# Chapter III :

## Overshadowing in Odour Mixtures and the Role of Antennal Lobe Projection Neurons as a Neuronal Correlate

# 1. Introduction

Throughout its life, the honeybee *Apis mellifera* is confronted with crucial odour information both within the hive and during foraging (Winston 1987). Its olfactory system is able to detect, learn and recognize a vast number of odours (Vareschi 1971). Especially, the odours produced by plants, which provide nectar and pollen necessary for the survival of the hive, can be learned and memorized very well, allowing bees to optimize food source exploitation (Menzel 1985; Menzel et al. 1993). Plants produce complex odour blends (Knudsen et al. 1993). Given the efficiency of honeybees for using odour cues during foraging, one could ask if the olfactory system is able to filter out irrelevant information and to concentrate on relevant signals. It could be for instance beneficial for the bees not to pay attention to odours that are common and to concentrate on odours that consistently correspond to valuable pollen and nectar sources. In this context, the overshadowing phenomenon could play an important role, the salience of particular odours in a mixture being ranked higher than other odours by the processing system.

Our earlier work (Chapter II) clearly showed that in honeybees, binary odour mixture identity (AB) is highly dependent on the characteristics of its odour components A and B. Quite often, these components have indeed different weights in the mixture, as observed by the overshadowing effect (Pavlov 1927; Kamin 1968, 1969): after mixture conditioning, generalization responses to the single components are then significantly different (e.g. $A_{AB}>B_{AB}$ according to our previous notation; (Staddon 1983; Gallistel 1990). We showed that differences in component odour salience, leading to differences in component acquisition or to asymmetrical cross-generalization promotes overshadowing. This effect is clearly predictable in two control experiments in which the single components are trained and both single odours are afterwards individually tested ($A_A$ and $B_A$ or $A_B$ and $B_B$). If one of the odours is better learned than the other ($A_A > B_B$) and/or if odour A receives relatively more generalisation responses after B conditioning than vice versa (cross-generalization asymmetry: $B_A/B_B > A_B/A_A$), then odour A is likely to overshadow B after mixture conditioning (Chapter II). How such an effect, which has received considerable evidence in behavioural work, can take place at the neurobiological level is still a mystery, which we aim at unravelling in the present study. The honeybee is a particularly suited model to address this question since its brain is well described and it grants good experimental access to the neurobiological study of odour processing (Menzel & Giurfa 2001; Galizia & Menzel 2001). We want to test the hypothesis that odour processing at the level of the first olfactory relay of

the odour pathway, the antennal lobe, is responsible for phenomena such as asymmetrical cross-generalization and overshadowing.

The honeybees' olfactory nervous system depends for odour detection on ~60000 olfactory receptor neurons (ORNs), organized in groups of 15 to 30 receptor cells innervating each sensillum placodeum (9 x 6 µm oval cuticula plate) (Esslen & Kaissling 1976) distributed over the first eight segments of each antenna (von Frisch 1921; Frings 1944). Each receptor type is sensitive to a wide range of odorants so that single receptor responses are not sufficient for odour characterisation, but only the response of the receptor ensemble provides reliable odour information. Minor odour processing seems to take place already within sensilla placodea (Akers & Getz 1992, 1993; Getz & Akers 1994) but its effect and impact on odour representation remain controversial. The axons of ORNs follow the antennal nerve and project to the ipsilateral antennal lobe (AL). Each AL consists of about 160 glomeruli, spherical structures organized in a single layer on the lobes surface (Arnold et al. 1985; Flanagan & Mercer 1989; Galizia et al. 1999). It is believed that ORNs expressing the same type of receptor project to the same glomerulus, as shown for mice (Vassar et al. 1994) and *Drosophila* (Vosshall et al. 2000). In the honeybee, optical imaging techniques allowed proving that olfactory information is represented by patterns of glomerular activation in the antennal lobe (Joerges et al. 1997). The organization of the glomeruli within the AL is conserved between animals (Arnold et al. 1985) which makes it possible to identify each glomerulus with the help of a standard anatomical atlas of the AL (Flanagan & Mercer 1989; Galizia et al. 1999a). Doing so, it was shown that odour-induced activity patterns are conserved between individuals (Galizia et al. 1999a; Sachse et al. 1999). Moreover, similarity relationships between odour signals in the AL correlate with bees' generalisation behaviour with these odours, so that we may treat these signals are neural representations of odours in the bee brain (Chapter I, Guerrieri, Schubert et al. 2005). Nevertheless, glomerular organization and odour representation can be plastic, since the size of individual glomeruli can change with experience (Brown et al. 2003) and glomerular odour-induced calcium signals can be modified by olfactory conditioning (Faber et al. 1999; Sandoz et al. 2003).

Within the glomeruli, the ORNs form synaptic contacts with ~4000 local interneurons (LNs) (Witthöft 1967) and ~800 projection neurons (PNs) conveying olfactory information to the mushroom bodies (MB) and the lateral protocerebral lobes (LPL). Using a technique that allows to stain the projection neurons specifically, Sachse and Galizia (2002) showed that odour representations at this stage are more contrasted than what is observed in standard calcium imaging experiments which reveal predominantly ORN-related activity (Sachse &

Galizia 2002, 2003). This contrast enhancement is thought to be caused by at least two inhibitory networks of LNs, a GABAergic type with a global inhibitory action and a second type (possibly histaminergic) promoting inhibition between specific glomeruli. Thus, because of the intense interconnectivity between ORNs, LNs and PNs, information processing within the AL is thought to shape the odour representation that will be conveyed to higher brain centres. The processed odour information leaves the ALs following three main tracts, the medial-, mediolateral- and lateral antennocerebral tracts (respectively m-, ml- and l-ACT) (Abel et al. 2001). Most of the glomeruli accessible to optical imaging (20 to 30 on the frontal part of the lobe) send their information to other brain centres via the l-ACT. Therefore, specifically staining l-ACT projection neurons, as done by Sachse and Galizia (2002), allows measuring the processed odour information leaving the antennal lobe towards other brain centres. If phenomena like asymmetric cross-generalization and overshadowing depend on odour processing within the AL, then measuring the responses of l-ACT projection neurones to odours in situations in which we observe the two phenomena behaviourally may provide us with their neural basis.

In our previous studies, we proposed a neurobiological model of asymmetrical generalization, based on similarity relationships between odour representation in the antennal lobe (Chapter I, Guerrieri, Schubert et al. 2005). In this model asymmetrical generalization between two odours A and B was explained by a differential change of A and B representations after A and B training. Such changes in odour representation through conditioning could appear, for instance, through modifications of the strength of relative inhibitions between glomeruli, as proposed in a previous study (Linster & Smith 1997). According to our model, if the neuronal representation of A after training becomes A', which is more dissimilar to B than the new representation original representation of A, and if the neuronal representation of B becomes B' after training, which is more similar to A than the original representation of B, then bees would show less generalization in behavioural tests from A to B than from B to A (cross-generalisation asymmetry), simply because their perception of odour similarity would be different in the two training situations. Likewise, overshadowing can be explained based on similarity relationships between neural representations, by asymmetries in the way two odours are represented in a mixture: thus, if after mixture conditioning (AB+) the representation of odour A is more similar to that of AB than the B representation, then the overshadowing effect would simply be based on higher similarity between A and AB than between B and AB. In fact, (Deisig et al. 2006) recently showed in unconditioned bees that components are indeed differentially represented in

mixtures, and that the more intense the AL signal induced by a component A when presented alone, the stronger this component will appear in the mixture representation. Therefore, the overshadowing effect could appear both with and without changes in odour representations through conditioning.

Given the predictions of our model, we devised the present calcium imaging experiment to test these predictions in a concrete case. We thus performed recordings of odour-responses in l-ACT neurons from 19 antennal lobe glomeruli. Four groups of bees were tested: two groups of bees were conditioned to the single odours 2-hexanol and hexanal. These two odours were chosen because (i) they showed asymmetrical generalization in Chapter I (Guerrieri, Schubert et al. 2005), which (ii) based on the results of Chapter II, suggested that overshadowing would be found after mixture conditioning. A third group was conditioned to the mixture 2-hexanol-hexanal to test the overshadowing effect and a last group was kept naïve. Twenty four hours after the conditioning phase, the bees were tested in imaging conditions with hexanal, 2-hexanol, their binary mixture and an air control. These experiments allowed us to test directly our hypotheses: first, we asked whether olfactory conditioning modifies odour-induced activity at the level of l-ACT neurons, as postulated by our model. Second, we asked if odour representations would change through conditioning in such a way that distance asymmetries could appear which would explain cross-generalization asymmetries. Lastly, we tested whether similarity relationships between the representations of the mixture and its components allows predicting the occurrence of overshadowing after mixture training. Because we included behavioural controls, the imaging results could be compared to the behavioural outcome of the different groups.

# 2. Material & Methods

## 2.1 Animals and preparation

Bees from an outdoor hive were captured at the hive entrance and were taken to the laboratory in ventilated glass vials. They were cooled down on ice to near immobility (about 5 min) and were harnessed into individual metal tubes, so that only movements of the antennae and the proboscis were possible. The back of the head was fixed with a drop of molten wax to prevent it from moving. Afterwards, the animals were left undisturbed for 2 hours. Fifteen minutes before the conditioning phase started, bees' motivation was tested by touching their antennae with 50% (w/w) sugar solution. Bees that did not show a clear proboscis extension (daily percentages varied strongly depending on season and weather conditions) were discarded. Each day, 18 bees were chosen for conditioning and 5 bees were kept for forming a naïve unconditioned control group. Thirty minutes after the last conditioning trial all animals (conditioned and naïve) were fed to repletion with 50% sugar solution. After the last bee was fed the animals were placed into an incubator (25°C) in which they spent the night until the 24 h retention tests. On the next day, before the tests began, 2-5 animals out of the 18 conditioned and 5 naïve bees were picked and prepared for imaging experiments. Only bees that had successfully learned the CS-US association on the previous day (i.e. that had responded already to the CS and before the US at conditioning trials 2 and 3) were chosen. Also, to be sure that bees for the imaging were appetitively motivated, conditioned as well as naïve bees had to show a clear PER after touching the antennae with sugar solution before being accepted for imaging preparation. The remaining conditioned bees were tested for retention (unrewarded presentation of the odours) in the same behavioural context as experienced during conditioning (see **2.4 Experimental design**).

The animals picked for imaging experiments were cooled down on ice to near immobility (about 5 min) and were transferred to individual plexiglass chambers adapted for optical imaging (Galizia et al. 1997). The bees were placed upright into the chamber with very thin horizontal plates separating the head from the rest of the animal's body without hurting physical structures (nerve strands or oesophagus). In that way, only the head capsule with the antennae and mouthparts were exposed (**Fig. 2.1**). The head was fixed very carefully with molten wax without touching the antennae. The same wax was used to fill the pool around the head with a thin layer to make it leak-proof. After fixing the head capsule the bees' antennae were restricted in movement by small needles. The first segments (*scapi*) of the antennae were fixed, pointing horizontally in front of the bee's head. Only the flagella carrying olfactory

sensilla were freely moveable. The mandibles were fixed with molten wax in a semi-open position, and the proboscis was extended and also fixed with a drop of wax to reduce muscle movements in the head capsule. Finally, a small plexiglass wall was glued on top of the bee's head to separate the antennae from the pool which was filled with Ringer solution (130 mM NaCl, 6 mM KCL, 4mM $MGCl_2$, 5 mM $CaCl_2$, 160 mM sucrose, 25 mM glucose, 10 mM HEPES, pH 6.7) right before surgery. A small window was then cut into the head capsule allowing access to the brain (**Fig. 2.2**, left part). Hypopharyngeal glands, tracheas and covering membranes were removed. Having access to the brain, crystals of the calcium-sensitive dye Fura-2 dextran were placed using a microelectrode into the tissue between the alpha lobes and the optical lobes, in order to severe the axonal tractus of l-ACT projection neurons going to the mushroom calyces (**Fig. 2.2**, right part). Then, the bees were left in a cool dark place for 3-8 hours, to allow the dye to migrate to the antennal lobe glomeruli.

**2.2 Apparatus**

**Odour stimulation (Behaviour)**. The stimulation device used in the experiments allowed single odorant and odour mixture presentations to individual bees (for details see Pelz et al. 1997; Gerber & Menzel 2000). A constant air stream was produced by an air pump (Rena Air 400, Annecy, France) and was divided into 8 identical channels which entered the stimulation device. Each single odour source was contained in a small plastic syringe (0.7 ml, see below for preparation) which was each inserted into one channel of the stimulation device. Thus, the stimulation device contained eight syringes each connected to a two-way electronic valve (LEE COMPANY S.A., Voisins-le-Bretonneux, France) which was controlled by the experimenter via a computer (**Fig. 2.3**). When activated, each valve directed its air stream through the syringe (containing an odour) into a mixing chamber and from there to the exit directed to the animal (**Fig. 2.3**, right part, green flash). When not activated, each valve directed the air stream to an outlet bypassing the bee to an air exhaust (**Fig. 2.3**, right part, blue flash). This allowed permanently extracting possible odour contaminations away from the system. Mixtures were presented when the valves corresponding to two odours were activated at the same time. A single odour was presented when the corresponding valve and a second empty one (i.e. which was connected to a syringe containing only a clean piece of filter paper) were simultaneously opened. Thus, air flow intensity was identical during mixture and single odour presentations. When no odour was presented, one valve was permanently active, directing a clean air stream to the bee. A permanent air exhaust placed behind the animal removed remaining odours from the experimental room.

**Odour stimulation (calcium imaging)**. The device used for odour stimulation during the recordings of neuronal activity in the antennal lobes consisted of a glass pipette connected to a silicon tube attached to an air pump (Rena Air 400, Annecy, France) providing a constant air stream. The orifice of a small glass tube was pointing towards the movable parts of the antennae of the bee in its recording chamber under the microscope. A small hole in the glass tube allowed placing the ends of two disposable Pasteur pipettes. These pipettes were each connected to computer-controlled two-way electronic valves.

During odour presentations, part of the permanent airflow flowed in equal amount through the two pipettes. For single odour presentations, one glass pipette contained a piece of filter paper saturated with an odour and one Pipette containing only filter paper. For mixture presentations, two odour-containing pipettes were used (**Fig. 2.4**). As for conditioning, this system allowed the use of a constant air stream without remarkable changes in the strength of the airflow during odour presentation. Just behind the animals an air extractor was installed to avoid odour contamination.

