrieval tests were performed 3 and 24 h after training for each animal.

Control and fipronil-treated bees learnt the task (ANOVA for repeated measurements, *conditioning trials* effect: $F_{5,570} = 66.6$, $p < 0.001$). Although the performance of the fipronil-treated animals appeared lower compared to controls, the difference between the two groups was not significant over all trials (Fig. 1 A and C: ANOVA for repeated measurements, *treatment effect*: $F_{1,114} = 2.10$, $p = 0.15$). However performance at the last trial was significantly higher in controls than in fipronil-treated animals (72% vs. 52.5%, $\chi^2 = 3.84$, $p = 0.05$). The evolution of responses during trials was not different between groups (ANOVA for repeated measurements, interaction (*conditioning trials × treatment*) effect: $F_{5,570} = 1.68$, $p = 0.14$). Thus, both groups learned

the task, with a slight, but not significant tendency for lower learning in fipronil-treated bees.

In the 3 h test phase (Fig. 1B) bees from the control group responded with the highest rate to the CS (A) on the trained side (58%) and with the lowest rate to the novel stimulus (B) on the untrained side (8.8%). The response rate to B on the trained side was equivalent to the response rate to A on the untrained side (respectively 26% and 24%). Statistical analyses indicate that response to the CS on the trained side was significantly higher than response to the other stimuli on both sides (Cochran's Q test, $Q = 52.1$, $p < 0.001$; all pairwise comparisons involving responses to A side 1: $S > 4.5$, $p < 0.001$). Fipronil-treated animals showed the same general trend, with higher responses to the CS on the trained side than to any other stimulus (Fig. 1D; Cochran's Q test, $Q = 22.2$, $p < 0.001$; all pairwise comparisons involving responses to A side 1: $S > 2.8$, $p < 0.05$). However, lower response rates were observed compared to the control group, especially for the CS on side 1 (Chi-square test, response to A side 1 for control vs. fipronil group, $\chi^2 = 5.79$, $p < 0.05$). This decrease is attributable to the somewhat lower performance acquisition, and possibly to additional impairment of memory consolidation.

After 24 h (supplementary materials, Fig. 5A and B), the results were essentially the same. Control honeybees maintained the same response profile with a slight increase of response to A on the trained side (Cochran's Q test, $Q = 40.0$, $p < 0.001$; all pairwise comparisons involving responses to A side 1: $S > 4.3$, $p < 0.001$). Fipronil-treated animals showed almost the same response to A and B on the trained side (Cochran's Q test, $Q = 15.5$, $p < 0.005$;
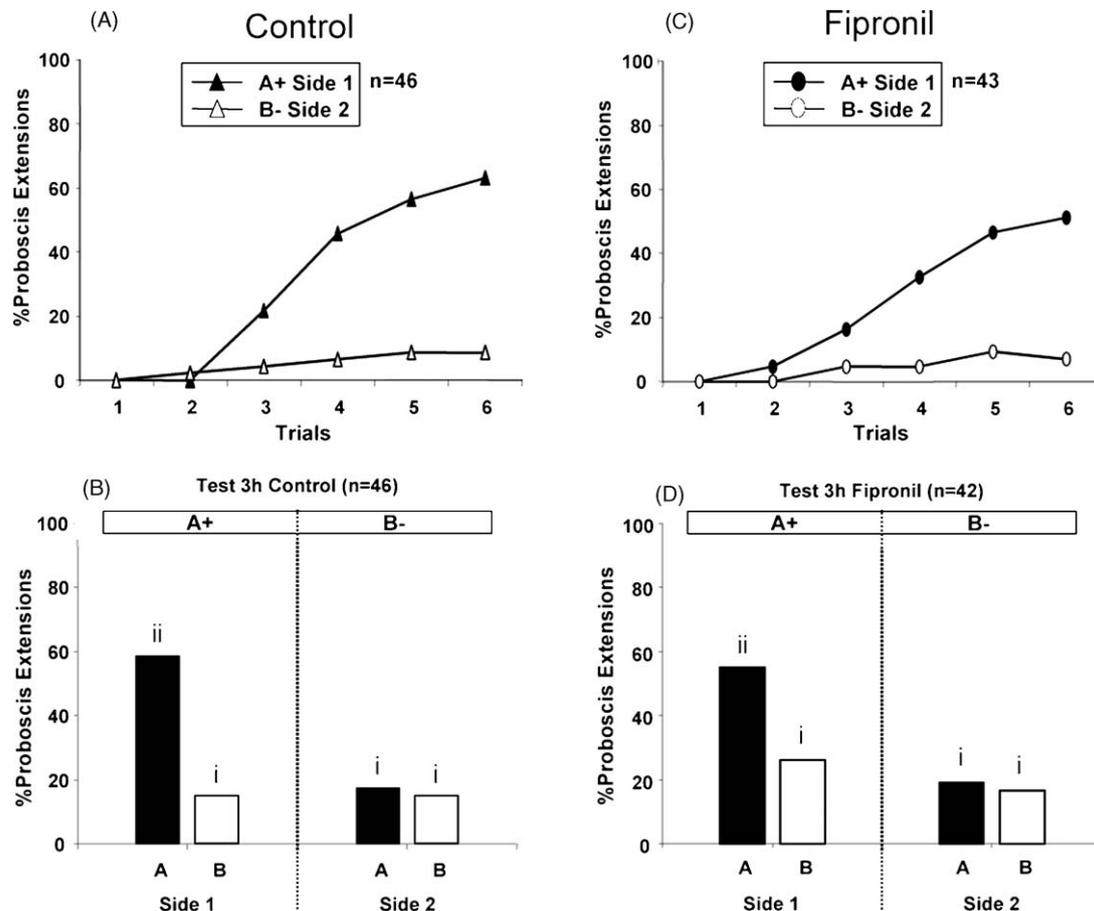
Fig. 2. Acquisition curves for control (A) and firponil-treated (C) bees in a bilateral differential conditioning procedure (A+/B−) and (B) and (D) in a test phase after 3 h retention. In the fipronil group, the response profile was equivalent to the one of control bees. Different letters indicate significantly different levels of responses (pairwise comparisons after Cochran's Q test).

