# Chapter III

## Partial unilateral lesions of the mushroom bodies affect olfactory learning in honeybees *Apis mellifera* L.

**Abstract**

The mushroom bodies (MBs) are central structures in the insect brain that have been associated with olfactory learning and memory. Here we used Hydroxyurea to treat honeybee larvae and induce partial MB ablations at the adult stage. We studied olfactory learning in honeybees with unilateral loss of the median calyces of their MBs and compared their ability to solve different forms of olfactory discrimination. When odorants were delivered in a side-specific manner, ablated bees could not solve either discrimination of the unambiguous problem (Paradigm 1: A+, B- / C+, D-) while they could solve at least one of both discriminations of the ambiguous problem (Paradigm 2: A+, B- / A-, B+), namely that proposed to their intact brain side. Non-ablated bees could learn both side-specific discriminations. MB lesions had no effect on a discrimination in which odorants were delivered simultaneously to both antennae (Paradigm 3: A+, B-, C+, D-). We suggest that MB lesions impair transfer of stored information between the two sides of the brain and that such transfer may or may not occur in normal circumstances, depending on the information stored in each brain side.

## Introduction

The mushroom bodies (MB) are prominent structures in the insect brain (Strausfeld et al. 1998) that have been associated with tasks requiring a certain level of behavioral plasticity (Menzel 1999, 2001; Heisenberg 2003; Giurfa 2003). Studies on insect olfactory processing and learning have played a key role for our current thinking about mushroom body function (Menzel 1999, 2001; Heisenberg 2003).

In honeybees (*Apis mellifera*), the MBs are believed to be centers of multimodal integration as they receive input from olfactory, visual, gustatory and probably mechanosensory pathways (Mobbs 1982, Strausfeld et al. 1998, Gronenberg et al. 1996, Schröter & Menzel 2003). They have been traditionally associated with olfactory learning and memory following studies principally using the olfactory conditioning of the proboscis extension reflex (PER - Erber et al. 1980; Menzel 1999, 2001; Hammer & Menzel 1998; Menzel & Giurfa 2001; Giurfa 2003). In this classical conditioning paradigm (Takeda 1961; Bitterman et al. 1983), restrained bees learn to associate an odorant (conditioned stimulus or CS) with a reward of sucrose solution delivered to the antennae and the proboscis (unconditioned stimulus or US) such that, after successful conditioning, they extend their proboscis to the mere presentation of the odorant. In this way, a link between CS and US is established. Bees also learn to respond to a rewarded odor (CS+) but not to a non-rewarded odor (CS-) in a differential conditioning, which is based on associations that link one CS with a US and the other CS with the absence of US.

Bees can also learn non-linear, olfactory discriminations (Deisig et al. 2001, 2002, 2003; Hellstern et al. 1995; Chandra & Smith 1998) in which each odorant appears rewarded as often as non-rewarded, thus creating ambiguity (e.g. *negative patterning*: A+, B+, AB-, where A and B stand for different odorants). Such forms of learning are termed 'non-elemental' in contrast to linear or 'elemental' forms in which the underlying associations are non ambiguous. It has been suggested that the MBs could be of fundamental importance for solving the more complex, non-linear learning problems (Giurfa 2003; Komischke et al. 2003). In fact, partial, unilateral lesions of the MBs have been found to be without effect on linear forms of learning (Malun et al. 2002b) as bees with such lesions can learn a simple differential conditioning (CS+ vs. CS-) both in the olfactory (Malun et al. 2002b) and in the tactile modality (Scheiner et al 2001). In olfactory learning, data already indicate that elemental associations can be established between odorants and reward of sucrose at the level of the antennal lobe (Hammer & Menzel 1998; Faber et al. 1999; Farooqui et al. 2003). The

explicit role of the mushroom bodies for non-linear forms of olfactory learning has not yet been tested directly.

Here, we asked whether partial, unilateral lesions of the mushroom bodies affect different forms olfactory learning in honeybees. We used a treatment in which first-instar larvae were fed with a solution containing hydroxyurea (HU) (Malun 1998, Malun et al. 2002a, b). HU kills dividing neuroblasts that give origin to the MBs, thus leading to a partial, but not complete, loss of the MBs in adult bees (Malun 1998). We trained HU-ablated and non-ablated bees with different forms of olfactory discriminations, ambiguous or non ambiguous. Odorants were delivered simultaneously to both antennae or in a side-specific manner by separating the antennal input with plastic walls between the antennae (Sandoz and Menzel 2001). Three discrimination problems were given to the bees, one using bilateral odor stimulation of the antennae, and two using side-specific stimulations. In the former case, we conditioned bees with a linear, unambiguous discrimination (A+, B-, C+, D-). In the latter case, we conditioned bees either with a side-specific, elemental and unambiguous double discrimination (A+, B- / C+, D-, where / stands for the separation between both antennae) or with a side-specific, non-elemental and ambiguous double discrimination (A+, B- / A-, B+) (Sandoz and Menzel 2001).

If the MBs do indeed play an important role in non-elemental forms of learning, we expect ablated bees to show deficiencies in the non-linear discrimination (side-specific conditioning A+ B- / A- B+) but not in the linear ones (side-specific A+B- / C+D- and bilateral A+B-C+D-). With respect to the use of olfactory information coming from one or both antennae, we expect that non-linear problems involving side-specificity (side-specific conditionings A+ B- / C+D- and A+B- / A-B+) will be solved less efficiently than those involving both antennae (bilateral conditioning A+, B-, C+, D-), as we found that honeybees were not able to learn another non-elemental olfactory discrimination, the negative patterning (see above) using only one antenna (Komischke et al. 2003).

## Material and Methods

### Hydroxyurea (HU) treatment

The procedure for generating MB-ablated bees after HU application at the larval stage follows that of Malun (1998) and Malun et al. (2002a, 2002b). First-instar larvae were taken out of their combs and placed on a food solution containing 0.5 µl of hydroxyurea per 100 ml

royal jelly. After 4 to 5 hours, the larvae were rinsed in water and placed back into their combs. The combs were then placed back in the hive to ensure the further development of the larvae. One day before the adult animals hatched, the combs were again removed from the hive and placed in an incubator. On the next day, freshly-emerged bees were put into small wire cages (20 cm x 5 cm x 1 cm), which were then brought back to the hive. These cages allow to isolate HU-treated bees, thus facilitating later recovery. They keep bees within the natural environment of the hive and permit therefore exchanges with hive mates. The cages were placed between adjacent combs for 11 days to allow full behavioral development of the bees, which ensures good odor-learning abilities (Laloi et al. 2001). On day 11 of the adult stage, the cages were removed from the comb and placed on ice for 3 min to immobilize the bees. The bees were then fixed in their individual harnesses for experiments using the olfactory conditioning of the proboscis extension reflex (PER).