**Optical imaging system.** Recordings were done with a T.I.L.L. photonics imaging system and an upright fluorescence microscope with a water immersion objective (UMPlanFL Olympus) with a magnification of 10x (numerical aperture: 0,3). A monochromator allowed producing light at a precise wavelength which was projected through the microscope onto the preparation below the objective lens. The reflection was filtered by a dichromatic mirror which allowed passage of all wavelengths above 410nm (the fluorescent emission of the excited calcium dye has a peak wavelength of ~510nm). To further restrict the reflected light, it passed another filter allowing only passage of wavelengths above 440nm. The emitted light of the dye which passed all filters was captured by a 12bit IMAGO camera (T.I.L.L. photonics) with a horizontal and vertical “binning” of 2. The “binning” process groups photon events from 2 x 2 pixels in order to reduce the necessary excitation time, thus allowing more rapid measurements and less photodamage of the preparation. This is however at the cost of image resolution which is reduced by 2 in each dimension.

For each measurement, a series of 100 double frames was acquired with a frequency of 5 Hz (200 ms interval). Each time, one frame was first acquired with monochromator excitation at 340nm, and then a second frame was acquired with 380nm excitation (see **Calcium-sensitive dye**). Odour presentation was given between double frames 15 and 19 (i.e. for 1 sec). The exposure time for stimulations was determined individually for each preparation in order to use a fluorescence index most efficiently adapted to the camera (about 3000 from a maximum

4096 grey levels). The mean exposure time with light of 340nm wavelength was 95ms and with light of 380nm wavelength it was 25ms.

**Calcium-sensitive dye.** The calcium sensitive dye Fura-2-dextran is a UV light-excitable, ratiometric $Ca^{2+}$ indicator. When fura-2 binds with intracellular $Ca^{2+}$ a shift in light absorption of this molecule can be seen (using exciting light with a wavelength from 300nm to 400nm), which results in a quantitative change of emitted light with a wavelength of 510nm. The maximum of excitation changes from 363nm when no $Ca^{2+}$ is present to 335nm when $Ca^{2+}$ is attached to the dye. A maximum of variation of excitation dependent on the $Ca^{2+}$ level was found between 340nm and 380nm. While rising $Ca^{2+}$ levels lead to rising light emission for excitation with 340nm, the light emission falls for excitation with 380nm (**Fig. 2.5**). The ratio between emission levels obtained for excitation with 340nm and with 380nm gives a good estimate of intracellular $Ca^{2+}$ levels. Because of the dextran group, Fura-2 dextran is a cell-impermeable dye which uses cellular machinery to be actively transported within neurons. It must however be delivered by microinjection directly into the cells or by damaging neurites

Dye crystals were dissolved on a cover slip in a small drop of 2% Bovine serum albumine which was allowed to dry, forming a gel that is easier to use than dye crystals. Before the injections, microelectrodes were scraped carefully on this medium to gather some dye at their tip.

**Injection.** The calcium sensitive dye (Fura-2-dextran) was applied to the lateral tract of the projection neurons (lateral antenno-cerebral tract, l-ACT). The l-ACT contains projection neurons which have their somata anteriorly in the dorsal and lateral region of the antennal lobe. Their dendrites have uniglomerular arborizations in the most frontal antennal lobe glomeruli (belonging to the T1 tract of olfactory sensory neurons) and their axons leave the antennal lobe at its ventromedial border. From the antennal lobe, the l-ACT projects laterally sending collaterals to the protocerebral lobe and continues between the α-lobe (αL) and the optic lobes (OLs), to the mushroom bodies. Multiple injections with Fura-2-dextran loaded glass micro electrodes were done bilaterally, on each side between the α-lobe and the optic lobes. Injection sites were marked with an X in **Fig. 2.2**. By injecting the dye into the tissue using glass electrodes, the axons of the l-ACT projection neurons were severed and by small rotations of the electrodes the dye was released at the injection site. As indicated above, migration during 3 to 8 hours allowed backfilled the projection neurons, so that we could measure changes in $Ca^{2+}$ levels in the glomeruli of the antennal lobe during odour stimulation.

### 2.3 Stimuli

The 2 odours used in our work were obtained from Sigma Aldrich (Deisenhofen, Germany) and were taken from the chemical groups of aldehydes (hexanal) and secondary alcohols (2-hexanol). The selected odours are usually present in floral volatile emissions (Knudsen et al. 1993). Previous experiments showed that responses to both odours in extinction tests after three-trial PER conditioning were equally high. Additionally, generalization between these odours was asymmetrical, generalization being stronger to hexanal after 2-hexanol training than the other way around. Therefore, according to the overshadowing model we developed in previous work (Chapter II) we expected dominance of hexanal when presented in a mixture with 2-hexanol, i.e. an OVS effect of hexanal over 2-hexanol (see **1. Introduction** and **2.4 Experimental design**). In both behavioural and imaging experiments, pure odours were used. To prepare an odour source, 4 µl of odour were applied onto a 1cm$^2$ piece of filter paper which was inserted into a 1 ml syringe, cut to 0.7 ml to make it fit into the odour stimulation device. As US, a 50 % w/w sucrose solution was used throughout.

### 2.4 Experimental design

The objectives of this work were: i) to evaluate possible learning-induced changes in calcium responses of projection neurons, and ii) to understand on the basis of glomerular activity patterns the occurrence of overshadowing. Overall, 3 different groups of bees were used, conditioned to hexanal (n = 123), 2-hexanol (n = 124) and their binary mixture (mix, n = 122), respectively. A fourth group was formed, which was treated exactly like the others but did not receive any conditioning. This group of naïve bees served as control in the imaging experiments (n = 4). Compared to our previous study on overshadowing (Schubert et al. Chapter II), the overshadowing group (OVS group) corresponds to the bees conditioned to the mixture hexanal/2-hexanol and the two control groups (Ctrl A and Ctrl B) to the bees conditioned to hexanal or 2-hexanol.

**Conditioning phase** Bees received three conditioning trials with 10 min inter-trial intervals. For each conditioning trial, one bee at a time was placed in front of the odour device. The total duration of each trial was 33 sec. After 10 sec of familiarisation to the experimental context, the odour CS was presented to the bee for 4 sec. Three seconds after CS onset, the antennae were stimulated with the US, leading to a proboscis extension. The bee was allowed to feed from the sucrose for 3 sec. The bee was then left in the same position for 17 sec until it was replaced by the next animal.

**Test phase of the behavioural experiments**. In all conditioned groups, 24 hours after the conditioning phase, bees were subjected to a test phase, during which the two odour components (hexanal and 2-hexanol) and the compound (hexanal/2-hexanol) were presented to the bees. A test trial was identical to a conditioning trial, except that each odour stimulus was presented for 4 sec without any US stimulation. The 3 tests were carried out in a randomised order with an ITI of 10 minutes. After the test phase, bees were stimulated with the US on the antennae to check their motivation to respond. Bees that did not respond at this stage were considered unmotivated and were discarded (< 15%).

**Test phase of the imaging experiments**. A few bees from the different groups (conditioned and naïve) were subjected on the next day to a test phase under imaging conditions [conditioned to hexanal (n = 4), to 2-hexanol (n = 4) and to the mix (n = 4) and naïve bees (n = 4)]. Due to the long duration of imaging experiments, the actual odour stimulations ranged from 26h to 32h after conditioning. After fixation, preparation and staining, the animals were placed under the microscope in front of the odour delivery system. All animals were tested 3 times each with hexanal, 2-hexanol, the binary mixture and a control air puff. Each stimulus was presented for 1 sec under 5Hz light stimulation schedule of the imaging recordings (see calcium imaging), without any stimulation with the US. These tests were carried out in a randomised order with an ITI of 1 minute.

### 2.5 Data analysis and statistics

**Behaviour**. Throughout the experiments, we recorded bees' behavioural responses to the odours. In the case of reinforced trials, a response was recorded whenever bees extended their proboscis after odour onset and before the presentation of sucrose solution (US). All through the test trials, a response was recorded only if it appeared during odour stimulation. Multiple responses during a CS were counted as a single PER. During conditioning, we also recorded whether bees responded to the US presentation to the antennae by a clear PER. When a bee showed no reaction to the US in any of the conditioning trials, this bee was considered as not sufficiently motivated for conditioning, and was discarded from further analysis. This happened in less than 3% of the cases. Acquisition performances were analysed using Cochran's Q test. Differences between groups at the 3$^{rd}$ trial acquisition trial were tested using $\chi^2$-tests. Bees which were dead (<1%) or which did not respond to the US after the test phase (<15%) were discarded, since such bees' lack of response during the tests could not be unambiguously attributed to a lack of learning or of generalisation. Test performances were

compared by $\chi^2$-tests (between-group comparisons) and Mc Nemar tests (within-group comparisons).

**Calcium imaging**. The data (emitted light) captured by the digital camera were stored by a TILL Photonics Imaging System Software (TILLvisION v4.01) and analysed with custom software (initially developed by Sachse and Galizia, 2002) written in IDL (Interactive Data Languages, Boulder, USA). First, to reduce photon (shot) noise, raw data at each wavelength were filtered in the spatial and in the temporal dimension using a median filter with a size of 5 pixels. Then, we applied a bleaching correction separately for the recordings at each wavelength, by subtracting from each pixel in each frame, the median of all the pixels of that frame. We then calculated at each pixel and at each frame the ratio of 340 and 380nm data, which was multiplied by 1000. Odour-induced activity maps were thus calculated as:

$$\Delta(F340/F380) = \left( \frac{F340_{17\text{-}19}}{F380_{17\text{-}19}} - \frac{F340_{12\text{-}14}}{F380_{12\text{-}14}} \right) \times 1000$$

This equation gives the change in the 340/380nm fluorescence ratio observed between just before the stimulus (frames 12 to 14) and at the moment when the amplitude of calcium signals is usually maximum after odor onset (frames 17 to 19, Sachse and Galizia 2003). To study the neural representation of the odorants, we related odour-induced neuronal activity to identified glomeruli by performing anatomical staining and glomerular reconstructions (see below). For the analyses, the activity from individual glomeruli was taken as an area of 9 x 9 pixels for each glomerulus. Thus, glomerular activity was always measured over the same area, independently of individual differences in glomerular size. For each active zone the amplitude of the change in fluorescence ratio (340/380nm) during each odour presentation was calculated by the equation:

$$A = \frac{F340_{(17\text{-}19)}}{F380_{(17\text{-}19)}} - \frac{F340_{(12\text{-}14)}}{F380_{(12\text{-}14)}}$$

To reduce between-stimulation variations, the mean amplitude of the three stimulations carried out with the same odour were used in the analyses as the response of each glomerulus to each odour.

**Anatomy (glomeruli) staining**. To relate odour-induce activity to identified antennal lobe glomeruli in each bee, we performed anatomical staining after the physiological recordings.

Thus, 20µl of a 125:1 mixture of liquid protease (from *Bacillus licheniformis*) and the fluorochrome RH 795 were applied onto the brain. After 1 to 3 hours, the protease had digested membranes and trachea on the AL, and the RH795 dye stained cell membranes of the glomeruli, revealing the glomerular structure of the AL. Images were done with light excitation between 500 and 550nm, with a 570nm dichroic filter and a long-pass 590nm emission filter. Then, a number of glomeruli were recognised in each bee, using the standard digital atlas of the honeybee AL (Galizia et al 1999a). Overall, 19 glomeruli belonging to the T1 region were recognised in all individuals and were taken in the analysis. Some additional glomeruli were recognised in only a few individuals and were therefore not included.

**Data normalisation.** As relatively strong heterogeneities appeared in the general response amplitudes of individual bees – which cannot be ascribed to learning - glomerulus responses were normalized within each animal to the strongest glomerulus response measured (100%) to all odours. Thus, each glomerular odour response is expressed as a proportion of the most active glomerulus in this bee's antennal lobe.

**Glomerular activation**. To study whether learning modifies PN activity, possibly leading to changes in odour representation, we first calculated the mean activation over the 19 glomeruli under investigation. Mean activation of different conditioning groups were compared by repeated measurements analysis of variance (ANOVA), with *conditioning group* as an independent factor and *test odour* as a repeated measurement factor. We specifically looked for interactions between the two factors, which would indicate that overall calcium signal intensity for the different tested odours is modified differentially by conditioning to different odours.

**Similarity of odour representations**. To investigate if conditioning modifies glomerular representation of odours so that they become more specific after training, we compared the similarity between activation patterns for the single odours and the mixture in the different groups. For this, we calculated Euclidian distances between patterns, using each glomerulus activation as a coordinate in a multi-dimensional space:

$d_{ij} = \sqrt{\sum_{k=1}^{p} (X_{ik} - X_{jk})^2}$ **,** with $i$ and $j$ indicating odours, $p$ the number of dimensions, i.e. glomeruli, and $X_{ik}$ the response in glomerulus $k$ to odour $i$.

Repeated measurement ANOVA were performed to see if the different conditioning groups showed heterogeneities in the similarity between glomerular odour representations (independent factor: *conditioning group*) and to see if similarity between odour pairs (2-

hexanol – mixture, hexanal - mixture and 2-hexanol – hexanal) differed (repeated measurement factor: *odour pair*). Scheffé post hoc comparisons were used to further investigate significance found by the ANOVAs

**Specific changes in CS glomeruli**. To detect changes in the relative contribution of individual glomeruli to AL activity, predominantly in the glomeruli activated by the CS during conditioning, we compared mean global responses (mean response to all tested odours) of the 19 glomeruli in each conditioning group with the mean global responses in naïve bees. Changes in each glomerulus' global response were then compared to the glomerular activation to the CS odour (2-hexanol, hexanal or the mixture in the three conditioning groups). To evaluate global odour response changes between conditioned and naïve bees as a function of the contribution of the glomeruli in the pattern of the CS, we carried out correlation analyses, and used the Pearson correlation coefficient to check for statistical significance.

**Overshadowing based on imaging signals**. We then evaluated if overshadowing can be explained by a simple model based on neural distances in the bee's putative olfactory space based on the 19 recorded glomeruli. We thus compared the 2-hexanol-mixture distance (d1) to the hexanal – mixture distance (d2). We also wanted to evaluate the relative contribution of each of the 19 glomeruli to these distances, to see if activity from a subset of the recorded glomeruli could provide a better prediction for the overshadowing effect. For this, we calculated the contribution of each glomerulus to each distance as $(A_{2\text{-hexanol}} - A_{mixture})^2$ or $(A_{hexanal} - A_{mixture})^2$ where $A_X$ is the amplitude of the glomerulus to odour X. We then subtracted one from the other $[(A_{2\text{-hexanol}} - A_{mixture})^2 - (A_{hexanal} - A_{mixture})^2)]$ to evaluate the relative contribution of this glomerulus to the squared distances between each odour and the mixture. If this value was positive, it meant that this glomerulus tended to increase the 2-hexanol-mixture distance more than the hexanal-mixture, i.e. making the mixture representation more similar to hexanal than to 2-hexanol.