pairwise comparison between responses to A and B on side 1: $S > 1.9$, NS) indicating a memory impairment at this period. As before, lower response rates were observed to the CS on side 1 between fipronil and control groups (Chi-square test, response to A side 1 for control vs. fipronil group, $\chi^2 = 6.08$, $p < 0.05$).

This experiment shows that tactile information (here A+/0) is not transferred between brain sides even after 24 h, and that fipronil induces slightly lower acquisition and significantly lower test performance.

Experiment 2 (A+/B−): in this paradigm, we tested if bees are able to learn that an explicitly non-reinforced stimulus is presented contralaterally to the reinforced stimulus and if this had an effect on lateralized learning performance. Honeybees had to discriminate between a reinforced tactile stimulus and a non-reinforced tactile stimulus, each on one side of the animal. Tactile stimulus A was presented to one antenna and was reinforced with sucrose (A+) while tactile stimulus B was presented to the other antenna without reinforcement (B−). Six A+ trials were interspaced with six B− trials with a 5 min ITI between right and left stimulation. The nature of the tactile stimulus and the stimulated antenna (right or left) were balanced within groups. Stimuli A and B were presented to each antenna during the 3 and 24 h tests in a random order and with a 5 min ITI.

Acquisition curves (Fig. 2A and C) show that both control and fipronil-treated bees learnt the task, responding to the CS+ and not to the CS− in the course of training [ANOVA for repeated measurements, interaction effect (*conditioned stimuli × conditioning trials*): $F_{5,435} = 34.9$, $p < 0.001$]. Although the slope of the learning curve to the CS+ was slightly lower for fipronil compared to control animals,

the two groups did not differ significantly in learning performances over all trials (ANOVA for repeated measurements, *treatment effect*: $F_{1,87} = 1.17$, $p = 0.28$) or at the end of training (A+ performances 51% vs. 65%, $\chi^2 = 1.27$, $p = 0.26$).

Responses in the 3 h test phase in control animals showed a clear heterogeneity (Fig. 2B) (Cochran's Q test, $Q = 52.6$, $p < 0.001$) with a high response rate to A on side 1 (59%) and a low response rate to A on the opposite side (17%; pairwise comparison involving responses between A side 1 and A side 2: $S > 5.5$, $p < 0.001$). This latter value was not different from responses to B on both sides (15%, pairwise comparison involving responses between A side 2 and B sides 1 and 2: $S < 0.3$, NS). These results indicate that each side behaved independently without any detectable transfer of tactile information. Very low response generalization was observed to B on side 1 (pairwise comparison involving responses between A and B side 1: $S > 5.8$, $p < 0.001$). In fipronil-treated animals the response profile was similar to that in the control group (Fig. 2D) (Cochran's Q test, $Q = 38.3$, $p < 0.001$; all pairwise comparisons involving responses to A side 1: $S > 4.1$, $p < 0.001$). Response rates for control and fipronil groups were almost the same (Chi-square test, response to A side 1 for control vs. fipronil group, $\chi^2 = 0.025$, NS).