**Experimental groups**

After finishing experiments we opened the head capsules of the conditioned bees and determined under a stereo microscope the presence or absence of MB lesions. The HU-treated bees were divided into two groups *a posteriori* of the conditioning experiments: "*HU-ablated bees*", which presented an ablation of one of the median calyces of the mushroom bodies (this is the predominant MB lesion that can be found in 11-day-old bees; see Fig. 1b), and "*HU-normal bees*, which despite the HU treatment did not show any MB ablation (Fig. 1c). Two other groups were used as controls throughout and were defined *a priori* of the conditioning experiments. The group of "*control bees*" contained animals which were treated exactly as the HU-treated bees with the difference that the feeding solution on which they were placed did not contain HU. Finally, the group of "*11-day-old bees*" contained bees which were not treated as larvae but were taken out of the hive one day before emergence. From then on, these bees were treated like the other three groups.

**Olfactory conditioning experiments**

**Subjects**

Bees were individually harnessed in metal holders so that they could only move their antennae and mouthparts, including the proboscis (Takeda 1961; Bitterman et al. 1983). They were kept in the dark and at high humidity for three hours. Fifteen minutes before starting the experiments, each subject was checked for intact proboscis extension reflex (PER) by lightly touching one antenna with a toothpick soaked with 30% sucrose solution without subsequent

feeding (unconditioned stimulus, US). Extension of the proboscis beyond a virtual line between the open mandibles was counted as PER (unconditioned response, UR). Bees that were subjected to side-specific olfactory conditioning and that therefore required separate olfactory inputs to the antennae had a plastic wall (40 x 50 mm) glued exactly between both antennae using low-temperature melting wax (Sandoz & Menzel 2001) (Figure 1a).

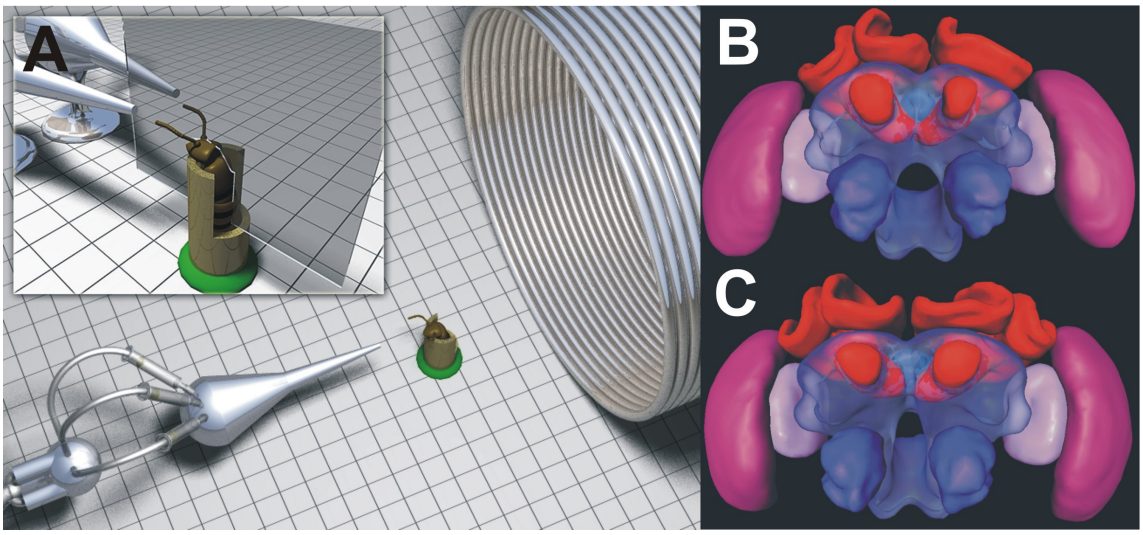


**Figure 1**: Experimental situation of an individual bee and bee brain models. A) Harnessed bees are placed in front of an odor-supplying device. In case of side-specific conditioning, two devices stimulate both antennae separately (see smaller picture). B) 3D computer model of a bee brain showing a lack of the right median calyx within the mushroom body. This ablation is caused by hydroxyurea treatment. C) Three dimensional computer model of a bee brain showing no ablations.

**Unconditioned and conditioned stimuli**

The unconditioned stimulus (US) was 30% (weight/weight) sucrose solution. As conditioned stimuli (CSs) we used the odorants limonene, 2-octanol, heptanal and 2-nonanone (SIGMA, Deisenhofen, Germany). These odors can be easily learned and differentiated by bees (Deisig et al. 2001, Komischke et al. 2002). On each experimental day, 4 µl of pure odorant was applied onto a fresh strip of filter paper. The paper strips were then inserted into a 1-ml plastic syringe and mounted in an odor-supplying device delivering a constant flow of clean air provided by a standard aquarium pump. Computer-driven solenoid valves (Lee Company, Essex, CT) controlled airflow delivery. For bilateral odor stimulations, the bee was placed in front of the device and received such a flow. During periods of odorant delivery, the airflow was shunted through a syringe containing the odorant. For side-specific odor stimulations, we used a bilateral stimulation device producing two distinct airflows directed to

the bees' antennae on the two sides, and which could provide odor stimulations independently of each other (Komischke et al. 2003). An exhaust system was arranged behind the bees to remove odor-laden air.

**Conditioning trials**

Each trial lasted 28 sec. At the beginning of each trial the subject was placed in front of the odor-supplying device for 15 sec to allow familiarization with the training situation. Thereafter the CS was presented for 4 sec. In reinforced trials, the US onset occurred 3 sec after CS onset. Both antennae were lightly touched with a toothpick soaked with sucrose solution and after proboscis extension the bee was allowed to feed for 3 sec. Thus, the inter-stimulus interval was 3 sec and the overlap between CS and US was 1 sec. The bee remained in front of the odor-supplying device until completing the 28 sec of the trial and then returned to its resting position. Another bee was then placed in the experimental setup. Such short trials have been used in previous works and did not impair learning of elemental and non elemental olfactory discriminations (Deisig et al. 2001, 2002, 2003).