# 3. Results

## 3.1 Conditioning

Bees learned odorants and significantly increased their responses to 2-hexanol, hexanal and the binary mixture during conditioning (**Fig. 3.1A**, black lines, Cochran's Q test, $P < 0.0001$ in all 3 cases). Conditioning success differed between odorants ($\chi^2$ test, $\chi^2 = 33.88$; df: 2; $P < 0.001$), hexanal and the mixture eliciting more responses than 2-hexanol at the third conditioning trial ($\chi^2$ test, hexanal – 2-hexanol: $\chi^2 = 27.55$; df: 1; $P < 0.001$; hexanal – mix: $\chi^2 = 2.18$; df: 1; $P = 0.14$; 2-hexanol – mix: $\chi^2 = 16.19$; df: 1; $P < 0.001$).

To determine the occurrence of overshadowing, we analyzed the performances of bees conditioned to the binary mixture when tested with the single odours and the mixture (Fig.3.1B, upper part). Bees responded significantly more to hexanal than to 2-hexanol (Mc Nemar test, $P < 0.005$) and responses to the single odours were both significantly lower than the responses to the conditioned mixture (Mc Nemar test, $P < 0.005$). Therefore, hexanal was the dominant odour in the mixture and overshadowed 2-hexanol. In a previous study, we found that two different component effects could explain overshadowing, i) different acquisition levels, and ii) cross-generalization asymmetries (see Chapter II). We thus first analyzed the test performances of bees conditioned to the single odours. In contrast to the different response levels reached at the third conditioning trial (see above), test responses to the different CSs 24h after conditioning showed similar response levels ($\chi^2$ test, $\chi^2 = 1.18$; df: 1; $P = 0.278$) (**Fig. 3.1B**, middle and lower part). The same pattern was already found in a previous study for these two odours tested 10 min after the last conditioning trial (Guerrieri et al. 2005) which shows, that at the time of CS testing both odours were equally well learned. This is probably the effect of the third odour-sucrose association which led to equal responding in the tests. Therefore, different conditioning levels for the single odours could not account for the overshadowing effect found after mixture training. We thus evaluated cross-generalization asymmetries. After hexanal conditioning the generalization responses to 2-hexanol were low compared to the CS responses (Mc Nemar test, $P < 0.001$), but after 2-hexanol conditioning, generalization responses to hexanal did not differ from CS responses (Mc Nemar test, $P = 0.383$). Thus, cross-generalization between the two odours appears asymmetrical, as bees responded more to hexanal after learning 2-hexanol, than vice versa. This observation is perfectly in line with the generalization asymmetry found between these two odours in our previous work (Chapter I, Guerrieri, Schubert et al. 2005). To provide a more controlled measure of asymmetrical cross-generalization, only generalization responses

of those bees that learned to respond to their respective CS were considered (see Chapter II for detailed explanation). After hexanal conditioning, 36 bees responded to the CS but not to 2-hexanol, whereas 22 bees responded to both odours. After 2-hexanol conditioning, 13 bees responded to the CS but not to hexanal, whereas, 37 bees responded to both. These frequencies revealed a significant cross-generalization asymmetry in which hexanal received more generalization ($\chi^2$ test, $\chi^2 = 14.09$; df: 1; $P < 0.001$). We conclude that in the binary mixture, hexanal overshadows 2-hexanol, and that this effect correlates to a higher salience of hexanal compared to 2-hexanol, as observed in particular by the analysis of cross-generalization asymmetry.

### 3.2 Imaging

We performed imaging experiments to evaluate i) the possibility that conditioning modifies response patterns in the bee antennal lobe, and ii) to find a neural correlate for the overshadowing effect and cross-generalization asymmetry. For these imaging experiments, only bees which responded to the CS from the second conditioning trial on were prepared for imaging experiments, to insure that they had learned the CS. These bees were not subjected to any behavioural tests to avoid extinction effects, but were instead prepared for the imaging 24 h after conditioning (hexanal: 27 from 123; 2-hexanol: 29 from 124; mixture: 23 from 122). From these bees 4 animals in each group (4 bees conditioned to hexanal, 2-hexanol and the mixture) and 4 naïve bees (in total n = 16) provided good quality recordings, and were used for analysis. In all bees, odour stimulations induced reproducible activity in a combination of AL glomeruli, as observed in other studies (Sachse et al. 1999; Sachse & Galizia 2002) while air stimulation seldom showed any activity. Odour-induced activity was either excitatory, calcium levels increasing during odour presentation, or inhibitory, calcium levels decreasing shortly, before returning to baseline (data not shown). The activation patterns were visually similar in the different conditioning groups and did not differ from the patterns found in naïve bees. The patterns found for hexanal and 2-hexanol were relatively similar as supported by previous data (Ditzen 2005). Mixture response patterns appeared to be similar to sums of the responses to the single odours (**Fig. 3.2**). After anatomical staining of each individual lobe, we were able to identify the same 19 glomeruli in all animals, and calculated the amplitude of the calcium response in each glomerulus for each odour.

### 3.2.1 Does learning modify odour representation?

*Glomerular activation*

We first asked whether conditioning modifies the intensity of odour representation at the level of the projection neurons of the bee antennal lobe. Such an effect was observed using a bath-applied calcium dye in previous work (Faber et al. 1999). **Fig. 3.3** shows the mean response amplitude of the 19 glomeruli for each odour in the different conditioning groups and the control group (n = 4 in each group). Using the raw data (**Fig. 3.3A**), a two-way ANOVA (training vs. test odour - repeated measure) yielded a non-significant training effect ($F_{3,12} = 0.69$, $p = 0.58$), a significant test odour effect ($F_{3,36} = 12.17$, $p < 0.001$) and most importantly no interaction ($F_{9,36} = 0.79$, $p = 0.63$). Therefore, training did not modify responding, neither in a general way (training effect term) nor in a differential way for the different odours (interaction term). As relatively strong heterogeneities appeared in the response amplitudes of individual bees – which cannot be ascribed to learning (see analysis above), further analysis was carried out on normalized data (see materials and methods, and Sachse & Galizia 2003). For this, glomerulus responses were normalized within each animal to the strongest glomerulus response (100%) to all odours. The effect of the normalization – less heterogeneity between groups - can be seen in **Fig. 3.3B**. The same analysis as above with the relative data also yielded a non-significant training effect ($F_{3,12} = 0.39$, $p = 0.76$), a significant test odour effect ($F_{3,36} = 22.76$, $p < 0.001$) and no interaction between effects ($F_{9,36} = 0.94$, $p = 0.50$). Thus, analyses using the mean activation of all glomeruli lead to the conclusion that calcium signal amplitudes do not show any significant differences when the animals were conditioned to different odours or were naive. Amplitudes were higher when tested with an odour compared to stimulation with the air control (Scheffé test, in all cases $p < 0.001$), but no difference between the single odours and the mixture were found (Scheffé test: hexanal – 2-hexanol, $p = 0.968$, NS; hexanal - mix, $p = 0.307$, NS; 2-hexanol – mix, $p = 0.135$, NS).

*Similarity of odour representations*

To detect more specific differences in glomerular response to the two odours and their mixture among conditioning groups, we focused on glomerular activity. **Fig. 3.4** and **3.5** show the normalized response of each glomerulus to all test odours for each conditioning group and for naïve bees. Over all conditioning groups, glomeruli did not respond to the air control (**Fig. 3.4A**), only glomeruli 28 and 37 showed some spontaneous activity during air control presentations. Comparing glomerular responses for 2-hexanol (**Fig. 3.4B**) and hexanal (**Fig.**

**3.5A**) showed several common glomeruli which were activated (e.g. 28, 33, 36), with only a few glomeruli (e.g. glomeruli 17, 37 and 60) exhibiting differences in response to the two odours. This underlines the similarity between the two single odours. The responses to the mixture (**Fig. 3.5B**) display the same active glomeruli as response patterns for 2-hexanol and/or hexanal and, therefore, are highly additive.

Then, we measured similarity between glomerular response patterns among the different conditioning groups by calculating Euclidian distances. For this, we used responses of the glomeruli as coordinates in a multi-dimensional space defining a point for each odour representation. The distances calculated between the points obtained for hexanal, 2-hexanol and the mixture thus represent how different these odours are to each other based on the recorded glomeruli (**Fig. 3.6**). An ANOVA (training x odour pair - repeated measure) showed no significant training effect ($F_{3,12} = 0.92$, $p = 0.46$) indicating that conditioning to different odours did not change distances between odour representations. Nevertheless, a significant odour pair effect ($F_{2,24} = 6.10$, $p < 0.01$) was found. Scheffé post hoc comparisons showed that the 2-hexanol – mixture distance (d1, mean over conditioning groups: 84.27) did not differ significantly from the hexanal – mixture distance (d2, mean over conditioning groups: 110.85) (Scheffé test, $p = 0.081$, NS), but from the distance 2-hexanol – hexanal (d3, mean over conditioning groups: 122.59) (Scheffé test, $p < 0.05$), whereas, the distance hexanal – mixture (d2) did not differ from 2-hexanol – hexanal (d3) (Scheffé test, $p = 0.586$, NS). From this, one could indirectly see that, although not significant, the distance 2-hexanol – mix (d1) was slightly shorter than the distance hexanal – mix (d2, see the triangle in **Fig. 3.6**). No interaction between effects was found ($F_{6,24} = 0.30$, $p = 0.93$) underlining that conditioning did not modify distances differentially for the different odours (i.e. like changing the shape of the triangle in **Fig. 3.6**).

*Specific changes in CS glomeruli*

Although we did not find any conditioning effects on response intensity or on odour similarity, we wanted to check the possibility that subtle changes could take place within a subset of glomeruli corresponding to the learned odour. For this we can use a prediction based on the results of a previous study: if conditioning induces higher calcium responses in the glomeruli activated by the CS, as found based on a compound calcium signal by Faber et al. (1999), we could expect that the change in responsiveness of these glomeruli would be observed for any odour activating this glomerulus. Thus, we would expect that the relative contribution of these glomeruli to odour responses over all presented odours would increase in

conditioned bees compared to naïve bees. To evaluate this possibility, **Fig. 3.7** depicts the mean global responses (mean responses to all odours) of each of the 19 glomeruli in bees conditioned to each CS (A, 2-hexanol, B, hexanal, C, mixture) and in naïve bees (**Fig. 3.7** dark blue and light blue bars respectively). For comparison, the activation in naïve bees of each of these glomeruli to the learned odour is given (dark red bars). We take here for comparison the responses of naïve bees, because they would represent the odour response pattern in the antennal lobe of bees when starting conditioning with each odorant (i.e. before conditioning, the bees are naïve). Comparing global glomerular responses for example in 2-hexanol-conditioned bees and in naïve bees (**Fig. 3.7A**, dark blue and light blue columns, respectively) shows increased responses for conditioned bees in some glomeruli (e.g. glomerulus 28, 36 and 52) but also decreases in others (e.g. glomerulus 43, 24 and 48). Comparing these changes in global responses with glomerular responses to 2-hexanol in naïve bees (**Fig. 3.7A**, dark red columns) does not show any clear relationship between the two variables: while glomeruli 43 and 52 were very important for representing the 2-hexanol odour, one increased its response to odours (glomerulus 52) and the other decreased it (glomerulus 43) after conditioning. Similarly, glomeruli 28, 33, 36 and 48 were also activated by 2-hexanol in naïve bees, but conditioning showed multiple effects, from no effect (glomeruli 33 and 48) to an increase (glomeruli 28 and 36). A similar pattern was found for the other conditioning groups (**Fig. 3.7B** and **C**). To represent global odour response changes between conditioned and naïve bees as a function of the contribution of the glomeruli in the pattern of the CS, we carried out correlation analyses (**Fig. 3.8**). For the 2-hexanol and mixture conditioning groups, data appeared as a cloud, and no significant correlations were found ($r < 0.27$; df: 17). Only the hexanal conditioned group (**Fig. 3.8B**) showed a significant effect in which low active or inhibited glomeruli of naïve bees in response to the CS (abscissa) increased their responses after conditioning and high active glomeruli decreased it (ordinate) ($r = 0.67$; df: 17). Therefore, it is not possible to predict general increase or decrease in responses of active glomeruli in all conditioning groups, based on the pattern of the learned odour.

### 3.2.2. Can we explain overshadowing based on imaging signals?

Our analyses aimed at identifying changes in the responses of projection neurons to odours after appetitive conditioning have not uncovered any significant changes. It is therefore not possible to explain generalization asymmetries based on the model proposed in the introduction (learning-induced asymmetry in odour representations). Overshadowing,

although it seems to be strongly related to generalization asymmetries (Chapter II), could nevertheless be explained by a simple model based on distances in the putative neural space, without any learning-induced changes: as behavioural generalization is correlated to similarity in glomerular patterns (Chapter I, Guerrieri, Schubert et al. 2005), we could explain overshadowing of hexanal over 2-hexanol if the distance between hexanal and mixture representations (**Fig. 3.6**, d2) was shorter than the distance between 2-hexanol and mixture representations (**Fig. 3.6**, d1). Bees would respond more to hexanal after mixture training, simply because the two neural representations are more similar to each other. We found exactly the opposing trend, suggesting that such a simple model cannot explain overshadowing. However, by calculating Euclidian distances using all glomeruli, we give each glomerulus a similar weight for calculating odour similarity. We do not know, however, how projection neuron information is read out by downstream neurons, i.e. by Kenyon cells. Some input channels may have more impact than others. Thus, we asked what the relative contribution of each of the 19 glomeruli is on the similarity between single odour and mixture representations (**Fig. 3.9**, n = 16 bees). In fact, in most glomeruli (**Fig. 3.9A**), calcium responses were more different between hexanal and the mixture, than between 2-hexanol and the mixture. And therefore, most glomeruli contribute to increasing the distance between hexanal and the mixture (**Fig. 3.9B**, bars going down), and only a few do the contrary (bar going up: glomerulus 43, and to a lower extent glomeruli 42, 49 and 38). Therefore, even if a measure of odour similarity by the brain was based on only a subset of the AL glomeruli recorded in this work, it is very unlikely that such calculation would make hexanal more similar to the mixture than 2-hexanol. We therefore conclude that measures of similarity based on the glomeruli recorded in this work cannot explain the overshadowing effect.