Over all, no major modification was found at 24 h, this very clear heterogeneity of the response profile being conserved in control (Cochran's Q test, $Q = 27.72$, $p < 0.001$) and fipronil-treated animals (Cochran's Q test, $Q = 18.3$, $p < 0.001$) (supplementary material, Fig. 5C and D). The profile of responses for both groups was conserved (Chi-square test, response to A side 1 for control vs. fipronil group, $\chi^2 = 0.134$, NS).
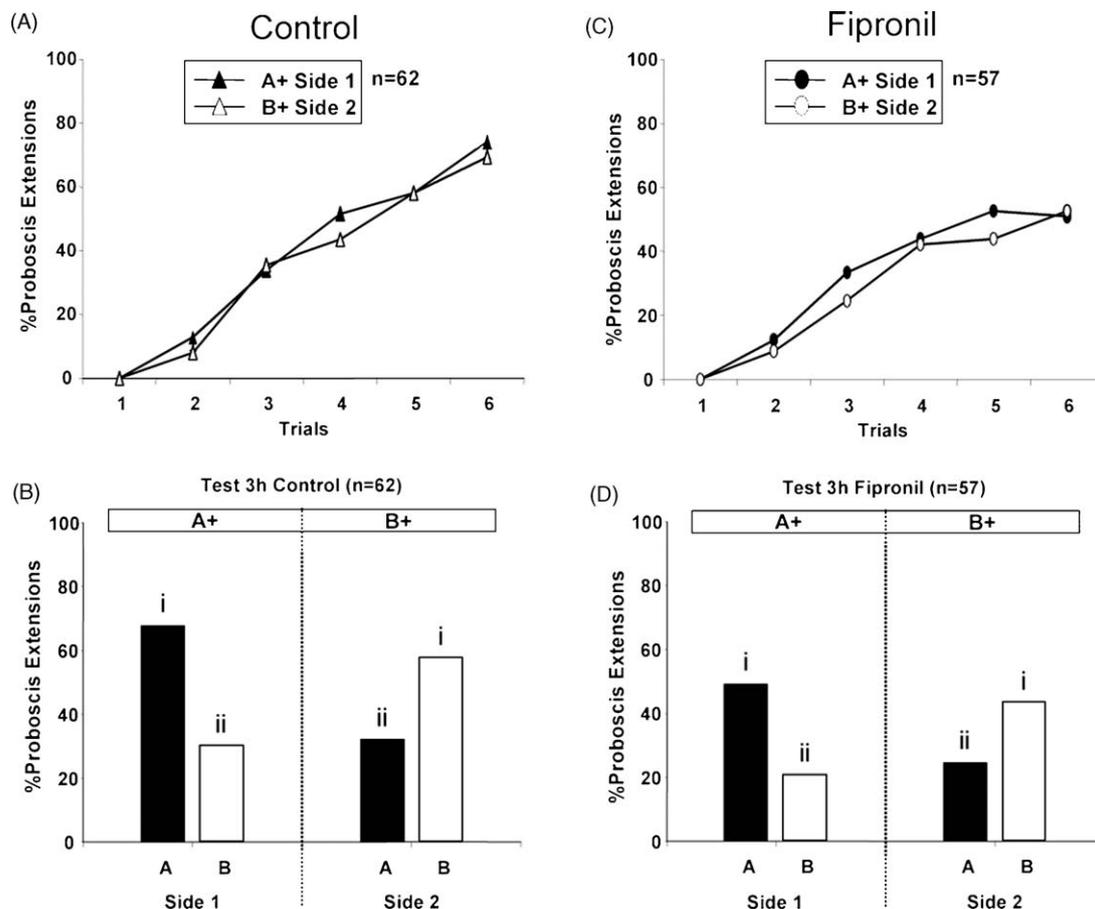
Fig. 3. Acquisition curves for bees conditioned to two different tactile stimuli on the two sides (A+/B+ training) for control (A) and fipronil-treated (C) animals; (B) and (D): performance in the test phase after 3 h retention. Bees show a specific response pattern, responding with a high rate to both CS+ on both trained sides and at a lower rate to the other stimulus. Different letters indicate significantly different levels of responses (pairwise comparisons after Cochran's Q test).

This experiment confirms that tactile information is not transferred between sides, and suggests that fipronil induces only slightly lower acquisition performance.

Experiment 3 (A+/B+): since tactile information gained on one antenna does not seem to be transferred to the other hemisphere, bees should be able to learn specifically a different plate on each antenna. This experiment was designed to test this possibility. Hence, each side received either A or B reinforced with sucrose.