Differential conditioning was used in all experiments. Bees had to learn to respond to rewarded odors (or binary mixtures of odors; henceforth CS+) but not to non-rewarded odors or binary mixtures of odors (CS presentations without US; henceforth CS-). The inter-trial interval (measured between successive CS presentations) was 10 min. CS+ and CS- trials were alternated. In all experiments (see below), bees had 4 different CSs and received 6 trials per CS, making a total of 24 trials. Each experiment lasted 240 min.

**Response measurement**

We recorded whether or not a bee extended its proboscis after onset of the odor (CS) and before presentation of the sucrose solution (US) in the case of reinforced trials, such that the anticipatory response recorded could only have been evoked by the CS. Multiple responses during a CS were counted as a single PER. After completing each experiment, all animals were again checked for proboscis extension reflex. Bees that did not show the PER at the end of the experiments were not included in the analyses (< 10%).

**Experimental paradigms**

Three different experimental paradigms were used. In two of them, the CSs were delivered in a separate, side-specific manner to the antennae (Sandoz & Menzel 2001). In the remaining one, the CSs were delivered to both antennae simultaneously (see Figure 1a).

**Delivering the CSs separately to each antenna**

In these two paradigms, the bees had a separating wall between the two antennae (see above) such that odorants reached each antenna separately.

In the first paradigm (*Paradigm 1*), bees were trained with four CSs, A, B, C and D, two of which were rewarded (A+, C+) and two were non-rewarded (B-, D-). Side-specificity was established by dispensing on separate trials one CS+ and one CS- on each side (A+B- on one side, C+D- on the other side). This means that bees trained in this paradigm had to learn two distinct differential conditionings, one on each side. Each CS was unambiguously associated with a US or with the absence of a US such that the discriminations underlying this problem could be solved elementally.

In the second paradigm (*Paradigm 2*) bees were trained with two odors A and B. Side-specificity was established by delivering a differential conditioning of opposed contingencies on each side (A+B- on one side, A-B+ on the other side). In this discrimination problem, the reversed contingencies for the same two odorants A and B generated ambiguity at the elemental level.

**Delivering the CS to both antennae simultaneously**

In this paradigm (*Paradigm 3*), the bees carried no separating wall between the antennae, so that odorants reached both antennae simultaneously. Bees were trained with four CSs, A, B, C and D, two of which were rewarded (A+, C+) and two were non-rewarded (B-, D-). Bees trained in this paradigm had to learn to respond to A and C but not to B and D. Each CS was unambiguously associated with a US or with the absence of US such that the discriminations underlying this problem could be solved elementally.

**Statistical analysis**

We measured the percentage of conditioned responses (%PER) in CS+ and CS- trials throughout the experiment. Such a *group performance* is reflected in the acquisition curves presented throughout. To assess *individual* learning success, we calculated the percentage of errors made by bees in the last block of trials. Such a block is particularly important as it shows to which extent bees learned to differentiated rewarded from non rewarded odorants *at the end* of conditioning. A block consisted of the four possible CS trials. As two of these were CS+ trials and two were CS- trials, 0% errors in the last block of trials means that a bee responded appropriately at all trials: it responded to the two CSs+ and did not respond to any

of the two CSs- ; 100% errors would mean that the bee responded wrongly in all four trials, which seldom happens. A bee that did not learn to differentiate CS+ from CS- usually responded to all stimuli (both CS+ and CS-) or to none of them, such that the average percentage of errors would be 50% in this case. We indicated the percentage of bees which solved the discrimination, i.e. which made 0% error in the last trial block, both for the side-specific experiments (Paradigms 1 and 2) and for the bilateral experiment (Paradigm 3). For between-side comparisons within animals, we used a Wilcoxon matched-pair test comparing correct responses (no error) to incorrect responses (one or two errors).

The Wilcoxon matched-pair test was also used for within-group comparisons. This test was performed only on the last three blocks of trials to focus on the discrimination reached at the end of training. In the side-specific experiments (A+ B- / A- B+ and A+ B- / C+ D-), comparisons were done between the response to CS+ and CS- within each side; in the bilateral experiment (A+, B-, C+, D-) we analyzed whether bees learned to respond to both CS+ and not to both CS-. An index was calculated to this end: in the last three blocks of trials, bees got six CS+ trials (three A+ and three C+ trials) and six CS- trials (three B- and three D- trials). If bees responded five or six times to both reinforced odors, the index had a value of 2; if they also responded five or six times to both non-reinforced odors, the index had also a value of 2. If bees performed one or zero responses to both reinforced odors the index was 0; if they performed one or zero responses to both non-reinforced odors, the index was also 0. All other response patterns (e.g. bees respond to one CS+ three times but they do not respond to the other CS+, and they do not respond to one CS- but they respond three times to the other CS-) yielded a value of 1. Thus, each bee got two index values: one for its CS+ responses and one for its CS- responses. The Wilcoxon matched-pair test was used to compare such index values and thus contrast CS+ vs. CS- responses.

In the case of side-specific conditioning in which the performance for CSs delivered to one antenna is compared with that for CSs delivered to the other antenna, we also used the Wilcoxon matched-pair test. Comparisons between groups in the case of side-specific conditioning were performed by means of a Chi-square test. In this case, we compared the number of animals that made no error in the last block of trials. Because of multiple comparisons between groups, the significance threshold was corrected using the Dunn-Sidak correction ($\alpha' = 1 - (1 - \alpha)^{1/k}$ where k is the number of two-by-two comparisons in which each data point is used). Comparisons between the numbers of bees that made 0% errors in the last block of 4 trials were performed by means of chi-square tests. The alpha level was set to 0.05 for all analyses except for multiple comparisons.

## Results

### Delivering the CSs to each antenna separately

#### Paradigm 1: Elemental side-specific conditioning (A+B- / C+D-)

Figure 2 presents the results of the four groups of bees trained with this paradigm (HU-ablated, HU-normal, control and 11-day-old bees; see upper row). The first two columns (Figure 2A) show the performance of the HU-ablated bees. These bees could not learn any of the two discriminations as shown by the acquisition curves (upper row). There were no statistical differences in learning performance between sides (Wilcoxon matched-pair test: $Z = 0.53$; df: 1; n.s.). On the intact side, bees did not discriminate the odors because the responses to CS+ and CS- were not significantly different (Wilcoxon matched-pair test: $Z = 1.83$; df: 1; n.s.). On the ablated side, bees also could not learn the discrimination (Wilcoxon matched-pair test: $Z = 1.28$; df: 1; n.s.).