# 4. Discussion

The learning phenomenon of overshadowing is generally seen as a rather cognitive process, because most of the early (Pavlov 1927) and later (Mackintosh 1971; Kehoe 1982; Couvillon & Bitterman 1980; Pelz et al. 1997, Brembs & Heisenberg 2001) overshadowing experiments were done on compound stimuli with components belonging to different sensory modalities (e.g. colours, achromatic patterns, tones, light and odours). As a consequence, overshadowing should take place in integration brain structures, after convergence of the different sensory pathways, rather than in sensory structures of the brain. But what about rarely shown intra-modal overshadowing effects (Derby et al. 1996; Smith 1998 and Chapter II of this work)? In intra-modal mixtures integration could take place already in sensory structures, as the olfactory bulb of vertebrates or the antennal lobe of invertebrates for the olfactory modality. Investigations in honeybees (Faber et al. 1999, Sandoz et al. 2003) showed that odour-evoked glomerular activation patterns in the antennal lobe may undergo certain plasticity after conditioning. It is further known that the AL network contains inhibitory connections (Sachse and Galizia 2002) which could channel such plasticity (Linster and Smith 1997). Both discoveries, an activation intensity increase in a sub-set of glomeruli after odour conditioning by Faber et al. (1999) and the existence of a heterogeneous inhibitory system in the AL network (asymmetrical inhibition between glomeruli) by Sachse and Galizia (2002) give reason to believe that odour pattern characteristics could produce after conditioning the neuronal correlate of overshadowing and/or asymmetric generalisation phenomena (see introduction).

Plasticity after conditioning could for instance accentuate specific inhibitory connections between glomeruli and, therefore, produce a different weighting of inhibitory connections between specific glomeruli when conditioning to different odours. Depending on the connectivity between active glomeruli, odour representations would thus change in a different way after conditioning to different odours. Such changes, taking place in a rather sensory structure, the AL, could easily explain the asymmetric generalisation and overshadowing phenomena observed behaviourally. To investigate this hypothesis, we tried to discover if odour experience could induce such physiological changes in the neuronal AL network of the honeybee.

The activation patterns of PNs for the odours used in our experiments in unconditioned honeybees have similar characteristics as the patterns found by others (Sachse & Galizia 2002; Ditzen 2005) but also significant differences. For example, the typical

activation pattern found for the odour 2-hexanol by the group of Prof. Galizia in the lab of Prof. Menzel at the "Freie Universität Berlin", Germany, includes mostly activation of glo28 followed by glo36, glo38, glo60 and glo52. With the exception of glo38 our imaging experiments carried out in the lab of Prof. Giurfa at the "Centre de recherches sur la cognition animal", Toulouse, France, resulted in the activation of the same glomeruli, but with different relative intensities. The pattern found in this work was dominated by activation of glo52 followed by glo48, glo43 and glo60. Glomeruli 28 and 36 were also active but were not as dominant as in the pattern obtained in Berlin. These differences could be due to several reasons. First of all, the honeybees under investigation in the two laboratories belong to two different subspecies: *Apis mellifera mellifera* (Toulouse, France) and *Apis mellifera carnica* (Berlin, Germany). Different subspecies are likely to show slight neuroanatomical and/or functional differences in their olfactory system. Second, the average age of the used animals could have been different in the two cases. It is well known that the olfactory system of bees maturates until 4-8 days after emergence, as shown electrophysiologically (electroantennograms, Masson & Arnold 1984; Allan et al. 1987). Recently, age has been shown to affect odour-evoked responses in calcium imaging experiments until 2 weeks after emergence, which could well explain differences in glomerular activations found in different groups (Wang et al. 2005). Third, the individual experience of bees at different periods of the year, and/or in two very different foraging environments (the south of France and the north of Germany) could also be responsible for different weighting of synapses between receptor-, excitatory and inhibitory inter- and projection-neurons, which would result in different response patterns at the lobe's output. In the light of our results, a rather short conditioning experience (here three conditioning trial) would not be enough to shape odour-evoked responses, but such an argument could be invoked for bees developing in hives containing different stored food and/or foraging for long periods of time in very different environments.

The odours used in this work were chosen on the basis of the results found in Chapter I and Chapter II. In Chapter I we found equal acquisition success in 3-trial PER conditioning for 2-hexanol and hexanal, and equal CS responses in unrewarded tests 10 min after training. However, these two odours had the property of showing asymmetric cross-generalization (higher generalization responses to hexanal after 2-hexanol conditioning than vice versa). In Chapter II we showed that asymmetric cross-generalization is a good indicator for overshadowing effects. It turned out in the present Chapter that the overshadowing effect predicted from the asymmetric cross-generalization between 2-hexanol and hexanal indeed

occurred in our behavioural experiment. This result thus confirms that asymmetric cross-generalization is a reliable predictor for the overshadowing effects. Moreover, the chosen odours, 2-hexanol and hexanal, produce very similar glomerular activation patterns in naïve bees as shown in imaging experiments in the antennal lobe (Sachse et al. 1999; Ditzen 2005). We hypothesized that due to such similar patterns, changes to odour-evoked responses and patterns after conditioning should be maximal.

We failed however to identify reliable changes to AL activity after conditioning. In early imaging experiments using a bath application of the calcium dye, Faber et al. (1999) showed that 10-30 min after differential PER conditioning (one odour rewarded and one odour unrewarded), odour experience did change glomerular activation intensity and reduced the correlation between the patterns obtained for the CS+ and the CS-. Later, Sandoz et al. (2003) used a side-specific conditioning procedure to compare the effect of two simultaneous differential conditioning tasks performed on each antenna. (A+B- on one side, B+A- on the other side). They found that 24 h after training, subtle changes in odour-induced patterns for the learned odours induced a decorrelation of the odour-evoked patterns between brain sides. In other words, differential conditioning made the representation of odour A on the left side different from that of odour A on the right side. Control odours were not affected. These works thus suggested that at different periods after conditioning and/or using different learning tasks could bring different kinds of changes in odour-evoked activity in the AL, either on signal intensity (Faber et al. 1999), or on the relative activity of the different glomeruli (Sandoz et al. 2003). In our work however, comparing intensities and odour similarity measures between three conditioning groups and the naïve group after 24h showed no significant differences, which could suggest that changes found in these two different kinds of differential conditioning experiments (Faber et al. 1999, Sandoz et al. 2003) did not occur in absolute conditioning. Absolute and differential conditioning procedures are thought to correspond to two different kinds of learning, because in contrast to absolute conditioning, in which a single CS is paired with a positive or negative US (Gallistel 1990), differential conditioning includes a CS+ paired with a positive US and a second CS- (differential inhibition) paired with a negative US (Domjan & Burkhard 1986; Mackintosh 1994) which could be a reason for the different results obtained in our work and other studies.

However, a more likely explanation to such differences relates to differences in the neural populations imaged in the different studies. Experiments using a bath-applied calcium dye reveal activity from all possibly stained cells: olfactory sensory neurons, local interneurons, projection neurons and even glial cells, although it is thought that these signals

mostly reflect OSN activity (Galizia et al. 1998, Sachse and Galizia, 2003). By contrast, we measured activity from a subpopulation of AL neurons. Using a specific staining method with the Fura-dextran dye, we recorded activity from uniglomerular projection neurons belonging to the l-ACT axonal tract. Using exactly the same staining method, (Peele 2005) showed very recently that these neurons, at least within 15 min after training, are highly plasticity-resistant, both after differential (using 3- and 5-trial conditioning designs) and after absolute appetitive training (using 1- and 3-trial conditioning designs). Although our data is still limited in terms of numbers of animals, our results seem to extend this finding at longer stages (24 h after training). It must be noted that this result does not mean that olfactory information relayed to brain integration centres like the mushroom bodies or the lateral protocerebral lobe is not plastic. As honeybee PNs constitute a heterogeneous group of uniglomerular m-, l-ACT and both uniglomerular and pluriglomerular ml-ACT neurons, plasticity could still be promoted by m- and/or ml-ACT neurons, which have not been imaged yet (Peele et al. 2006).

**Neural correlates of overshadowing and asymmetrical cross-generalisation**

To find a neuronal correlate for overshadowing, we investigated odour similarity measuring Euclidian distances between activation patterns for single odours and the mixture (2-hexanol – mixture and hexanal – mixture) in mixture-conditioned animals. If the activation pattern of hexanal was more similar to the mixture pattern than the 2-hexanol pattern, one would understand why the animals respond more to hexanal than to 2-hexanol after mixture training (overshadowing effect), because then the animals would simply make their decision based on the similarity between odour-evoked patterns in the AL. We know already that this is a common strategy used by bees during behavioural generalisation: the more similar AL activity patterns are, the more bees generalise between odours (Chapter I, Guerrieri, Schubert et al. 2005). The astonishing result showed that, contrarily to our prediction, the distance 2-hexanol – mixture was slightly shorter than the distance hexanal – mixture. Thus, although, the distance 2-hexanol – mixture is the shorter one, hexanal is the odour which overshadowed 2-hexanol in the behavioural experiment. The results showed the opposite of the expected situation and, therefore, overshadowing cannot be explained by a simple similarity rule between glomerular activity patterns of single odours and the mixture.

To find a neuronal correlate for asymmetric cross-generalization we looked for asymmetrical changes after conditioning to single odours, calculating Euclidian distances between activation patterns for 2-hexanol and hexanal, after either 2-hexanol or hexanal

conditioning. As explained in the introduction, changes in odour-induced glomerular activation patterns after conditioning could be responsible for better discrimination between two odours, and if such changes are asymmetric it could well lead to asymmetric cross-generalisation. According to such a model, calculation of Euclidian distances between glomerular activation patterns for 2-hexanol and hexanal after conditioning to 2-hexanol or hexanal should reveal asymmetrical changes after conditioning. Because the behavioural results confirmed on our sample an asymmetric generalization between the two odours in the control groups (generalization responses to hexanal after 2-hexanol conditioning were higher than in the opposite situation) we expected a higher similarity between hexanal and 2-hexanol pattern after 2-hexanol conditioning than after hexanal conditioning. Again, contrarily to our prediction, the results showed that the distance between both odours was higher after 2-hexanol than after hexanal conditioning. Therefore, asymmetric cross-generalization cannot be explained by different similarities between glomerular patterns of single odours after conditioning to one or the other odour in the AL.

In a last analysis, we wanted to investigate if conditioning effects were restricted to the glomerular sub-population activated by the learned odour. One would expect if only odour activated glomeruli change their intensity, that they would all do it in the same direction (increase or maybe decrease) after conditioning. Such a scenario would therefore show a correlation between activation intensity before conditioning and a change in intensity after conditioning (e.g. highly activated glomeruli would show the strongest changes of intensity after conditioning). We did not find such a trend. Except in the case of hexanal, correlations between odour induced glomerular activity in naïve bees and changes in the same glomeruli after conditioning were not significant. In the case of hexanal, the correlation was significant but the inclination of the line of best fit was negative, meaning that the higher the glomerular activation in naive bees the lower the activation changes after conditioning of this odour. This was an unexpected result and moreover, not a general observation. In general single glomeruli increased or decreased their activity after conditioning independently of their activation intensity. These results show that conditioning did not change neuronal activation unidirectional. Odour conditioning effects seem to influence both, in- and decrease of neuronal activation. Underlining that excitation and inhibition as well as inactivity of neurons is crucial for glomerular odour patterns (Freeman 1999).

Summarising the results in this chapter, we can state that conditioning does not change overall-activity intensity of the glomerular activation patterns in the AL, that it does not change the odour induced glomeruli activation pattern and that the changes in activation intensity of individual glomeruli do not follow a common pattern, they in- or decrease their activity independently. Moreover, we were not able to substantially enforce our model, which included asymmetrical changes in the glomerular activation patterns for two odours A and B after conditioning to these odours, foretelling the more salient odour which would be dominant when conditioned in a mixture AB and would therefore overshadow the second one. A new model should thus be found.

One interesting possibility we have studied, is that similarity between glomerular activation patterns could be evaluated downstream of the olfactory pathway by reading out only part of the glomerular information leaving the AL, i.e. integrating only information from a part of the AL glomeruli. According to our analysis most of the glomeruli contributed to a stronger difference between hexanal and the mixture than between 2-hexanol and the mixture. Only a few glomeruli showed the opposite effect. Thus, very few glomeruli should be taken into account to obtain a higher similarity between hexanal and the mixture than between 2-hexanol and the mixture, which would allow predicting OVS (hexanal overshadowing 2-hexanol, as in the behavioural experiment). But such a selective integration would also reduce the quality of specific odour coding because only a few units would be used. This possibility is therefore not probable in our view.

If conditioning does not modify the olfactory information transmitted to higher brain centres, then an asymmetrical measure of similarity should be used by the brain to predict asymmetrical cross-generalisation. In such an alternative model, asymmetrical cross-generalization could be explained if the distance measure from A to B representations is not equal to that from B to A. For instance, a measure based only on a comparison of the glomeruli activated by the learned odour (and not the inactive ones) would mean measuring similarity on *different sets of glomeruli* in the two cases. This could easily lead to asymmetrical similarity measures, as could a number of slightly different models. In order to substantiate any such model, new experiments should be done. First, an important number of odours, which are known to produce asymmetrical cross-generalization should be used, so that the different models can be tested on an important number of odour pairs. This will be the subject of future work.

For interpretation of the results in this calcium imaging study, we are naturally aware of the problem that we imaged only the glomeruli at the dorsal surface of the AL. The contribution of the rest of the AL glomeruli and their network to the plasticity of odour representation remains therefore unanswered. Thus, although not accessible with our techniques – or techniques used until now in insects – conditioning related plasticity could still take place in the AL. Our former work (Chapter I, Guerrieri, Schubert et al. 2005) and the work of others (Galizia et al., 1999) showed that the investigated glomeruli are sufficient for odour coding but apparently they are not sufficient to show training induced plasticity. In addition, we keep in mind that the relative small sample size obtained in each experimental group (n = 4) is not yet sufficient for reliable interpretation of our results and further experiments will have to be done.