Acquisition curves (Fig. 3A and C) show that control and fipronil-treated bees learnt the task (ANOVA for repeated measurements, *conditioning trials* effect: $F_{5,585} = 99.2$, $p < 0.001$). Control and fipronil animals learnt to respond to both CS+ (Fig. 3A and C) on each side without any difference between sides/stimulus (ANOVA for repeated measurements, *conditioned stimuli* effect: $F_{1,117} = 2.33$, $p = 0.13$). Moreover, the performance of control and fipronil groups did not differ over all trials (ANOVA for repeated measurements, *treatment effect*: $F_{1,117} = 1.77$, $p = 0.19$) but there was a significant interaction between treatment and conditioning trials (ANOVA for repeated measurements, interaction (*treatment × conditioning trials*) effect: $F_{5,585} = 2.35$, $p < 0.05$). Thus learning efficacy was different in the two groups. Indeed, final performance was significantly higher for controls with respect to fipronil-treated bees for the A+ stimulus (74.2% vs. 50.9%, $\chi^2 = 5.96$, $p = 0.015$) and near-significantly so for the B+ stimulus (69.3% vs. 52.6%, $\chi^2 = 2.83$, $p = 0.092$) (Fig. 3A and C).

In control animals, the 3 h retrieval test showed a rather specific and symmetrical distribution of responses between sides, with a high response rate to the CS+ on each side and a significantly lower response to the other stimulus (Fig. 3B) (Cochran's Q test, $Q = 40.2$, $p < 0.001$; pairwise comparisons involving responses between A and B side 1: $S > 5.1$, $p < 0.001$ and side 2: $S > 3.5$, $p < 0.005$). Each side thus responded preferentially to the stimulus reinforced on this side. In fipronil-treated animals, the profile of responses was the same as in controls (Fig. 3D) (Cochran's Q test, $Q = 25.45$, $p < 0.001$; pairwise comparisons involving responses between A and B side 1: $S > 4.1$, $p < 0.001$ and side 2: $S > 2.8$, $p < 0.05$). However, general response levels in the fipronil group appeared lower, but this effect was only near-significant for A on side 1 (49% vs. 68%, $\chi^2 = 3.51$, $p = 0.06$) and not significant for B on side 2 (44% vs. 58%, $\chi^2 = 1.86$, $p = 0.17$).

At 24 h the test showed a good retention of the discriminative learning for both control (Cochran's Q test, $Q = 20.2$, $p < 0.001$) and treated animals (Cochran's Q test, $Q = 30.92$, $p < 0.001$) (supplementary material, Fig. 5E and F). In fipronil animals the responses to A and B on each side were more contrasted (pairwise comparisons involving responses between A and B side 1: $S > 3.3$, $p < 0.05$ and between A and B side 2: $S > 4.4$, $p < 0.001$).

This experiment shows that bees can learn two different tactile stimuli, one on each antenna, and that fipronil only tends to decrease acquisition performance.

Testing an adverse effect of the solvent: to test an effect of the solvent (acetone + water) on learning and memory that could mask the fipronil effect we compared the performances of non-treated animals used for the preliminary experiment (Fig. 4) to those of control animals receiving the solvent in the closest learning situation A+/B− (see Fig. 2). We found no difference between the groups for learning performances [ANOVA for repeated measurements, *treatment effect* (non-treated animals vs. control animals receiving the solvent): $F_{1,82} = 0.135$, $p = 0.71$] and for memory performances at 3 h [A+ and B+ responses (non-treated animals) vs. A side 1 responses (control animals) $\chi^2 = 0.02$, $p = 0.88$] and at 24 h [A+ and B+ responses (non-treated animals) vs. A side 1 responses (control animals) $\chi^2 = 0.25$, $p = 0.61$]. This comparison established on the worse case (compare Figs. 1–3 to Fig. 4) allows us to ensure that diluted acetone topically applied to honeybees did not modify learning abilities and responses to memory tests.

Global analysis of the effect of fipronil on learning rates: in the three different protocols studied above, fipronil-treated animals showed a general tendency for lower acquisition curves compared to control honeybees. Although the samples' size was large enough ($43 \leq N \leq 62$) we could not detect a significant difference between control and fipronil groups when analyzing each experiment alone and this pattern was consistent for all of the experiments (A+/0, A+/B− and A+/B+). To test the tendency of fipronil to systematically decrease the response to CS+ we performed a global analysis by pooling honeybees' responses to all CS+ (A) for the three experiments. Data were analysed using an ANOVA for repeated measurements with *conditioning trials* as the repeated factor and *treatment* (control and fipronil) and *experiment* (1, 2 and 3) as fixed factors. Both groups (control and fipronil) learnt the task (ANOVA for repeated measurements, *conditioning trials* effect: $F_{5,1590} = 186.6$, $p < 0.001$) but, in this case, the performance of the fipronil-treated animals was significantly lower compared to control animals (ANOVA for repeated measurements, *treatment* effect: $F_{1,318} = 4.65$ $p < 0.05$). No effect of the experiment was found (ANOVA for repeated measurements, *experiment* effect: $F_{2,318} = 1.76$, $p = 0.17$). Thus, when all the results of the three experiments are used, the global analysis shows that fipronil-treated animals have significantly lower acquisition performance than control animals.