To assess *individual* learning success, we calculated first the percentage of bees that solved both discriminations simultaneously. This percentage was only 5% in the case of HU-ablated bees (Table 1). We also calculated the percentage of errors made by HU-ablated bees in the last block of trials (Fig. 2A, lower row). On the intact side, bees made 45% errors because they responded to the CS- or did not respond to the CS+; on the ablated side, they made 42.5% errors for similar reasons.

The second two columns from the left (Figure 2B) show the performance of the HU-normal bees. There were no statistical differences in learning between sides, as shown by the acquisition curves (upper row; left vs. right: Wilcoxon matched-pair test: $Z = 1.89$; df: 1; n.s.). On the left side, bees did discriminate the odors because the responses to CS+ and CS- were significantly different (Wilcoxon matched-pair test: $Z = 4.21$; df: 1; $p < 0.001$). On the right side, bees did also discriminate the odors significantly (Wilcoxon matched-pair test: $Z = 4.78$; df: 1; $p < 0.001$).

The analysis of *individual* learning success showed that the percentage of bees that solved both discriminations simultaneously was 11.4% (Table 1). The percentage of errors made by HU-ablated bees in the last block of trials (Fig. 2B, lower row) was 40% for the left side and 32.9% for the right side.

The third two columns from the left (Figure 2C) show the performance of the control bees. There were no statistical differences in learning between sides, as shown by the acquisition curves (upper row; left vs. right: Wilcoxon matched-pair test: $Z = 0.21$; df: 1; n.s.). On the left side, bees did discriminate the odors because the responses to CS+ and CS- were significantly different (Wilcoxon matched-pair test: $Z = 4.31$; df: 1; $p < 0.001$). On the right side, a significant discrimination was also found (Wilcoxon matched-pair test: $Z = 2.76$; df: 1; $p < 0.01$).

The analysis of *individual* learning success showed that the percentage of bees that solved both discriminations simultaneously was 14.3% (Table 1). The percentage of errors made by HU-ablated bees in the last block of trials (Fig. 2C, lower row) was 34.7% for the left side and 36.7% for the right side.

Finally, the last two columns from the left (Figure 2D) show the performance of the 11-day-old bees. There were no statistical differences in learning between sides, as shown by the acquisition curves (upper row; left vs. right: Wilcoxon matched-pair test: $Z = 0.56$; df: 1; n.s.). On the left side, bees did discriminate the odors because the responses to CS+ and CS- were significantly different (Wilcoxon matched-pair test: $Z = 2.17$; df: 1; $p < 0.05$). On the right side, a significant discrimination was also found (Wilcoxon matched-pair test: $Z = 2.67$; df: 1; $p < 0.01$).

The analysis of *individual* learning success showed that the percentage of bees that solved both discriminations simultaneously was 16.7% (Table 1). The percentage of errors made by HU-ablated bees in the last block of trials (Fig. 2D, lower row) was 27.1% for the left side and 33.3% for the right side.

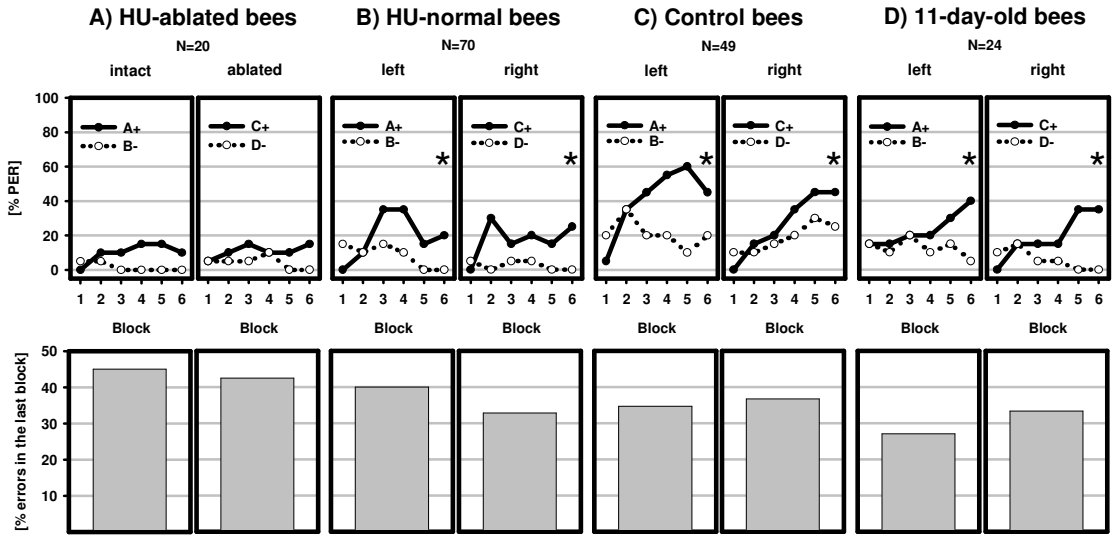


**Figure 2**: Elemental side-specific conditioning (A+B- on one side, C+D- on the other). Four experimental groups of bees are shown in columns A - D. Acquisition curves for each antenna are presented in the first row of graphs. Ablated bees do not learn to discriminate the stimuli in any of the tasks, neither on their ablated nor on their intact side. All other groups fulfill the discrimination tasks at the end of the training procedure. The second row of graphs illustrates the percentage of errors with respect to PER responses bees made in the last block of trials. Significant discriminations between rewarded and non-rewarded stimuli given to one antenna are marked by an asterisk.

Thus, HU-ablated bees did not learn any of the two differential conditioning tasks while the other three groups learned at least one of them. On average, there were only 12.3% of the bees responding correctly both to the CS+ and to the CS- on both sides in the last trial (Table 1). No statistical differences were detected between these groups (Chi²-Test: $\chi^2 = 1.01$; df: 3; n.s.). Nevertheless, Paradigm 1 (A+B- / C+D-) shows a strong influence of MB ablations on an unambiguous side-specific olfactory learning. MB-ablated bees were impaired in solving elemental olfactory discriminations even on their intact brain side.

**Paradigm 2: Non-elemental side specific conditioning (A+B- / A-B+)**

Figure 3 presents the results of the four groups of bees trained with this paradigm (HU-ablated, HU-normal, control and 11-day-old bees; see upper row). The first two columns (Figure 3A) show the performance of the HU-ablated bees. Ablated bees solved the problem on their intact brain side but not on their ablated side, as shown by the acquisition curves (upper row). Due to this, we found statistical differences in learning between sides (ablated

vs. intact; Wilcoxon matched-pair test: Z = 2.02; df: 1; p<0.05). On the intact side, bees learned to discriminate the odors (Wilcoxon matched-pair test: Z = 2.93; df: 1; p < 0.01) while discrimination was not possible on the ablated side (Wilcoxon matched-pair test: Z = 1.83; df: 1; n.s.)