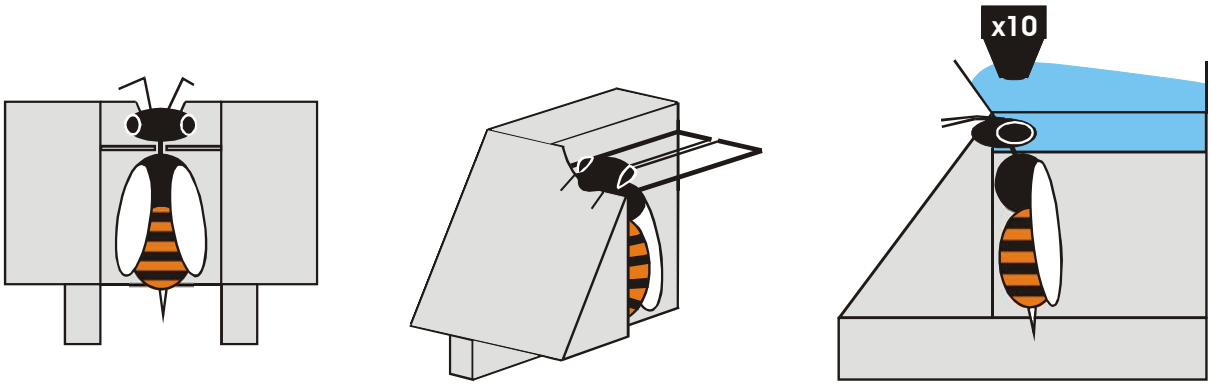


**Figure 2.1:**

Animal preparation for the imaging experiments. The bees were fixed to the imaging chambers exposing the head (but not the antennae) to the pool which was filled with ringer (blue liquid in the right figure) allowing brain surgery without dry out and imaging with an upright fluorescence microscope with a water immersion objective lens (x10).

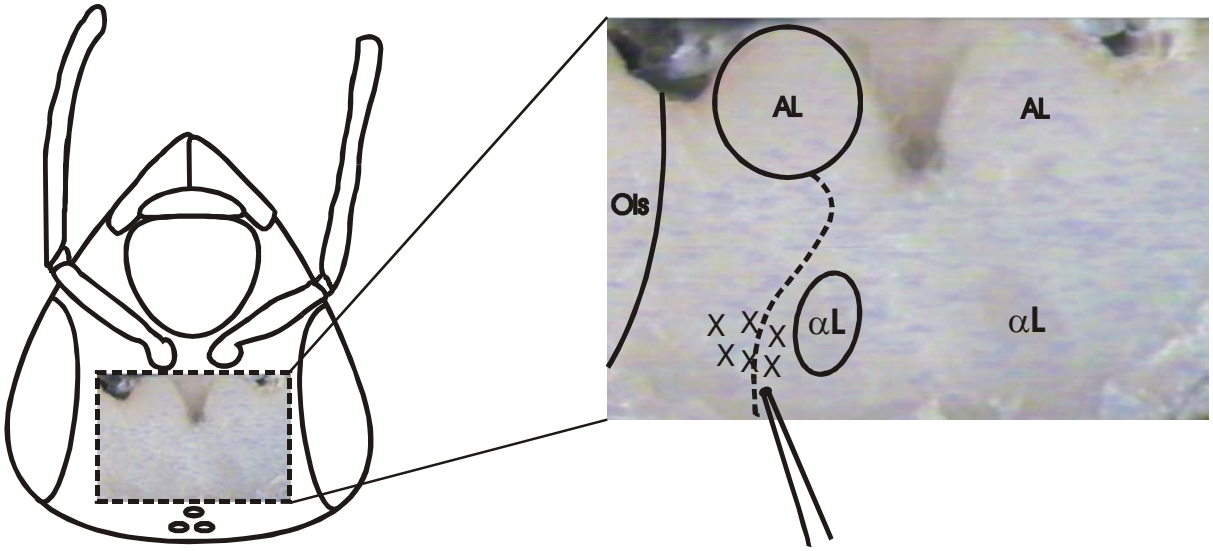


**Figure 2.2:**

An opening was cut in the head capsule with his outer margins between the eyes, dorsal to the bases of the antennae and ventral to the ocelli (dotted line in the left part of the figure) to gain access to the brain tissues. In the right part of the figure one can see the enlarged brain with his α-lobes (αL), optical lobes (OLs), antennal lobes (AL) and the expected track of the projection neurons (dotted line in the right part of the figure). Multiple injections (1-6) were done at the spots which were marked with an X.

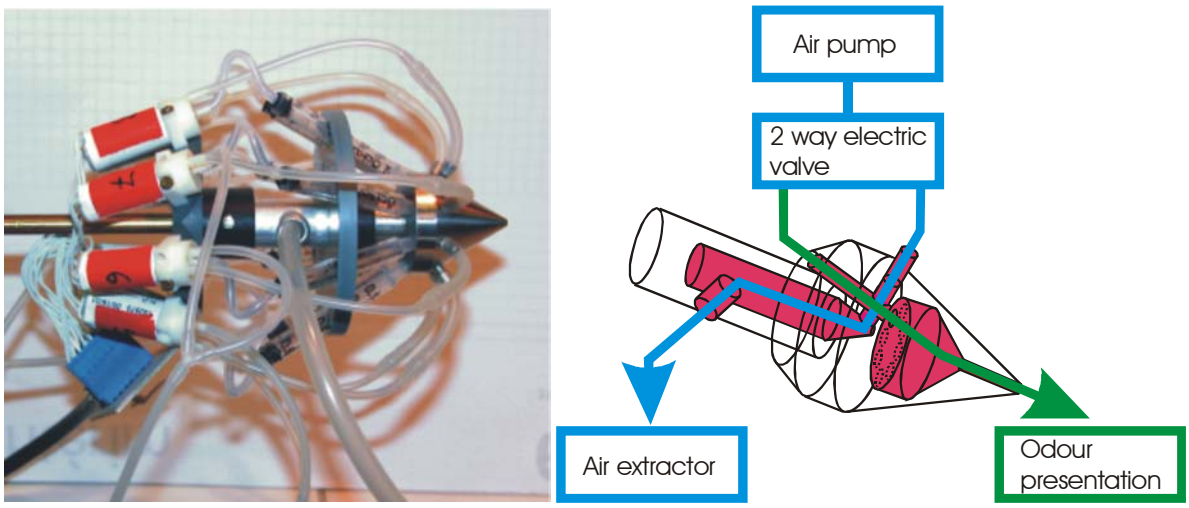


**Figure 2.3:**

The odour cannon. An air pump delivered the air stream which was connected to 8 computer controlled electronic valves (numbered 1-8, left photo). When activated, a valve directed the air stream into an odour laden syringe. The air stream pushed the odour molecules out of the syringe, into the mixing chamber and out of the cannon exit to present the odour to an animal (green flash, right scheme). When inactivated, a valve directed the air stream to an alternative entrance, through a tube, crossing the tube used for odour presentations, and through an alternative exit leading to an air extractor (blue flash, right scheme).

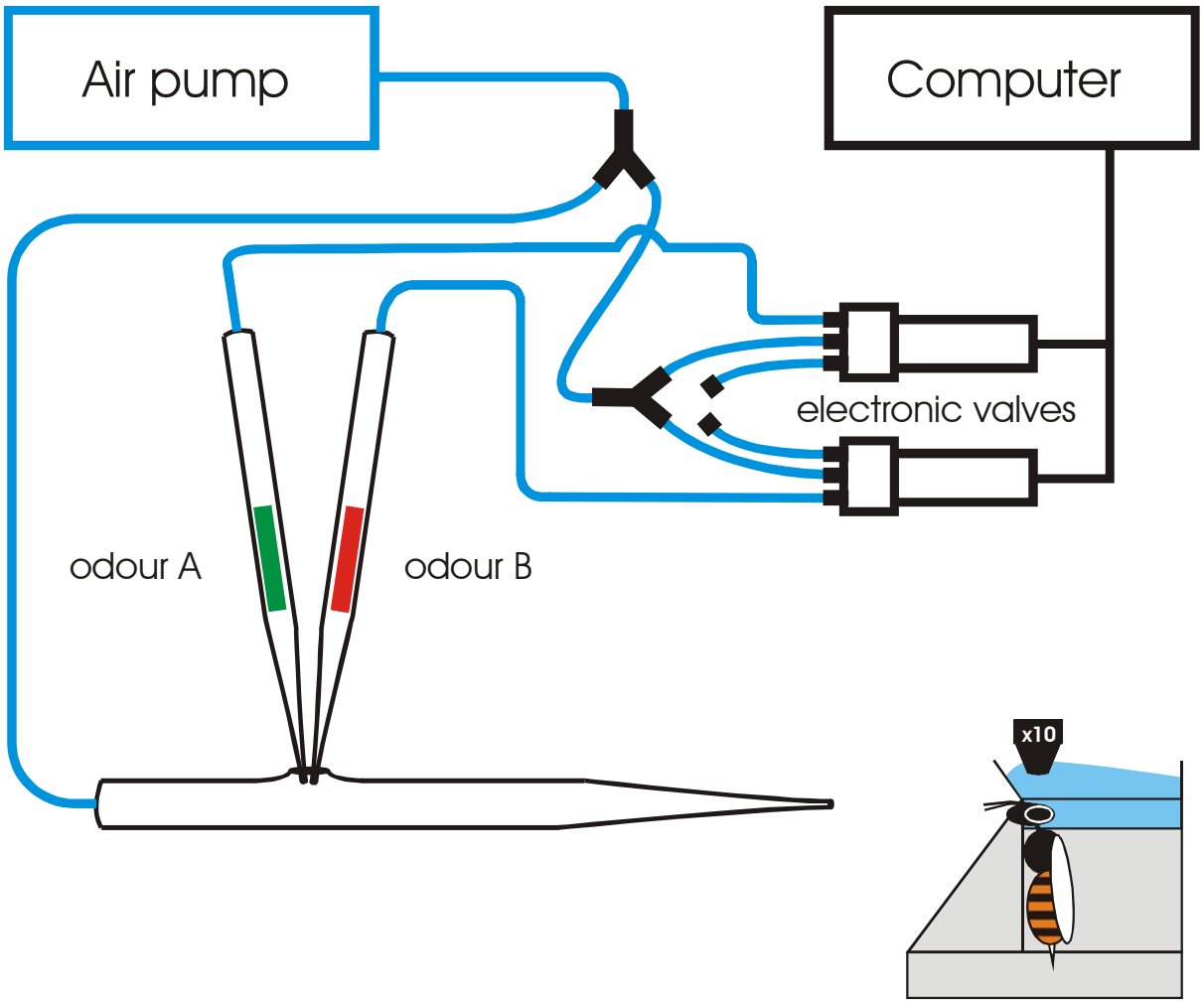


**Figure 2.4:**

Odour stimulation set up for the imaging experiments. An air pump delivered the air stream permanently presented to the animals through an empty Pasteur pipette (horizontal pipette). When activated by a computer two electronic valves let pass the stream through two Pasteur pipettes mounted with filter paper from which either only one hold an odour and the other one was clean (component presentation) or both filter papers hold an odour (compound presentation). Different odours were shown as differently coloured (red and green) filter papers.

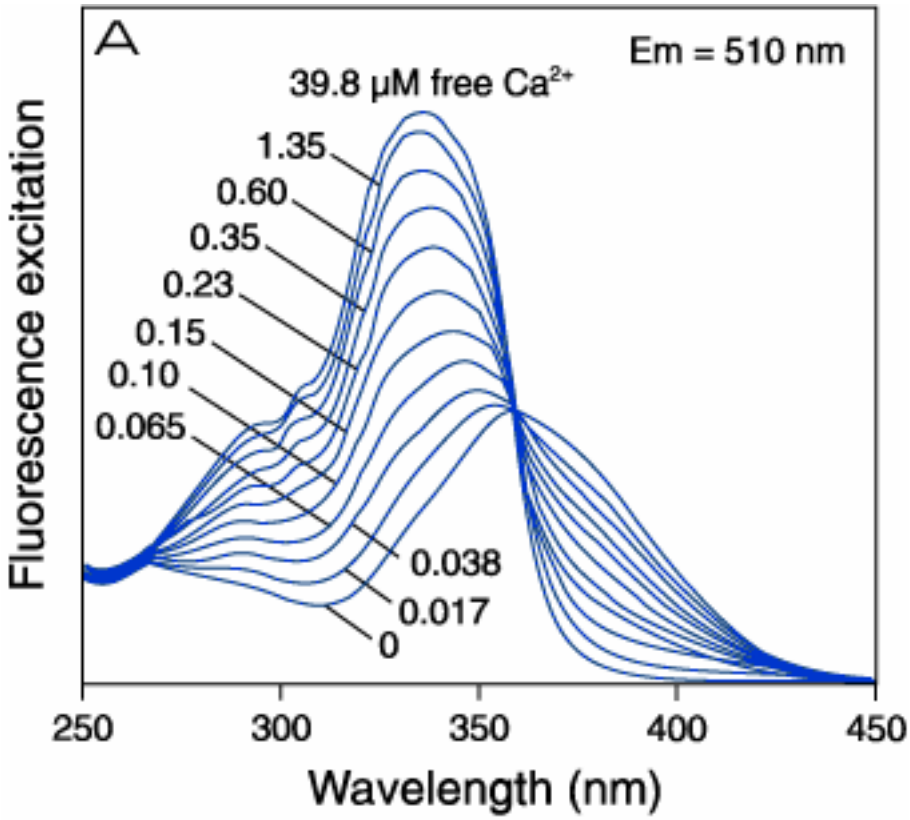


**Figure 2.5:**

Fluorescence excitation spectra of fura-2 in solutions containing 39.8 µM free Ca2+ (from "Handbook of Fluorescent Probes and Research Products" by Richard P. Haugland, 8th edition).