As for acquisition performance, a global analysis was realized on the 3 and 24 h retrieval tests. This analysis was performed by pooling honeybees' responses to the CS+ (A) at each test. Fipronil-treated bees showed significantly lower responses than controls both at the 3 h test ($\chi^2 = 8.58$, $p = 0.003$) and at the 24 h test ($\chi^2 = 7.49$, $p = 0.006$).

Although the effects of fipronil are weak and non-significant in the experimental conditions recommended by EPPO, the global analysis shows the coherence of the fipronil effect through the three learning situations and indicates that the weak detrimental effect is not linked to chance.

4. Discussion

This work shows that bees are able to learn side-specific tactile tasks and that tactile information is not transferred between sides. Topical application of a sublethal dose of fipronil induced in all three experiments a slight decrease of acquisition success, and in some cases lower test performance. This trend was confirmed when performing a global analysis of the data.

4.1. Side-specific tactile learning

Control honeybees are able to learn a specific tactile stimulus on one side (A+/0), even while another stimulus is presented either unreinforced (A+/B−) or reinforced (A+/B+) on the opposite side. The A+/0 situation, with the reinforced stimulus balanced on each antenna, indicates that bees are able to learn a tactile stimulus with one antenna only, and that both brain sides learn equally well. Moreover, in all our experiments, we found no indication that bees may share or retrieve tactile information between sides. This observation fits with the results of Erber et al. (1997), Scheiner et al. (2001) and Giurfa and Malun (2004). In the same way, side-specific operant conditioning of antennal movements is retrievable from the conditioned side only (Haupt, 2007; Kisch and Haupt, 2009). The transfer of information tested as the retrieval performance to A on the contralateral side was not null in our case but very low, as seen by the significant difference between the level responses to A on each side, whatever the test delay (3 or 24 h, see supplementary results). This was verified in all conditioning situations. These results suggest a clearly different situation for tactile information and for olfactory information. Indeed, olfactory

experiments showed that a unilaterally learned olfactory association is retrievable from the other brain side after a retention period (Sandoz and Menzel, 2001). These observations were interpreted as indicating either a distributed memory trace on both sides of the brain or a memory trace limited to one side of the brain and accessible contralaterally during retrieval. The difference existing between side-specific odorant and tactile learning is not in the capability of bees to acquire information from one side of the brain but in the way of storing unilaterally or bilaterally the learned information or to have access to it. Our experimental paradigms were rather similar to those used in the olfactory experiments but important differences still reside both concerning the US and the CS used. The two sensory modalities employed as CS rely on different neuronal pathways that may show differences in their interhemispheric connections. Concerning the US, a compound antennal and proboscis sucrose stimulation was used in the olfactory conditioning and in tactile conditioning (Erber et al., 1998; Kisch and Haupt, 2009; Scheiner et al., 2001), while in our case, the US was applied to the proboscis only. However, an extensive study of the impact of the US input site showed that bees can also learn a unilateral odor CS with a proboscis US (Sandoz et al., 2002), a result equivalent to the one we observed in our experiments.

4.2. US pathway

The US sucrose delivered to the proboscis stimulates gustatory receptors present on the labium. Through labial nerves, these receptors project ipsilaterally to the dorsal lobes (Barbara et al., 2005a) and ipsi- and contralaterally to the subesophageal ganglia (SEG) (Rheder, 1989). Neurons that connect the SEG to the calyces of the mushroom bodies have been described. The Ventral Unpaired Median neuron 1 of the maxillary neuromere (VUM-mx1) in the SEG responds to gustatory stimuli and innervates the basal ring and the lip of the calyces (Hammer, 1993). This neuron probably takes its information from the gustatory primary afferents through direct connections or via interneurons. More recently, a subesophageal-calycal tract (SCT) that connects the SEG to the MB has been described (Schröter and Menzel, 2003). The neurons at the origin of the tract have their somata located in the dorsal lobe. In the SEG their dendritic fibres overlap with terminals of the sensory neurons from the proboscis and the axonal projections of the SCT neurons end in a small portion of the collar and in the lip regions of the calyces. These anatomical data indicate that the proboscis US pathway is largely bilateral. When the sucrose is delivered to the proboscis, the reinforcement reaches the right as well as the left part of the brain, in the SEG, the dorsal lobes and at the level of the calyces of the MB. Questions arise then to know where in the brain does the association between the CS and the US take place and if sites for associative processes overlap with sites for information storage?