The analysis of *individual* learning success showed that the percentage of bees that solved both discriminations simultaneously was 12.5% (Table 1). The percentage of errors made by HU-ablated bees in the last block of trials (Fig. 3A, lower row) was 35.4% on the intact side, and 45.8% on the ablated side.

The second two columns from the left (Figure 3B) show the performance of the HU-normal bees. There were no statistical differences in learning between sides, as shown by the acquisition curves (upper row; left vs. right: Wilcoxon matched-pair test: Z = 0.91; df: 1; n.s.). On the left side, bees learned to discriminate the CS+ and the CS- (Wilcoxon matched-pair test: Z = 4.98; df: 1; p < 0.001). On the right side, a significant discrimination was also found (Wilcoxon matched-pair test: Z = 4.87; df: 1; p < 0.001).

The analysis of *individual* learning success showed that the percentage of bees that solved both discriminations simultaneously was 7.5% (Table 1). The percentage of errors made by HU-ablated bees in the last block of trials (Fig. 2D, lower row) was 40% for the left side and 35.7% for the right side.

The third two columns from the left (Figure 3C) show the performance of the control bees. There were no statistical differences in learning between sides, as shown by the acquisition curves (upper row; left vs. right: Wilcoxon matched-pair test: Z = 0.00; df: 1; n.s.). On the left side, bees did discriminate the odors since responses to CS+ were significantly higher different than those to the CS- (Wilcoxon matched-pair test: Z = 3.24; df: 1; p < 0.01). On the right side, a significant discrimination was also found (Wilcoxon matched-pair test: Z = 2.76; df: 1; p < 0.01).

The analysis of *individual* learning success showed that the percentage of bees that solved both discriminations simultaneously was 7.7% (Table 1). The percentage of errors made by HU-ablated bees in the last block of trials (Fig. 3C, lower row) was 37.4% for the left side and 37% for the right side.

Finally, the last two columns from the left (Figure 3D) shows the performance of the 11-day-old bees. There were no statistical differences in learning between sides as shown by

the acquisition curves (upper row; left vs. right: Wilcoxon matched-pair test: Z = 0.53; df: 1; n.s.). On the left side, bees did discriminate the odors because they responded significantly more to the CS+ than those to the CS- (Wilcoxon matched-pair test: Z = 3.29; df: 1; $p < 0.01$). On the right side, a significant discrimination was also found (Wilcoxon matched-pair test: Z = 3.24; df: 1; $p < 0.01$).

The analysis of *individual* learning success showed that the percentage of bees that solved both discriminations simultaneously was 41.7% (Table 1). The percentage of errors made by HU-ablated bees in the last block of trials (Fig. 3D, lower row) was 20% for the left side and 22.5% for the right side.

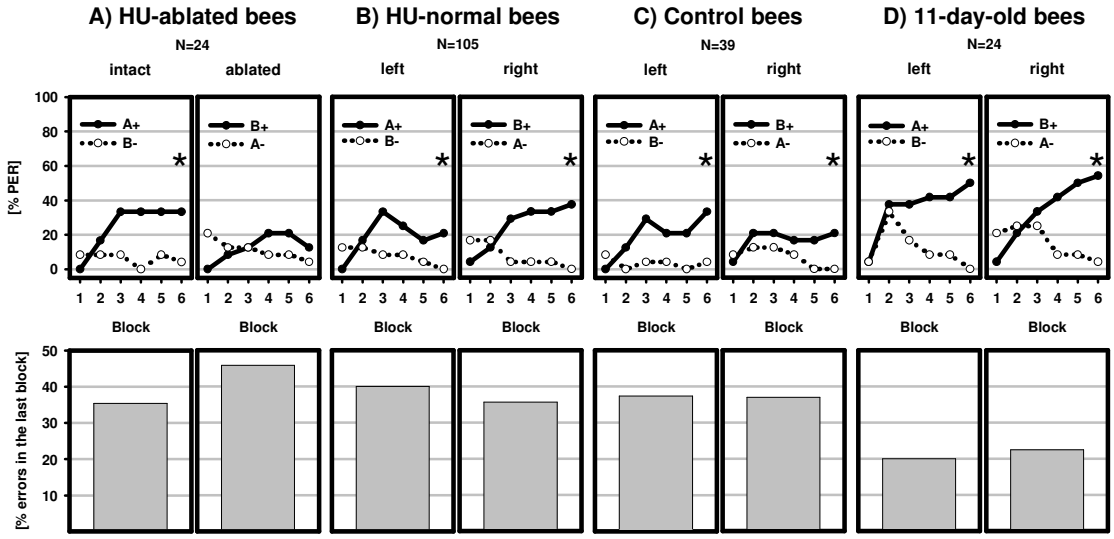


**Figure 3**: Non-elemental side-specific conditioning (A+B- on one side, A-B+ on the other). Four experimental groups of bees are shown in columns A - D. Acquisition curves for each antenna are presented in the first row of graphs. Ablated bees do not learn to discriminate the stimuli on their ablated side, whilst they do discriminate the stimuli on their intact side. All other groups fulfill the discrimination tasks at the end of the training procedure. The second row of graphs illustrates the percentage of errors with respect to PER responses bees made in the last block of trials. Significant discriminations between rewarded and non-rewarded stimuli given to one antenna are marked by an asterisk.

This paradigm shows, therefore, a clear-cut effect of unilateral MB lesions, namely that the ablated side performs worse in learning an A+ vs. B- discrimination than the intact side, which receives reciprocal training (A- vs. B+). In average, 11.8% of the bees responded correctly both to the CS+ and to the CS- on both sides in the last trial (Table 1). In this case, the 11-day-old bees performed significantly better than the other experimental groups in

Table 1 (Chi²-Test: $\chi^2 = 19.54$; df: 1; $p < 0.001$), which did not differ between them (Chi²-Test: $\chi^2 = 0.01$; df: 2; n.s.).