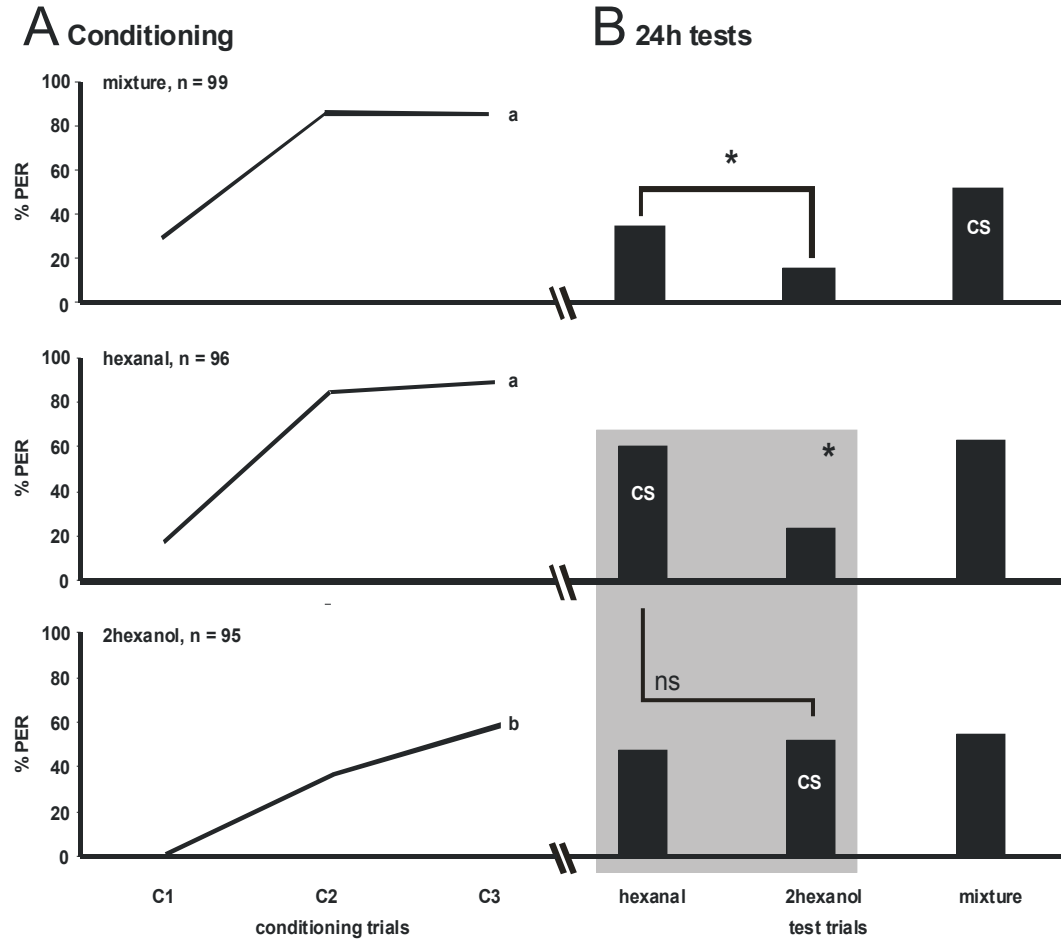


**Figure 3.1:**

**A** Acquisition and **B** 24h tests for the three conditioning groups (mixture, hexanal and 2-hexanol in the upper, middle and lower graph, respectively). All bees learned the CS, but acquisition was higher for the mixture (a) and hexanal (a) than for 2-hexanol (b). After conditioning to the mixture (upper graph) bees showed significant overshadowing, responding more to hexanal than to 2-hexanol. Although, conditioning performance significantly differed between hexanal and 2-hexanol in the third trial (middle and lower graph, respectively) CS responses after 24h revealed similar response levels. Moreover, cross-generalization between hexanal and 2-hexanol was asymmetric (see grey box).

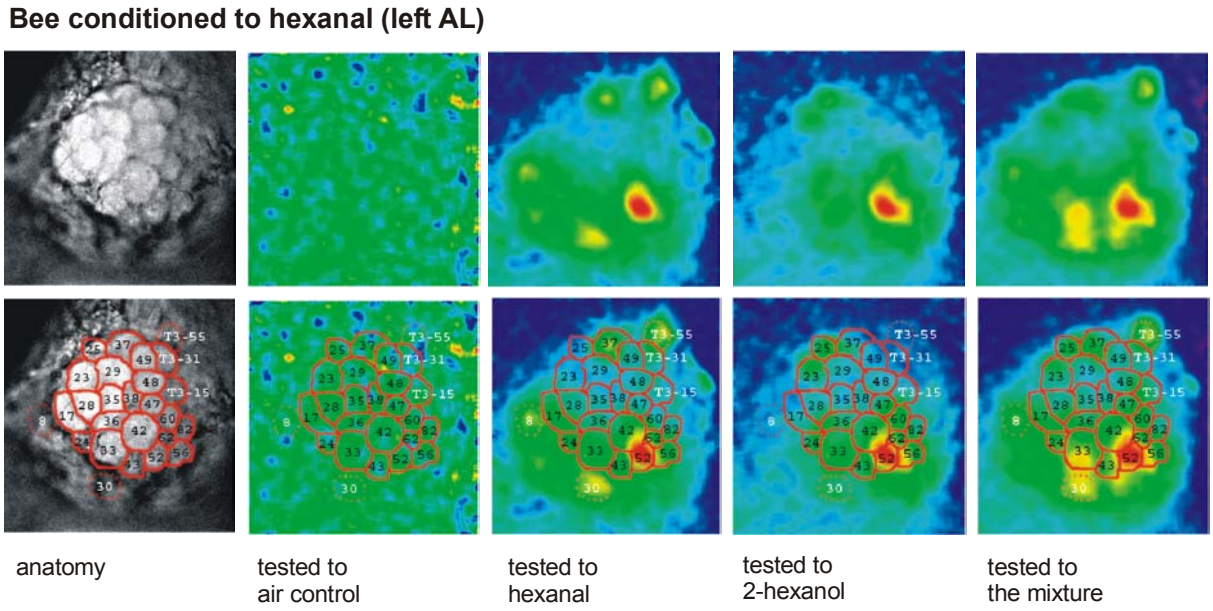


**Figure 3.2:**

Anatomy and activation patterns for a bee conditioned to hexanal and tested with an air control, hexanal, 2-hexanol and the mixture of both odours (from left to right). The lower row shows the same images as the row above but with a mask added showing the identified glomeruli.

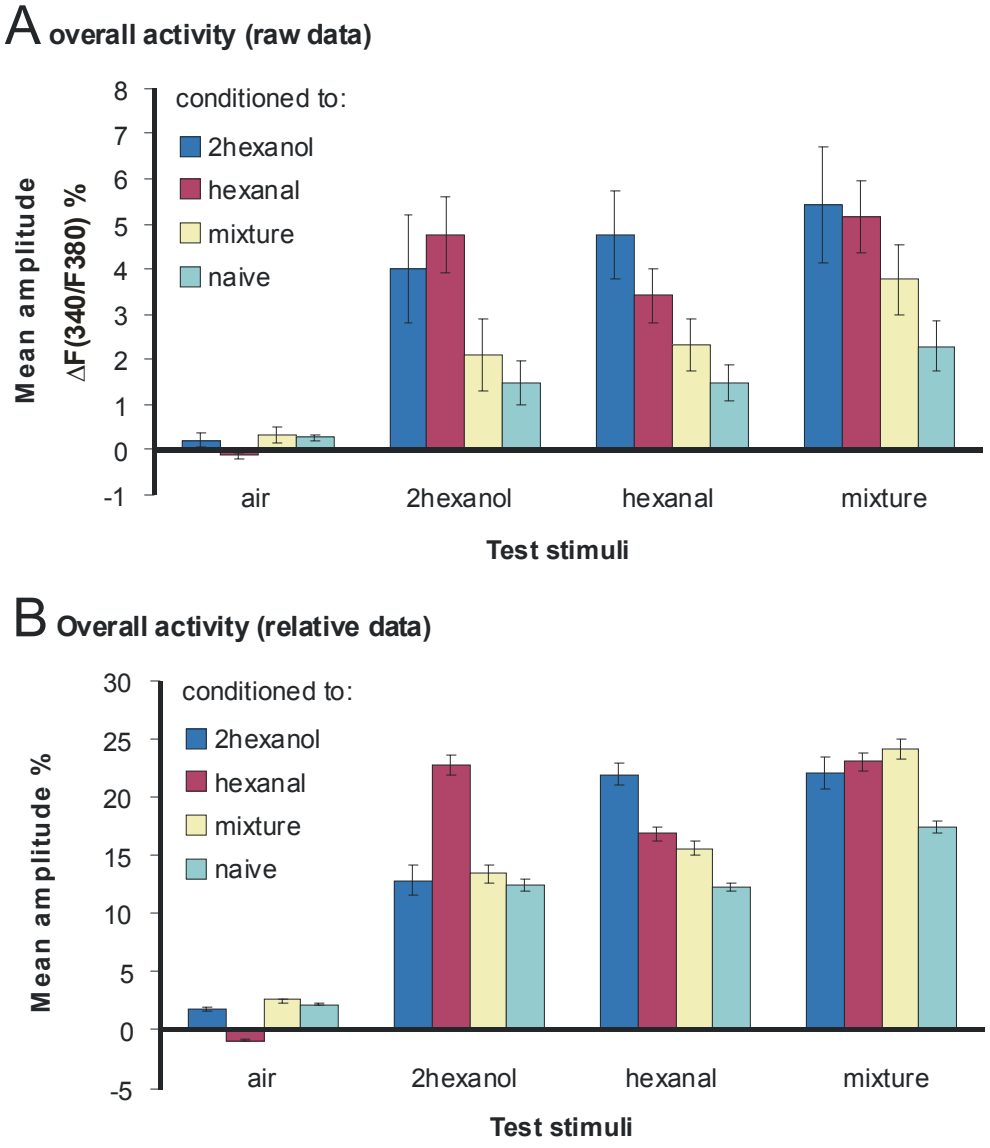


**Figure 3.3:**

Mean calcium response amplitudes in the 19 glomeruli for each conditioning group and for naive bees (n = 4 in each group). A For the raw data a two-way ANOVA (training vs. test odour repeated measure) yielded a non-significant training effect ($F_{3,12} = 0.69$, $p = 0.58$), a significant test odour effect ($F_{3,36} = 12.17$, $p < 0.001$) and no interaction ($F_{9,36} = 0.79$, $p = 0.63$). B For the relative data the same analysis also yielded a non-significant training effect ($F_{3,12} = 0.39$, $p = 0.76$), a significant test odour effect ($F_{3,36} = 22.76$, $p < 0.001$) and no interaction between both effects ($F_{9,36} = 0.94$, $p = 0.50$). Error bars show standard errors.

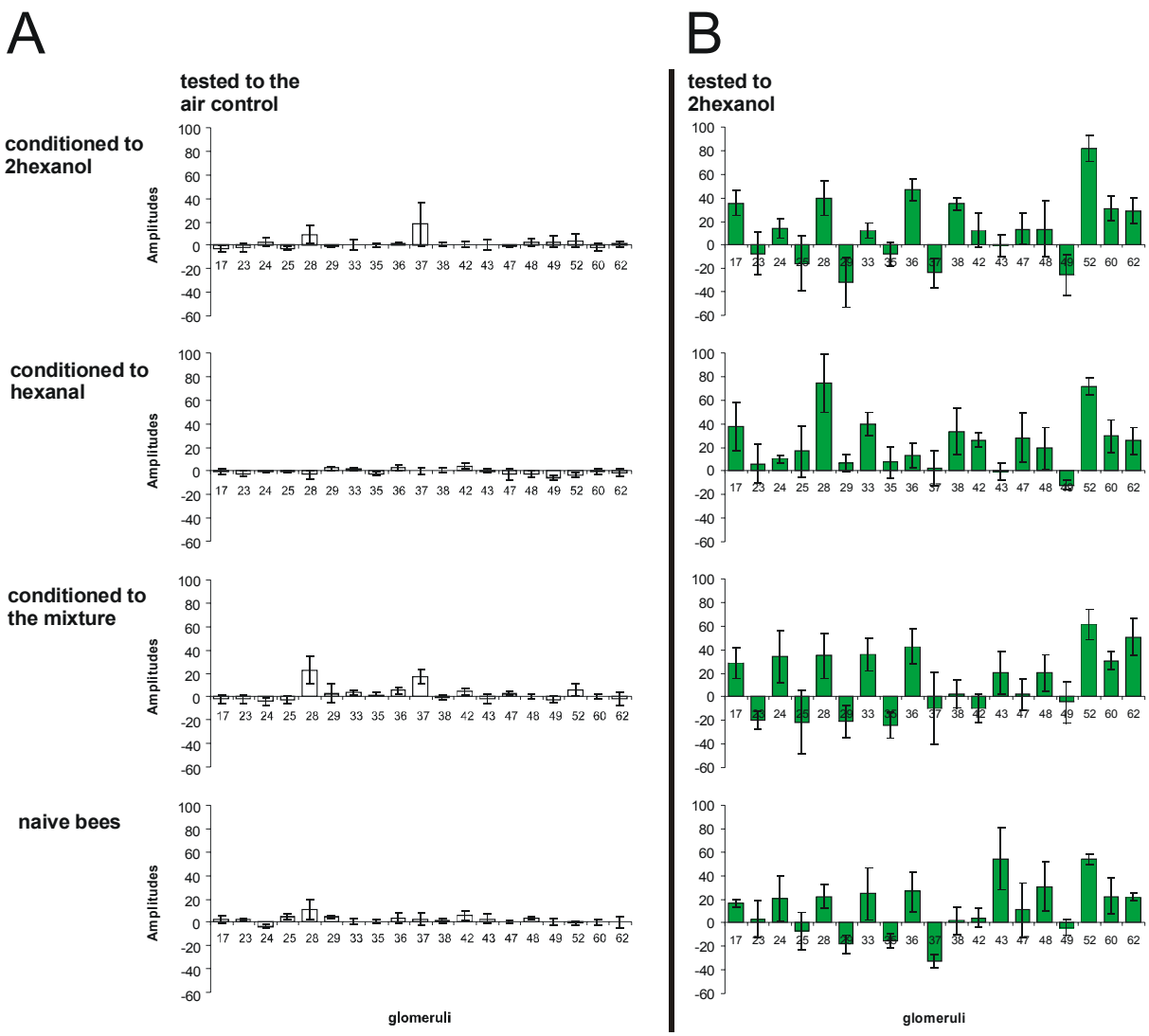


**Figure 3.4:**

Normalized calcium response (%) of each glomerulus when tested with the air control (left column) and 2-hexanol (right column) for each conditioning group and naïve bees (n = 4 in each group).