4.3. CS pathway

The tactile and mechanosensory neurons present on each antenna send axons forming the ventral part of the antennal nerve to the ipsilateral dorsal lobe (Haupt, 2007). Anatomical and functional description of the deutocerebrum (Rospars, 1988; Homberg et al., 1989) did not indicate the presence of commissural fibres between the right and the left dorsal lobes. Contrary to olfactory information that is conveyed from the antennal lobes to the MB through the important lateral and median antenno-cerebral tracts (Abel et al., 2001), only some fibres from the dorsal lobe are gathered into the median antenno-cerebral tract and reach the basal ring of median and lateral calyces of the ipsilateral MB (Mobbs, 1982). The presence of several commissures between the

MB lobes might assure some bilateral transfer of output information (Mobbs, 1982; Rybak and Menzel, 1993).

4.4. Hypothesis for unilateral representation of acquired and stored information

The anatomical data on the CS and US pathways indicate a first convergence of both information types in the dorsal lobe and another convergence site at the level of the basal ring of the MB calyces. These regions could be potential sites for associative processes between the CS and the US during conditioning and thus acquisition processes could well be restricted to the stimulated brain hemisphere. Several insect data demonstrate the role of the MB lobes in retrieval processes, suggesting a role of α and β lobes in the retrieval of information (Cano Lozano et al., 2001; Dubnau et al., 2001; McGuire et al., 2001). As several commissures link symmetrical lobe regions of the mushroom bodies, it is conceivable that stimulating the contralateral non-learning brain could elicit the conditioned response during the test. This was observed for the olfactory learning with a transfer of learned information between sides, but this does not hold true for tactile learning. We advance the hypothesis that the difference between olfactory and tactile retrieval processes is linked to the weak projections of tactile stimuli to the MBs, making the MB network weakly involved in tactile acquisition and retrieval. As a consequence, we propose that the dorsal lobe, in addition to being a potential associative site between the CS and the US, would also be a storage site for learned tactile information. This would explain the mainly unilateral retrieval from the conditioned side.

4.5. Effects of fipronil

The effects of fipronil are rather limited on side-specific tactile learning. A non-significant decrease of acquisition performance was observed in each learning situation compared to control animals. Pooling the results of the three experiments shows a low detrimental effect of fipronil on acquisition performance. As a consequence of lower acquisition, retrieval performance was lower in fipronil-treated animals compared to controls for both tests (3 and 24 h). Interestingly, the same fipronil treatment (topical application of 0.5 ng/bee) decreased learning and memory performance in an olfactory conditioning task (El Hassani et al., 2005), indicating that the detrimental effect was not linked to the tactile learning procedure. More interestingly, fipronil treatment did not abolish the response pattern profile during the retrieval tests and no generalization of the response to a non-learned stimulus was observed on the conditioned side. Fipronil acts as an antagonist of GABA and GluCl receptors (Barbara et al., 2005a,b), thus blocking inhibitory networks in the honeybee brain. The role of these networks in memory processes has been recently underlined (El Hassani et al., 2008) and seems particularly important in olfactory coding (Sachse and Galizia, 2002) and in olfactory discrimination (Stopfer et al., 1997; Hosler et al., 2000). For example, chronic administration of fipronil was responsible for unspecific olfactory responses in honeybees subjected to an olfactory learning procedure (Aliouane et al., 2009). As stated above, such an effect of fipronil on tactile discrimination was clearly not observed in our experiment. Together with the work of El Hassani et al. (2005) these results indicate that processes of tactile information are less susceptible to fipronil than olfactory processes, a result that can be related to the rather large distribution of GABA-immunoreactivity in the antennal lobes compared to the dorsal lobes (Schäfer and Bicker, 1986).

Although we detect only a small noxious effect in the performance of fipronil-treated animals, it is difficult to predict the impact of this effect at the colony level, as subtle inter-

individual differences in the probability of performing any behaviour may have important consequences at the colony level due to amplification processes (Camazine et al., 2001). This hypothesis should be tested in future work as it is known that social behaviors as labor division and foraging activity in honeybees are dependent upon internal factors at the individual level (Ben-Shahar et al., 2003; Schulz and Robinson, 2001).

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jinsphys.2009.08.019.

References

Abel, R., Rybak, J., Menzel, R., 2001. Structure and response patterns of olfactory interneurons in the honeybee, Apis mellifera. Journal of Comparative Neurology 437, 363–383.

Ai, H., Nishino, H., Itoh, T., 2007. Topographic organization of sensory afferents of Johnston's organ in the honeybee brain. Journal of Comparative Neurology 502, 1030–1046.

Aliouane, Y., El Hassani, A.K., Gary, V., Armengaud, C., Lambin, M., Gauthier, M., 2009. Subchronic exposure of honeybees to sublethal doses of pesticides: effect on behavior. Environmental Toxicology and Chemistry 54, 653–661.