All in all, the comparison of the results of Paradigms 1 and 2 suggests that an ambiguous side-specific conditioning for two odors (Paradigm 2) is easier to solve than an elemental one with four odors (Paradigm 1; compare Figs. 3A vs. 2A). This suggestion is based on the fact that HU-ablated bees were successful on the intact side in the ambiguous problem (Fig. 3A, upper row) while they were unsuccessful on the same side in the non-ambiguous problem (Fig. 2A, upper row). Despite this obvious difference , a statistical comparison between Paradigms 1 and 2 involving all four bee groups yielded no significant difference (Kruskal-Wallis Anova: $\chi^2 = 0.14$; df: 1; n.s.), thus indicating that the difference between paradigms was restricted to the group of HU ablated bees.

**Delivering the CS simultaneously to both antennae**

**Paradigm 3: Elemental differential conditioning with four odors (A+, B-, C+, D-)**

Figure 4 presents the results of the four groups of bees trained with this paradigm (HU-ablated, HU-normal, control and 11-day-old bees; see upper row). The first column (Figure 4A) shows the performance of the HU-ablated bees. Ablated bees solved the problem and learned to respond to A+ and C+ but not to B- and D-, as shown by the acquisition curves (Wilcoxon matched-pair test: $Z = 2.37$; df: 1; $p < 0.05$).

The analysis of *individual* learning success showed that the percentage of bees that responded correctly to all four stimuli in the last block of trials was 36.4%. (Table 1). The percentage of errors made by HU-ablated bees in the last block of trials (Fig. 4A, lower row) was 22.5%.

The second column from the left (Figure 4B) shows the performance of the HU-normal bees. Bees in this group also learned to discriminate the two CSs+ from the two CSs-, as shown by the acquisition curves (Wilcoxon matched-pair test: $Z = 5.71$; df: 1; $p < 0.001$).

The analysis of *individual* learning success showed that the percentage of bees that responded correctly to all four stimuli in the last block of trials was 50.9%. (Table 1). The percentage of errors made by HU-ablated bees in the last block of trials (Fig. 4A, lower row) was 19.3%.

The third column from the left (Figure 4C) shows the performance of the control bees. These bees also learned to discriminate the two CSs+ from the two CSs-, as shown by the acquisition curves (Wilcoxon matched-pair test: $Z = 4.78$; df: 1; $p < 0.001$).

The analysis of *individual* learning success showed that the percentage of bees that responded correctly to all four stimuli in the last block of trials was 22.0%. (Table 1). The percentage of errors made by HU-ablated bees in the last block of trials (Fig. 4A, lower row) was 29.5%.

Finally, the last column from the left (Figure 4D) shows the performance of the 11-day-old bees. As bees in all other groups, 11-day-old bees learned to discriminate the odors because the responses to the CSs+ differed significantly from those to the CSs-, as shown by the acquisition curves (Wilcoxon matched-pair test: $Z = 3.72$; df: 1; $p < 0.001$).

The analysis of *individual* learning success showed that the percentage of bees that responded correctly to all four stimuli in the last block of trials was 25.9%. (Table 1). The percentage of errors made by HU-ablated bees in the last block of trials (Fig. 4A, lower row) was 26.9%.

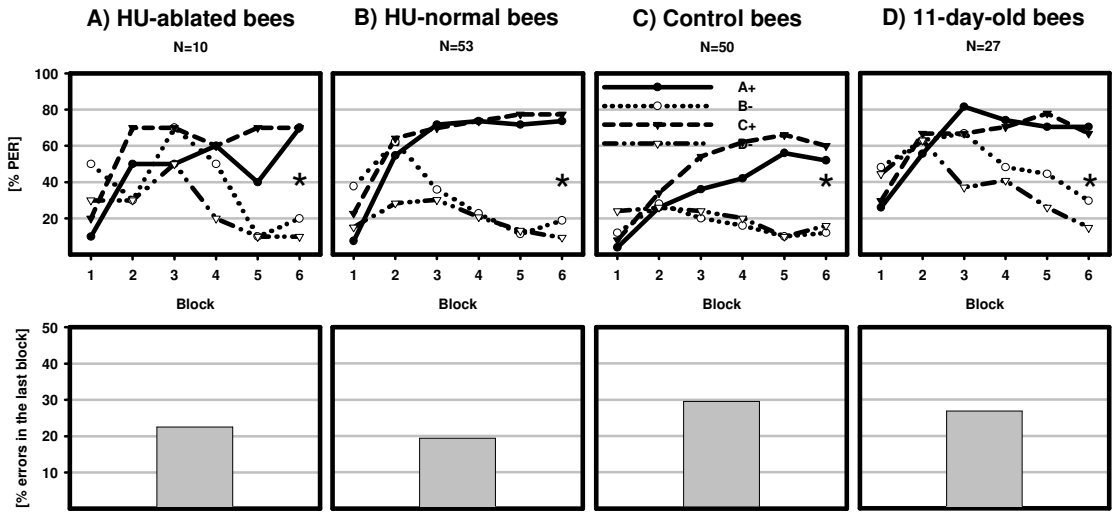


**Figure 4**: Elemental differential conditioning with four odors (A+, B-, C+, D-). Four experimental groups of bees are shown in columns -A to D. Acquisition curves for each group are presented in the first row of graphs. All experimental groups learn to discriminate the olfactory stimuli at the end of the training procedure. They learn to respond to the reinforced odors but not to the non-reinforced ones. The second row of graphs illustrates the percentage of errors with respect to PER responses bees made in the last block of trials. Significant discriminations between rewarded and non-rewarded stimuli are marked by an asterisk.

Thus, when each odorant was delivered simultaneously to both antennae in an unambiguous discrimination, MB ablations did not impair the bees' performance. All groups learned equally well to respond appropriately to the rewarded and the non-rewarded odorants. Statistical differences between groups could be detected (Chi²-Test: $\chi^2 = 10.72$; df: 3; $p < 0.05$). This finding is based on the performance of the HU-normal bees, which performed significantly better than bees of the other groups. These groups showed no statistical differences with respect to their performance in the last block of trials (Chi²-Test: $\chi^2 = 1.44$; df: 2; n.s.).