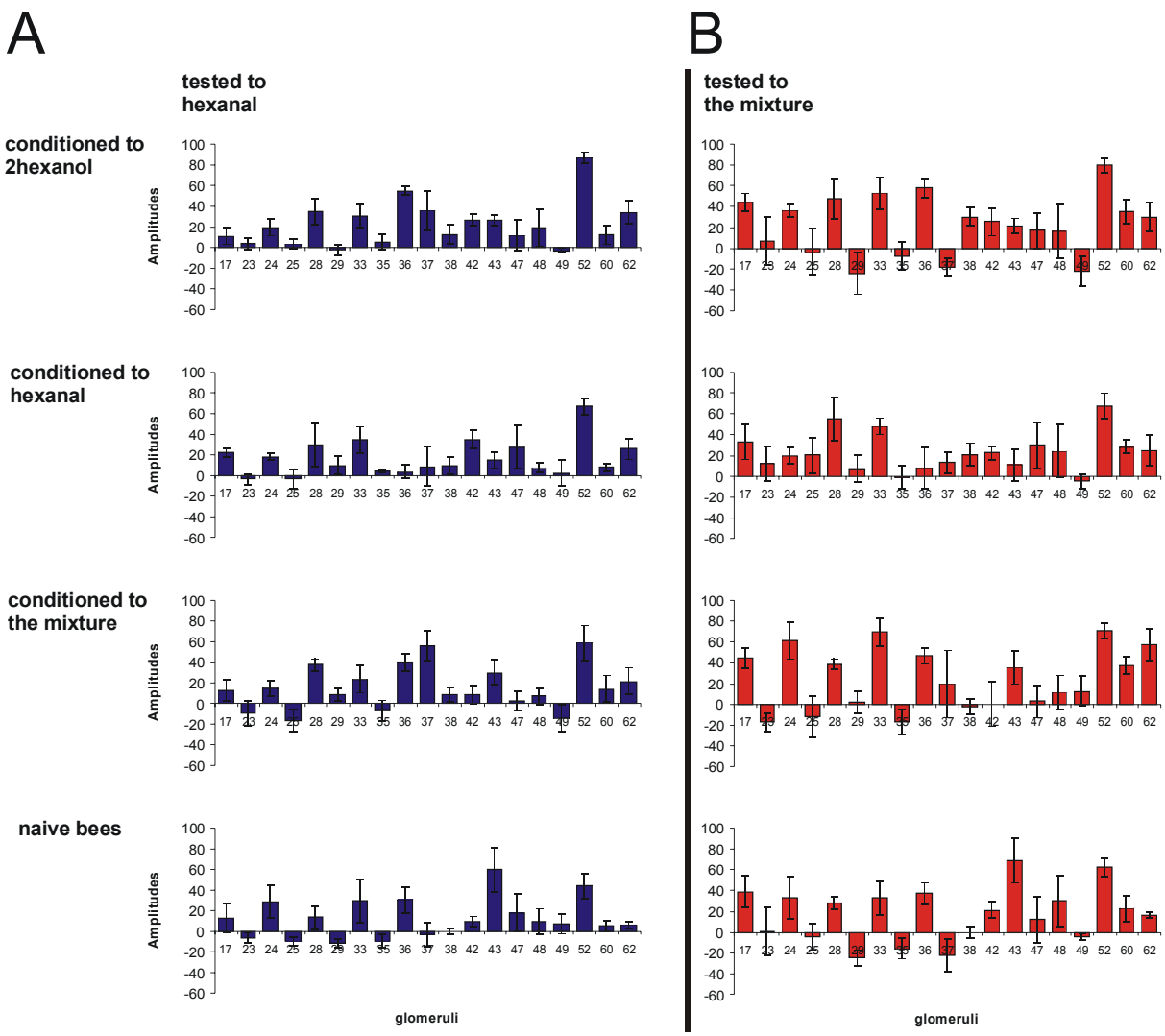


**Figure 3.5:**

Normalized calcium response (%) of each glomerulus when tested with hexanal (left column) and the mixture (right column) for each conditioning group and naïve bees (n = 4 in each group).

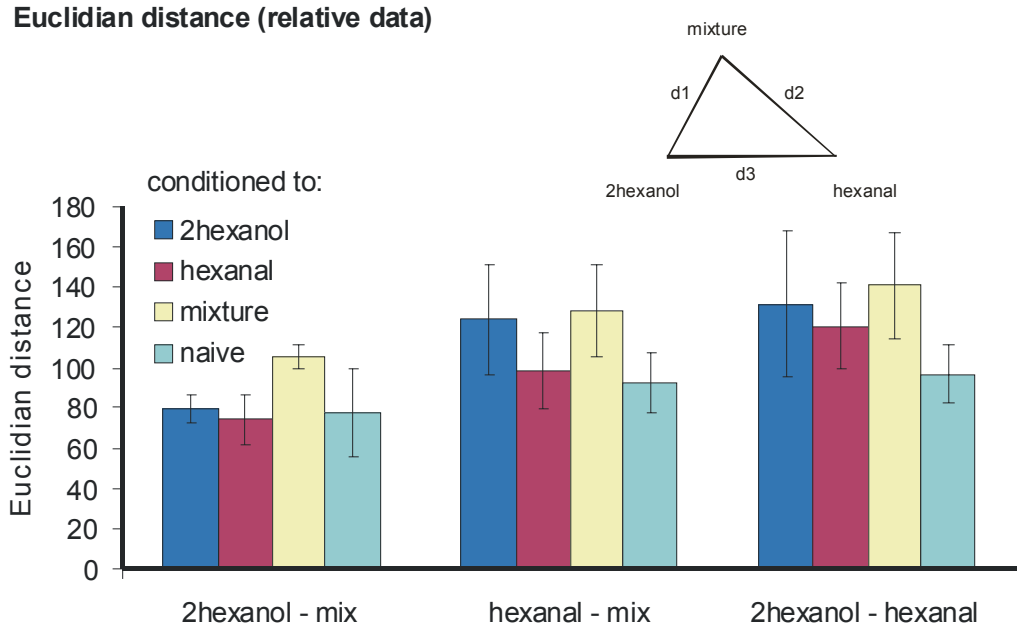


**Figure 3.6:**

Euclidian distances between the representations of the single odours (2-hexanol and hexanal) and their mixture in a putative neural space in which the activity of each glomerulus represents a dimension. An ANOVA (training x odour pair - repeated measure) shows a significant odour pair effect ($F_{2,24} = 6.10$, $p < 0.01$), but no significant conditioning effect ($F_{3,12} = 0.92$, $p = 0.46$) and no interaction ($F_{6,24} = 0.30$, $p = 0.93$). The triangle in the upper right corner represents the average distances (d1, d2 and d3) found between the single odours and their mixture.

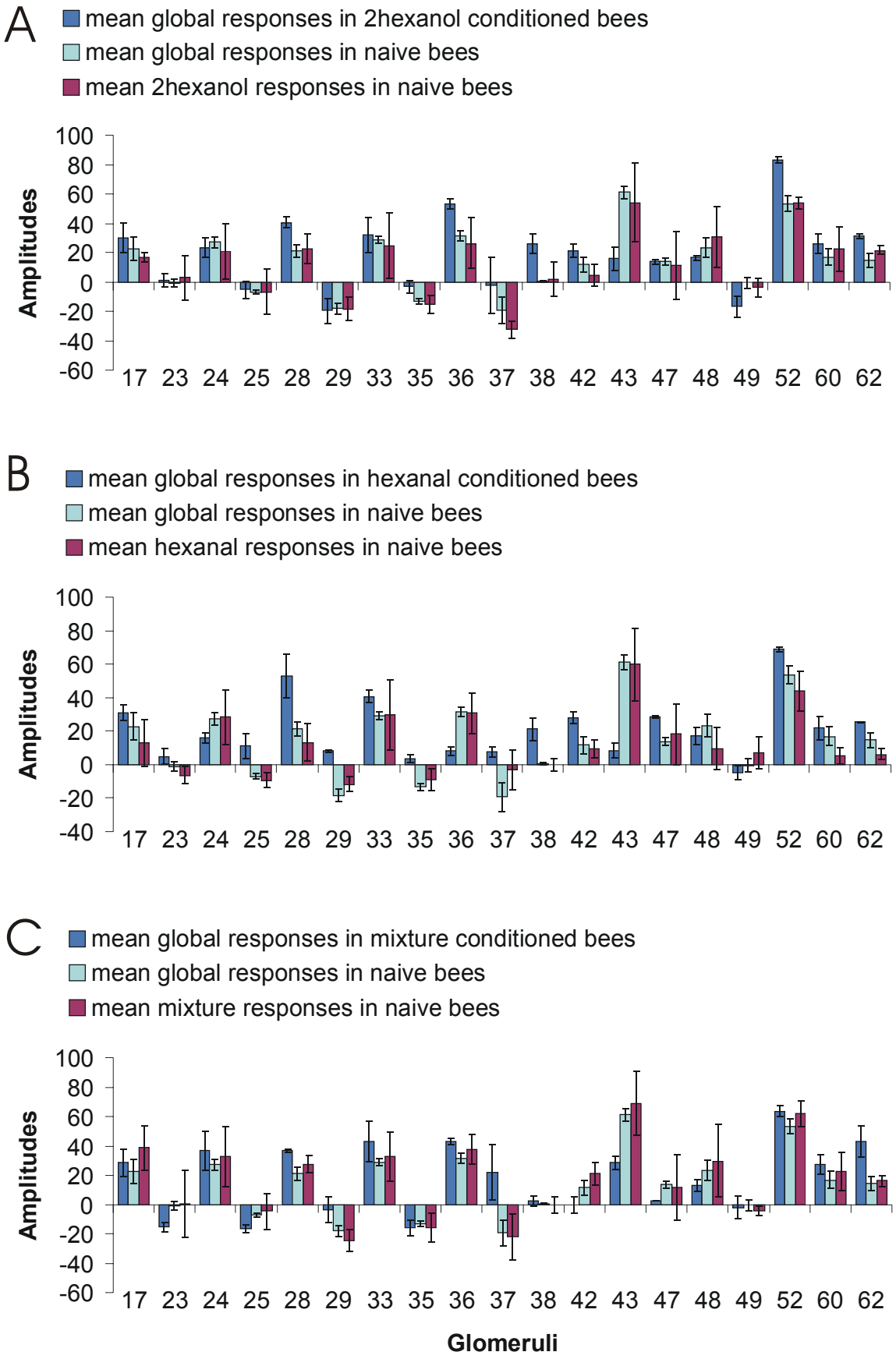
A
mean global responses in 2hexanol conditioned bees
mean global responses in naive bees
mean 2hexanol responses in naive bees
Amplitudes
100
80
60
40
20
0
-20
-40
-60
17 23 24 25 28 29 33 35 36 37 38 42 43 47 48 49 52 60 62
B
mean global responses in hexanal conditioned bees
mean global responses in naive bees
mean hexanal responses in naive bees
Amplitudes
100
80
60
40
20
0
-20
-40
17 23 24 25 28 29 33 35 36 37 38 42 43 47 48 49 52 60 62
C
mean global responses in mixture conditioned bees
mean global responses in naive bees
mean mixture responses in naive bees
Amplitudes
100
80
60
40
20
0
-20
-40
-60
17 23 24 25 28 29 33 35 36 37 38 42 43 47 48 49 52 60 62
Glomeruli

**Figure 3.7:**

Mean global responses (mean responses to all tested stimuli) for each glomerulus in bees conditioned to A) 2-hexanol, B) hexanal and C) the mixture (dark blue bars) and in naïve bees (light blue bars). For comparison, the response of naïve bees to the odour that is the CS in each case is given (red bars). Comparing global responses in conditioned and naïve bees allows identifying specific changes in glomerulus responses after conditioning. Comparing these changes in global responses with the responses to each CS odour in naïve bees allows checking the possibility that changes take place within the glomeruli belonging to the pattern of the CS.

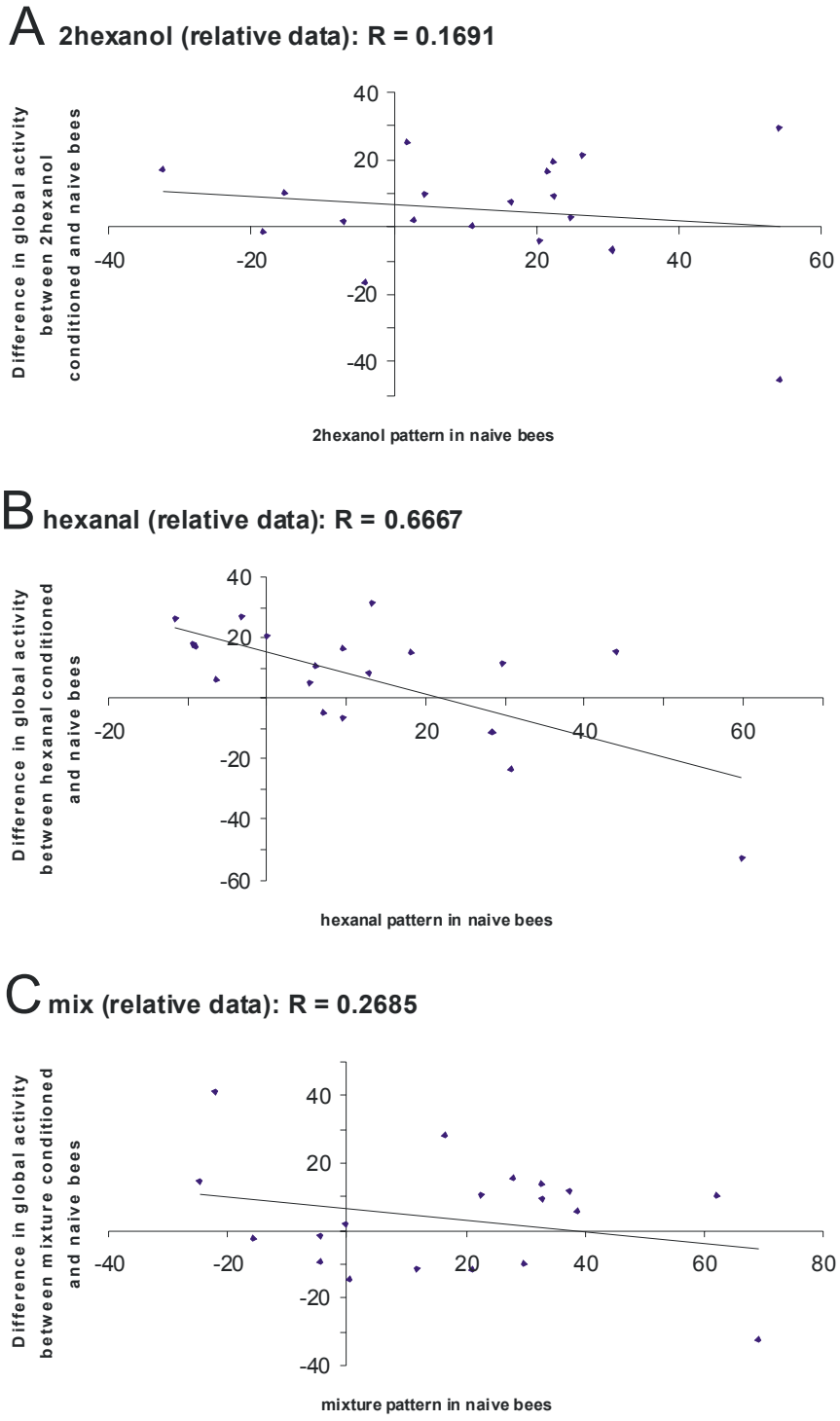


**Figure 3.8:**

Difference in global contribution of each glomerulus between conditioned and naïve bees (ordinate) as a function of the contribution of these glomeruli in naive bees to the CS pattern (abscissa, A: 2-hexanol, B: hexanal and C: mixture).