Barbara, G.S., de Brito Sanchez, G., Haupt, S., Giurfa, M., Gauthier, M., 2005a. Central projections and electrophysiological characterization of taste hairs in the honeybee, Apis mellifera. In: Proceedings of the 3rd European Congress on Social Insects (IUSSI), St. Petersburg, p. 132.

Barbara, G.S., Zube, C., Rybak, J., Gauthier, M., Grünewald, B., 2005b. Acetylcholine, GABA and glutamate induce ionic currents in cultured antennal lobe neurons of the honeybee, Apis mellifera. Journal of Comparative Physiology A 191, 823–836.

Ben-Shahar, Y., Leung, H.T., Pak, W.L., Sokolowski, M.B., Robinson, G.E., 2003. cGMP-dependent changes in phototaxis: a possible role for the foraging gene in honeybee division of labor. Journal of Experimental Biology 206, 2507–2715.

Bitterman, M.E., Menzel, R., Fietz, A., Schafer, S., 1983. Classical conditioning of proboscis extension in honeybees (Apis mellifera). Journal of Comparative Psychology 97, 107–119.

Camazine, S., Deneubourg, J.-L., Franks, N.R., Sneyd, J., Theraulaz, G., Bonabeau, E., 2001. Self-organization in Biological Systems. Princeton University Press, Princeton, NJ.

Cano Lozano, V., Armengaud, C., Gauthier, M., 2001. Memory impairment induced by cholinergic antagonists injected into the mushroom bodies of the honeybee. Journal of Comparative Physiology A 187, 249–254.

Dacher, M., Lagarrigue, A., Gauthier, M., 2005. Antennal tactile learning in the honeybee: effect of nicotinic antagonists on the memory dynamics. Neuroscience 130, 37–50.

Decourtye, A., Devillers, J., Genecque, E., Le Menach, K., Budzinski, H., Cluzeau, S., Pham-Delègue, M.H., 2005. Comparative sublethal toxicity of nine pesticides on olfactory learning performances of the honeybee Apis mellifera. Archives of Environmental Contamination and Toxicology 48, 242–250.

Devaud, J.M., Blunk, A., Podufall, J., Giurfa, M., Grünewald, B., 2007. Using local anaesthetics to block neuronal activity and map specific learning tasks to the mushroom bodies of an insect brain. European Journal of Neuroscience 26, 3193–3206.

Dubnau, J., Grady, L., Kitamoto, T., Tully, T., 2001. Disruption of neurotransmission in Drosophila mushroom body blocks retrieval but not acquisition of memory. Nature 411, 476–480.

Durham, E.W., Scharf, M.E., Siegfried, B.D., 2001. Toxicity and neurophysiological effects of fipronil and its oxydative sulfone metabolite on European corn borer larvae. Pesticides Biochemistry and Physiology 71, 97–106.

El Hassani, A.K., Dacher, M., Gauthier, M., Armengaud, C., 2005. Effects of sublethal doses of fipronil on the behavior of the honeybee (Apis mellifera). Pharmacology Biochemistry and Behaviour 82, 30–39.

El Hassani, A.K., Giurfa, M., Gauthier, M., Armengaud, C., 2008. Inhibitory neurotransmission and olfactory memory in honeybees. Neurobiology of Learning and Memory 90, 589–595.

Erber, J., Mashur, T.H., Menzel, R., 1980. Localization of short-term memory in the brain of the bee, Apis mellifera. Physiological Entomology 5, 343–358.

Erber, J., Pribbenow, B., Grandy, K., Kierzek, S., 1997. Tactile motor learning in the antennal system of the honeybee (Apis mellifera L.). Journal of Comparative Physiology A 181, 355–365.

Erber, J., Kierzek, S., Sander, E., Grandy, K., 1998. Tactile learning in the honeybee. Journal of Comparative Physiology A 183, 737–744.

European Mediterranean Plant Protection Organisation (EPPO), 1992. Guideline on test method for evaluating the side effects of plant protection products on honeybees. EPPO Bulletin 22, 203–215.

Giurfa, M., Malun, D., 2004. Associative mechanosensory conditioning of the proboscis extension reflex in the honeybee. Learning & Memory 11, 294–302.

Grünewald, B., 1999. Morphology of feedback neurons in the mushroom body of the honeybee, Apis mellifera. Journal of Comparative Neurology 404, 114–126.

Hammer, M., 1993. An identified neuron mediates the unconditioned stimulus in associative olfactory learning in honeybees. Nature 366, 59–63.

Haupt, S.S., 2007. Central gustatory projections and side-specificity of operant antennal muscle conditioning in the honeybee (Apis mellifera L.). Journal of Comparative Physiology A 193, 523–535.

Homberg, U., Chritensen, T.A., Hildebrandt, J.G., 1989. Structure and function of the deutocerebrum in insects. Annual Review of Entomology 34, 477–501.