**Table 1**: Percentage of bees which made no errors in the last block of trials are shown for all experimental groups and paradigms. Groups that differ statistically from the other groups within a paradigm are marked by an asterisk. Results of Chi-square-tests are presented in the rightmost column. With respect to animals making no error in the last block of trials, paradigm 3 has been solved by significantly more animals then the other two paradigms (Chi²-Test: $\chi^2 = 37.85$; df: 1; $p < 0.001$) which do not differ statistically (Chi²-Test: $\chi^2 = 0.80$; df: 1; n.s.).

| | HU ablated | HU normal | Control | 11-day-old | $\chi^2$ |
|---|---|---|---|---|---|
| Paradigm 1 | 5.0% | 11.4% | 14.3% | 16.7% | 1.01; df: 3; n.s. |
| Paradigm 2 | 12.5% | 7.5% | 7.7% | 41.7% * | 19.54; df: 1; p<0.001 |
| Paradigm 3 | 36.4% | 50.9% * | 22.0% | 25.9% | 10.72; df: 1; p<0.05 |

**Discussion**

MB lesions had no effect on the discrimination in which odorants were delivered simultaneously to both antennae. In this case, all bees, ablated or not, could solve the elemental, unambiguous discrimination (A+, B-, C+, D-) (see Fig. 4). When odorants were delivered in a side-specific manner, ablated bees could not solve any of the discriminations of the non-ambiguous problem (A+, B- / C+, D-) (see Fig. 2), while they could solve at least one of the two discriminations of the ambiguous problem (A+, B- / A-, B+) (see Fig. 3). Our results show a clear-cut effect of unilateral MB lesions, namely that the ablated side performs worse in learning an A+ vs. B- discrimination than the intact side, which receives reciprocal training A- vs. B+.

Our results can therefore be analyzed in the light of three main factors that could have influenced the bees' performance: 1) the number of odorants processed and learned; 2) elemental vs. non-elemental discriminations; and 3) side-specific vs. bilateral olfactory stimulation.

**Number of odorants processed and learned**

Differences in performance between paradigms might arise from the amount of differential information that bees had to process. In other words, discriminations involving two odorants could be easier than those involving four odorants. Although comparing Paradigms 1 (A+B- / C+D-: 4 odorants) and 2 (A+B- / A-B+: 2 odorants) seems to support this hypothesis, since discrimination was better in Paradigm 2 than in Paradigm 1 (see in particular performance of HU ablated and 11-day-old bees), several arguments question it. Firstly, a global statistic analysis comparing both paradigms yielded no significant differences. Secondly, it is not clear that in Paradigm 2 (A+B- / A-B+), the bees processed A and B only as two odors. As these odors had different contingencies on each side, we think that bees have to suppress information transfer between sides (see below) and to build separate representations of these odorants on each side, resulting in a situation in which the brain would cope in fact with four different representations, i.e. four different stimuli. Thirdly, in Paradigm 3 (A+, B-, C+, D-), bees were trained with four odorants and could solve the task, thus showing that a discrimination involving four stimuli is not necessarily more demanding.

In fact, all of our three paradigms (including Paradigm 2; see above) could be viewed as involving four stimuli, thus making it difficult to say whether the amount of odorants involved in the discrimination affected bees' performance. Further experiments should be performed to compare the performance of different groups of bees conditioned with increasing numbers of odorants. To this end, stimuli should be delivered bilaterally to avoid the cofactor of side-specificity. In doing this, generalization between odorants should be kept to a minimum, which is possible by using perceptually distinct odorants. We thus conclude that differences between groups and/or paradigms could not be ascribed directly to differences in the number of odorants used.

**Elemental vs. non-elemental discriminations**

We assumed that the ambiguity underlying non-elemental discriminations could be an important factor determining the intervention of the MBs (Giurfa 2003). We conjectured that ablated bees should show deficiencies in the non-elemental, ambiguous discrimination (Paradigm 2: A+ B- / A- B+) but not in the elemental, unambiguous ones (Paradigm 1: A+B- / C+D- and Paradigm 3: A+, B-, C+, D-).

Comparing Paradigms 1 and 3 (elemental) vs. 2 (non-elemental) introduces, however, a confounding factor that should be eliminated to appreciate the impact of the linearity of the discrimination task under study. This factor is the side-specificity of stimulus delivery, which is present in Paradigms 1 and 2 but not in Paradigm 3. In order to focus on the effect of linearity, we thus focus on a comparison between Paradigms 1 and 2, which are both side-specific.

In Paradigm 1 (A+, B- / C+, D-), bees had to learn an unambiguous, elemental discrimination while in Paradigm 2 (A+, B- / A-, B+), they had to learn a non-elemental, side-specific discrimination in which each odorant was rewarded as often as non-rewarded. We found a MB-lesion-specific effect in Paradigm 2, since the performance of the HU-ablated bees was different with respect to the intact vs. the ablated side, the ablated side being deficient for solving the discrimination while the intact not. In any case, HU-ablated bees in Paradigm 2 solved at least one of both discriminations. This was not the case in Paradigm 1 where HU-ablated bees could not learn any of the side-specific discriminations. These results were counterintuitive from the perspective that the MBs may contribute essentially to non-elemental, ambiguous forms of learning (Giurfa 2003; Komischke et al. 2003). From this perspective, the HU-ablated bees should have been more impaired in Paradigm 2 than in Paradigm 1. The opposite was the case.

Malun et al (2002b) showed that a single A+ vs. B- olfactory discrimination can be learned in a side-specific manner, even on the ablated side. Thus, the fact that bees were incapable of learning the same discrimination in Paradigms 1 and 2 was related to the presence of an additional discrimination on the other side. Crosstalk and information exchange occurs between both brain sides and it is believed that the MBs play a prominent role in this communication (Mobbs 1982) . Bees trained in a side-specific manner transfer the learned olfactory information to the contralateral brain hemisphere and react to the conditioned odor when presented to the contralateral antenna (Erber et al. 1980; Sandoz and Menzel 2001). Also, to be able to solve particular olfactory learning tasks, bees need input to the two brain sides (Thorn and Smith 1997, Komischke et al. 2003). We suggest that this transfer of stored information between the brain sides, which may or may not occur, depending on the kind of information stored, was disturbed by MB lesions.

In the non-elemental, side-specific olfactory conditioning (Paradigm 2: A+ B- / A- B+), normal, adult bees learn to respond appropriately to each odorant on its correct side (Sandoz and Menzel 2001, Sandoz et al. 2003). Bees trained in this paradigm and tested 24 h later, still respond appropriately thus showing that they have access to a mechanism that

allows avoiding confusion between sides and keeping the representations of the odorants distinct and separate. This can be achieved if the transfer mechanism between brain sides mentioned above is actively inhibited during this task. In Paradigm 2, transferring information between sides may impair appropriate odor discrimination, as each side would end up with four representations A+ A- B+ B-. We therefore suggest that whenever contradictory information between the two sides is detected (i.e. $A+_{left}$ $A-_{right}$; $B-_{left}$ $B+_{right}$ ), the transfer between sides is inhibited. As a result, each side would end up with two unconnected representations: A+ B-, in one case and A- B+ in the other case. The inhibition of crosstalk between sides would force the bees to build four separate representations (cf. the discussion about the number of stimuli above), which would facilitate discrimination on both sides. One possibility is that MB ablations could have an effect on this inhibition process such that the ablated side cannot block the transfer from the intact side. As a result, HU-ablated bees would be left with a single discrimination on the intact side (say, A+ B-) but with a double, ambiguous discrimination on the ablated side (A+ A- B+ B-). Under these circumstances, bees should achieve the discrimination on the intact side but not on the ablated side. This is exactly what we found in Paradigm 2.