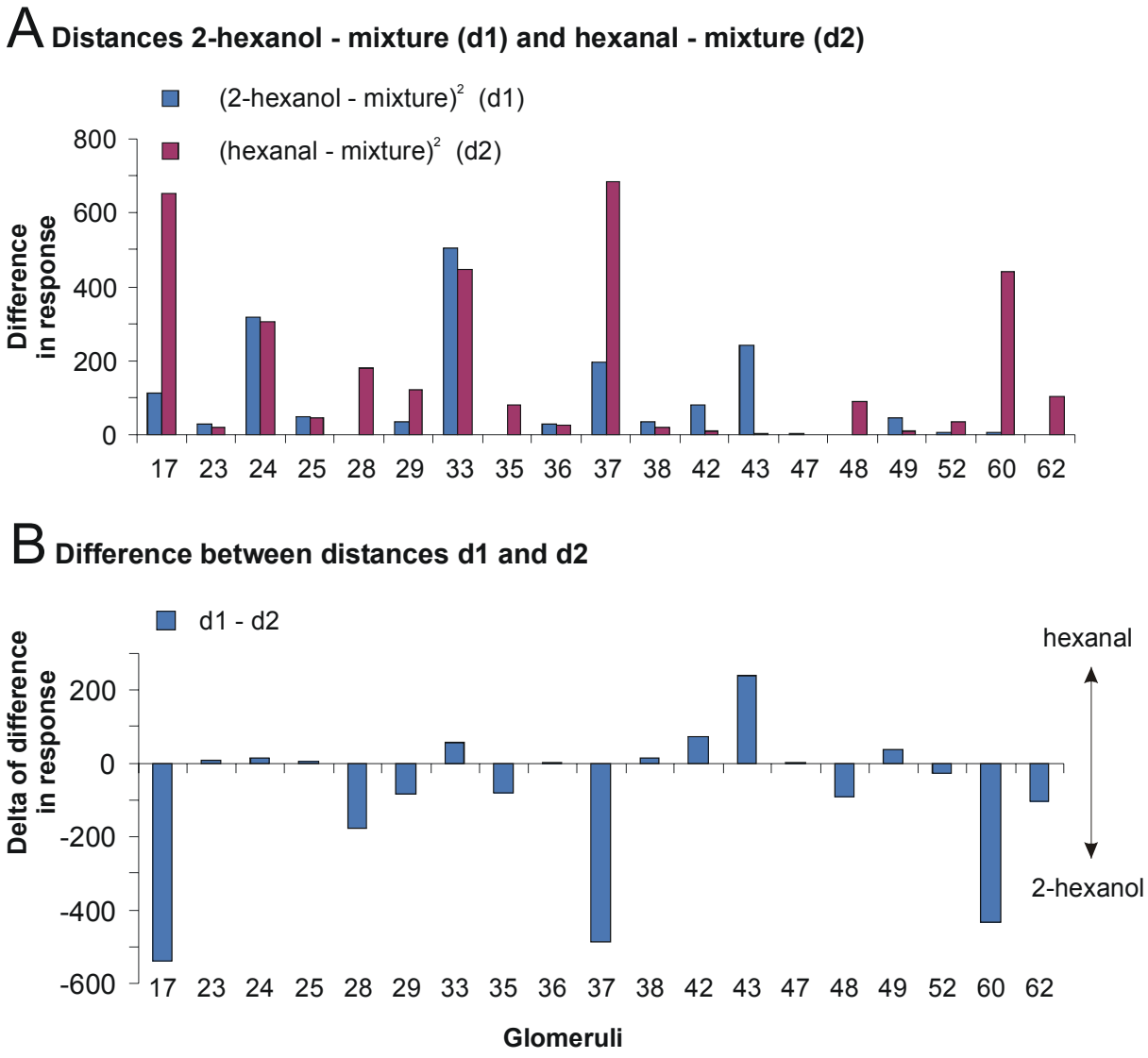


**Figure 3.9:**

A Difference in response to the single odours and the mixture (d1 and d2) over all glomeruli (relative data, mean for 16 bees). B Delta of differences between d1 and d2 over all glomeruli (relative data, mean for 16 bees).

## References

Abel,R., Rybak,J. & Menzel,R. 2001. Structure and Response Patterns of Olfactory Interneurons in the Honeybee, Apis mellifera. The Journal of Comparative Neurology 437, 363-383.

Akers,R.P. & Getz,W.M. 1992. A test of identified response classes among olfactory receptor neurons in the honey-bee worker. Chemical Senses 17, 191-209.

Akers,R.P. & Getz,W.M. 1993. Response of olfactory receptor neurons in honey bees to odorants and their binary mixtures. Journal of Comparative Physiology [A] 173, 169-185.

Allan, S.A., Slessor, K.N., Winston, M.L. & King, G.G.S. 1987. The influence of age and task specialization on the production and perception of honeybee pheromones. Journal of Insect Physiology 33, 917-922.

Arnold,G., Masson,C. & Budharugsa,S. 1985. Comparative study of the antennal lobes and their afferent pathway in the worker bee and the drone (Apis mellifera). Cell and Tissue Research 242, 593-605.

Brembs,B. & Heisenberg,M. 2001. Conditioning with compound stimuli in Drosophila melanogaster in the flight simulator. J Exp Biol 204, 2849-2859.

Brown,S.M., Napper,R.M. & Mercer,A.R. 2003. Foraging experience, glomerulus volume, and synapse number: a stereological study of the honey bee antennal lobe. Activity-dependent structural changes 4-49.

Couvillon,P.A. & Bitterman,M.E. 1980. Some phenomena of associative learning in honey bees. Journal of Comparative and Physiological Psychology 94, 878-885.

Deisig,N., Giurfa,M., Lachnit,H. & Sandoz,J.-C. 2006. Neural representation of olfactory mixtures in the honeybee antennal lobe. Eur. J. Neurosci. 24, 1161-1174.

Derby,C.D., Hutson,M., Livermore,B.A. & Lynn,W.H. 1996. Generalization among related complex odorant mixtures and their components: Analysis of olfactory perception in the spiny lobster. Physiology & Behavior 60, 87-95.

Ditzen,M. 2005. Odor Concentration and Identity Coding in the Antennal Lobe of the Honeybee Apis Mellifera. Doctorate in Biology, FU Berlin.

Domjan,M. & Burkhard,B. 1986. The Principles of Learning and Behavior. 2nd edn. Pacific Grove, CA: Brooks/Cole.

Esslen,J. & Kaissling,K.E. 1976. Zahl und Verteilung antennaler Sensillen bei der Honigbiene (Apis mellifera L.). Zoomorphology 83, 227-251.

Faber,T., Joerges,J. & Menzel,R. 1999. Associative learning modifies neural representations of odors in the insect brain. nature neuroscience 2, 74-78.

Flanagan,D. & Mercer,A.R. 1989. An atlas and 3-D reconstruction of the antennal lobes in the worker honey bee, Apis mellifera L. (Hymenoptera: Apidae). International Journal of Insect Morphology and Embryology 18, 145-159.

Freeman,W.J. 1999. Olfactory System: Odorant Detection and Classification. In: Building Blocks for Intelligent Systems: Brain Components as Elements of Intelligent Function, Vol. III, Part 2 (Ed. by D.Amit & G.Parisi) New York, Academic Press.

Frings,H. 1944. The loci of olfactory end-organs in the honey-bee, Apis mellifera Linn. J. exp. Zool. 88, 65-93.

Galizia,C.G., Joerges,J., Küttner,A., Faber,T. & Menzel,R. 1997. A semi-in-vivo preparation for optical recording of the insect brain. Journal of Neuroscience Method 76, 61-69.

Galizia,C.G., McIlwrath,S.L. & Menzel,R. 1999. A digital three-dimensional atlas of the honeybee antennal lobe based on optical sections acquired by confocal microscopy. Cell Tissue Res. 295, 383-394.

Galizia,C.G. & Menzel,R. 2001. The role of glomeruli in the neural representation of odours: results from optical recording studies. Journal of Insect Physiology 47, 115-129.

Gallistel,C.R. 1990. The Organization of Learning. Cambridge, Mass., London: MIT Press.

Gerber,B. & Menzel,R. Contextual modulation of memory consolidation. Learning & Memory 7, 151-158. 2000.

Getz,W.M. & Akers,R.P. 1994. Honeybee olfactory sensilla behave as integrated processing units. Behav. Neural Biol. 61, 191-195.

Guerrieri,F., Schubert,M., Sandoz,J.C. & Giurfa,M. 2005. Perceptual and neural olfactory similarity in honeybees. PLoS Biol 3, e60.

Joerges,J., Küttner,A., Galizia,C.G. & Menzel,R. 1997. Representation of odours and odour mixtures visualized in the honeybee brain. Nature 387, 285-288.

Kamin,L.J. 1968. Attention-like processes in classical conditioning. In: Miami Symp. Predictability, Behavior and Aversive Stimulation (Ed. by M.R.Jones), pp. 9-32. Miami, Univ.Miami Press.

Kamin,L.J. 1969. Selective association and conditioning. In: Fundamental Issues in Associative Learning. (Ed. by W.K.Honig) Halifax, Dalhousie University Press.

Kehoe,E.J. 1982. Overshadowing and summation in compound stimulus conditioning of the rabbit's nictitating membrane response. J Exp Psychol Anim Behav Proc 8, 313-328.

Knudsen,J.T., Tollsten,L. & Bergstrom,L.G. 1993. Floral scents - A checklist of volatile compounds isolated by headspace techniques. Phytochemistry 33, 253-280.

Linster,C. & Smith,B.H. 1997. A computational model of the response of honey bee antennal circuitry to odor mixtures: overshadowing, blocking and unblocking can arise from lateral inhibition. Behavioral Brain Research 87, 1-14.

Mackintosh,N.J. 1971. An Analysis of Overshadowing and Blocking. Q J Exp Psychol 23, 118-125.

Mackintosh,N. 1994. Animal Learning and Cognition: Handbook of Perception and Cognition. 2nd edn. San Diego, CA: Academic Press.

Masson,C. & Arnold,G. 1984. Ontogeny, maturation and plasticity of the olfactory system in the workerbee. Journal of Insect Physiology 30, 7-14.

Menzel,R. 1985. Learning in honey bees in an ecological and behavioral context. In: Experimental Behavioral Ecology and Sociobiology (Ed. by B.Hölldobler & M.Lindauer), pp. 55-74. Stuttgart, Gustav Fischer Verlag.

Menzel,R. & Giurfa,M. 2001. Cognitive architecture of a mini-brain: the honeybee. Trends Cogn Sci. 5, 62-71.

Menzel,R., Greggers,U. & Hammer,M. 1993. Functional organization of appetitive learning and memory in a generalist pollinator, the Honey Bee. In: Insect Learning: Ecological and Evolutionary Perspectives (Ed. by D.Papaj & A.C.Lewis), pp. 79-125. New York, Chapman & Hall.

Pavlov,I.P. 1927. Conditioned Reflexes: An Investigation of the Physiological Activity of the Cerebral Cortex. Oxford University Press.

Peele,P. 2005. Stable Odor Coding Is Ensured by Uniglomerular Output Neurons Throughout Olfactory Learning in the Honeybee Apis Mellifera. Doctorate in Biology, FU Berlin.

Pelz,C., Gerber,B. & Menzel,R. 1997. Odorant intensity as a determinant for olfactory conditioning in honeybees: Roles in discrimination, overshadowing and memory consolidation. Journal of Experimental Biology 200, 837-847.

Sachse,S. & Galizia,C.G. 2002. The Role of Inhibition for Temporal and Spatial Odor Representation in Olfactory Output Neurons: A Calcium Imaging Study. Journal of Neurophysiology 87, 1106-1117.

Sachse,S. & Galizia,C.G. 2003. The coding of odour-intensity in the honeybee antennal lobe: local computation optimizes odour representation. European Journal of Neuroscience 18, 2119-2132.

Sachse,S., Rappert,A. & Galizia,C.G. 1999. The spatial representation of chemical structures in the antennal lobe of honeybees: steps towards the olfactory code. Eur. J. Neurosci. 11, 3970-3982.

Sandoz,J.C., Galizia,C.G. & Menzel,R. 2003. Side-specific olfactory conditioning leads to more specific odor representation between sides but not within sides in the honeybee antennal lobes. Neuroscience 120, 1137-1148.

Smith,B.H. 1998. Analysis of interaction in binary odorant mixtures. Physiol Behav. 65, 397-407.

Staddon,J.E. 1983. Adaptive Behavior and Learning. New York: Cambridge University Press.

Vareschi,E. 1971. Duftunterscheidung bei der Honigbiene - Einzelzell-Ableitungen und Verhaltensreaktionen. Zeitschrift fuer vergleichende Physiologie 75, 143-173.

Vassar,R., Chao,S.K., Sitchran,R., Nuñez,J.M., Vosshall,L.B. & Axel,R. 1994. Topographic organization of sensory projections to the olfactory bulb. Cell 79, 981-991.

von Frisch,K. 1921. Über den Sitz des Geruchssinnes bei Insekten. Zoologische Jahrbücher Physiologie 38, 1-68.

Vosshall,L.B., Wong,A.M. & Axel,R. 2000. An olfactory sensory map in the fly brain. Cell 102, 147-159.

Wang,S., Zhang,S., Sato,K. & Srinivasan,M.V. 2005. Maturation of odor representation in the honeybee antennal lobe. Journal of Insect Physiology 51, 1244-1254.

Winston,M.L. 1987. The Biology of the Honey Bee. Cambridge, Mass.: Harvard University Press.

Witthöft,W. 1967. Absolute Anzahl und Verteilung der Zellen im Hirn der Honigbiene. Zeitschrift für Morphologie der Tiere 61, 160-184.