Hosler, J.S., Buxton, K.L., Smith, B.H., 2000. Impairment of olfactory discrimination by blockade of GABA and nitric oxide activity in the honeybee antennal lobes. Behavioral Neuroscience 114, 514–525.

Janssen, D., Derst, C., Buckinx, R., Van den Eynden, J., Rigo, J.M., Van Kerkhove, E., 2007. Dorsal unpaired median neurons of Locusta migratoria express invermectin- and fipronil-sensitive glutamate-gated chloride channels. Journal of Neurophysiology 197, 2642–2650.

Kevan, P.G., 1987. Texture sensitivity in the life of the honeybees. In: Menzel, R., Mercer, A. (Eds.), Neurobiology and Behavior of Honeybees. Springer-Verlag, Berlin, pp. 96–101.

Kevan, P.G., Lane, M.L., 1985. Flower petal microtexture is a tactile cue for bees. Proceedings of the National Academic Science USA 82, 4750–4752.

Kisch, J., Haupt, S.S., 2009. Side-specific operant conditioning for antennal movements in the honeybee. Behavioural Brain Research 196, 131–133.

Kloppenburg, P., 1995. Anatomy of the antennal motoneurons in the brain of the honeybee (Apis mellifera). Journal of Comparative Neurology 363, 333–343.

Lunney, G.H., 1970. Using analysis of variance with a dichotomous dependent variable: an empirical study. Journal of Educational Measurement 7, 263–269.

McGuire, S., Le, P.T., Davis, R.L., 2001. The role of Drosophila mushroom body signaling in olfactory memory. Science 293, 1330–1333.

Menzel, R., 1999. Memory dynamics in the honeybee. Journal Comparative Physiology A 85, 323–340.

Mobbs, P.G., 1982. The brain of the honeybee Apis mellifera. I. The connections and spatial organization of the mushroom bodies. Philosophical Transactions of the Royal Society London B 298, 309–354.

Rheder, V., 1989. Sensory pathways and motoneurons of the proboscis reflex in the suboesophageal ganglion of the honeybee. Journal of Comparative Neurology 279, 499–513.

Rospars, J.P., 1988. Structure and development of the insect antennodeutocerebral system. International Journal of Insect Morphology and Embryology 17, 243–294.

Rybak, J., Menzel, R., 1993. Anatomy of the mushroom bodies in the honeybee brain: the neuronal connections of the alpha-lobe. Journal of Comparative Neurology 334, 444–465.

Sachse, S., Galizia, C.G., 2002. Role of inhibition for temporal and spatial odor representation in olfactory output neurons: a calcium imaging study. Journal of Neurophysiology 87, 1106–1117.

Sandoz, J.C., Menzel, R., 2001. Side-specificity of olfactory learning in the honeybee: generalization between odors and sides. Learning & Memory 8, 286–294.

Sandoz, J.C., Hammer, M., Menzel, R., 2002. Side-specificity of olfactory learning in the honeybee: US input side. Learning & Memory 9, 337–348.

Schäfer, S., Bicker, G., 1986. Distribution of GABA-like immunoreactivity in the brain of the honeybee. Journal of Comparative Neurology 246, 287–300.

Scheiner, R., Schnitt, S., Erber, J., 2005. The functions of antennal mechanoreceptors and antennal joints in tactile discrimination of the honeybee (Apis mellifera L.). Journal of Comparative Physiology A 191, 857–864.

Scheiner, R., Weiss, A., Malun, D., Erber, J., 2001. Learning in honeybees with brain lesions: how partial mushroom-body ablations affect sucrose responsiveness and tactile antennal learning. Animal Cognition 4, 227–235.

Schröter, U., Menzel, R., 2003. A new ascending sensory tract to the calyces of the honeybee mushroom body, the suboesophageal-calycal tract. Journal of Comparative Neurology 465, 168–178.

Schulz, D.J., Robinson, G.E., 2001. Octopamine influences division of labor in honeybee colonies. Journal of Comparative Physiology A 187, 53–61.

Stopfer, M., Bhagavan, S., Smith, B., Laurent, G., 1997. Impaired odour discrimination on desynchronisation of odour-encoding neural assemblies. Nature 390, 70–74.

Suzuki, H., 1975. Convergence of olfactory inputs from both antennae in the brain of the honeybee. Journal of Experimental Biology 62, 11–26.

Vergoz, V., Roussel, E., Sandoz, J.C., Giurfa, M., 2007. Aversive learning in honeybees revealed by the olfactory conditioning of testing extension reflex. Plos One 2 (3), e288 doi:10.1371/journal.pone0000288.

Zar, J.H., 1999. Biostatistical Analysis. Prentice-Hall International, London.