Another explanation would argue that transfer between sides is not the critical factor to consider but that the representations on the intact side (say, A+ B-) are more salient than those in the ablated side (say, A- B+). If in Paradigm 2 transfer between sides occurs despite contradictory information, bees would be left on each side with representations of the type A+ A- B- B+. They would therefore focus on the more salient discrimination A+ B-. This possibility implies that bees would always respond correctly on the intact side and would always respond wrongly on the ablated side. But in both cases, bees would respond differentially to odorants A and B. This was, however, not the case as bees in Paradigm 2 did not discriminate the odorants on the ablated side. Thus, we conclude that the lesions at the level of the MBs essentially affected the normal transfer of stored information between mushroom bodies and that such transfer may or may not occur in normal circumstances depending on the information stored in each brain side.

**Side-specific vs. bilateral olfactory stimulation**

In order to focus on the effect of side-specific vs. bilateral stimulation, we focus on a comparison between Paradigms 1 (A+ B- / C+ D-) and 3 (A+, B-, C+, D-) which involved both the same odorants A, B, C and D, with same contingencies but with different spatial distribution. HU-ablated bees could solve Paradigm 3 but not Paradigm 1 despite the common

factors mentioned above. Consistent with previous results (Thorn & Smith 1997; Komischke et al 2003), bilateral delivery of olfactory stimulation allows better discrimination performances. Our previous work (Komischke et al. 2003) led us to the conclusion that bilateral olfactory input was necessary to solve a non-elemental discrimination such as the negative patterning (A+, B+, AB-; see Introduction). The present results allow extending this conclusion to elemental discriminations such as those involved in Paradigms 1 and 3.

In Paradigm 1, bees were trained with A+ B- on one side and C+ D- on the other side. We have proposed that transfer between sides is impaired or inhibited when the side-specific information is contradictory, which is not the case in Paradigm 1. Accordingly, transfer should not be affected and each brain side would end up with four representations A+ B- C+ D-, similarly to what happens in Paradigm 3 where the same four odorants with the same contingencies were conditioned bilaterally. Thus, the impossibility of learning both side-specific discriminations in HU-ablated bees trained in Paradigm 1 may be related to an unknown distortion of the transfer mechanism between brain sides. More experiments are necessary to test this idea and unravel its mechanistic basis. One possible way to test it is the use of a reversible blocking procedure of mushroom body activity in a side-specific manner. Contrarily to lesions, selective, reversible blocking has the advantage of showing whether distortion of the transfer between brain sides can be restored when the effect of blocking is no longer present. Reversible blocking of mushroom body function can be achieved using local anesthetics (Müller et al. 2003) or TTX (work in progress) in a side-specific manner. The experiments mentioned, which are under way, will allow to clarify the hypotheses mentioned above.

**Neural correlate of transfer between mushroom bodies**

In the honeybee brain, odor processing involves different stages and is symmetrical between sides. Axons of the chemoreceptors on each antenna project to the 160 glomeruli of each antennal lobe, the primary olfactory center, where they synapse with about 4000 local interneurons and about 800 projection neurons (Arnold et al. 1985, Abel et al. 2001, Mobbs 1982). Projection neurons convey information to higher brain centers, the mushroom bodies and the lateral protocerebral lobes (Abel et al. 2001, Mobbs 1982). At the anatomical level, the olfactory pathways of the two brain sides are mainly connected at the level of the output of the MBs, the α-lobes, although a few connecting neurons between antennal lobes have also been reported (Mobbs 1982). Placed at an intensive information crossway in the bee brain, each MB α-lobe receives direct information from the antennal lobe (Abel et al. 2001), as well

as processed information from the MB calyces ipsilaterally and from the contralateral α-lobe (Rybak & Menzel 1993, Abel et al. 2001). The tracts connecting both MBs could therefore allow the transfer of information between brain sides and allow the joint activity of the two brain sides. The effect of MB ablations on such connecting tracts is still unknown, although we believe that, since the medial calyces are usually missing in ablated bees and the volume of the α-lobes is strongly reduced on the ablated side (Malun et al. 2002a), developmental deficiencies must appear at this level. Indeed, previous work evaluated the anatomical impact of median calyx ablations (Malun et al. 2002a, b) and made two important observations. Firstly, ablation of such a central brain structure strongly influences overall brain wiring. Although no volumetric or odour-evoked activity differences have been found at the AL level (Malun et al. 2002b), the wiring pattern of projection neurons leaving the AL to relay information to the MBs was clearly modified. Such wiring changes are to be expected in the case of connecting neurons. Indeed, in some bilateral ablations of median calyces, β output lobes on both sides are fused together, with Kenyon cells projecting to the contralateral brain side (Malun et al. 2002a). We thus believe that inter-hemispheric neuronal wiring must be disturbed in ablated bees. Secondly, determination of the amount of proteins involved in neural plasticity, learning and memory on the two brain sides of bees with a median calyx missing, showed increased protein levels on the *intact* MB side, protein levels on the ablated side being similar to those of control animals (Malun et al. 2002a). This means that during development, compensation processes appear to take place, which can have important processing consequences not only on the ablated side, but also on the intact side (Malun et al. 2002a). Therefore, we believe that learning deficits, which can be either limited to the ablated side (Paradigm 2), or can attain both brain sides (Paradigm 1), can be explained on the basis of such unilateral and bilateral anatomical deficiency. A more detailed analysis of possible modifications in the wiring of MB extrinsic neurons, in particular of neurons connecting the two α-lobe areas would be required to understand such effects better.

In summary, our data show that both MBs of the bee brain seem to work as a functional unit with respect to olfactory learning. Separate processing between brain sides can only be achieved in special cases in which the spatial separation of olfactory information has to be achieved as a part of the learning problem. In this case each brain side forms its own, exclusive memory by using only information from its ipsilateral antenna.

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