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Early olfactory experience modifies neural activity in the antennal lobe of a social insect at the adult stage

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Abstract

In the antennal lobe (AL), the first olfactory centre of the insect brain, odorants are represented as spatiotemporal patterns of glomerular activity. Whether and how such patterns are modified in the long term after precocious olfactory experiences (i.e. in the first days of adulthood) remains unknown. To address this question, we used *in vivo* optical imaging of calcium activity in the antennal lobe of 17-day-old honeybees which either experienced an odorant associated with sucrose solution 5–8 days after emergence or were left untreated. In both cases, we imaged neural responses to the learned odor and to three novel odors varying in functional group and carbon-chain length. Two different odor concentrations were used. We also measured behavioral responses of 17-day-old honeybees, treated and untreated, to these stimuli. We show that precocious olfactory experience increased general odor-induced activity and the number of activated glomeruli in the adult AL, but also affected qualitative odor representations, which appeared shifted in the neural space of treated animals relative to control animals. Such effects were not limited to the experienced odor, but were generalized to other perceptually similar odors. A similar trend was found in behavioral experiments, in which increased responses to the learned odor extended to perceptually similar odors in treated bees. Our results show that early olfactory experiences have long-lasting effects, reflected in behavioral responses to odorants and concomitant neural activity in the adult olfactory system.

Introduction

Early life experiences can profoundly and permanently alter the development of brain function and influence behavior in the long term, as shown by the phenomenon termed imprinting (Lorenz, 1935). Long-term influences of early life experiences have been shown in both mammals (Oitzl *et al.*, 2000; Iso *et al.*, 2007) and insects (Caubet *et al.*, 1992; Sandoz *et al.*, 2000; Ichikawa & Sasaki 2003, Devaud *et al.*, 2003). In honeybees, a standard animal model for the study of behavioral and neural experience-dependent plasticity (Giurfa, 2007), precocious learning of a particular odor associated with reward during a relatively short time window (when bees are 5–8 days old) results in better olfactory retention at the adult stage (when bees are 17 days old) than when the same exposure occurs before (1–4 days old) or after (9–12 days old) this critical period (Arenas & Farina, 2008).

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In the insect olfactory system, the antennal lobe (AL) is the primary integration centre of odor information. Its anatomy and function resemble those of the olfactory bulb in vertebrates (Hildebrand & Shepherd,1997). Like the olfactory bulb, the AL is made up of glomeruli, globular structures where olfactory receptor neurons synapse with local interneurons and second-order neurons. Physiological studies have shown that odors are encoded in terms of spatiotemporal patterns of glomerular activity (Friedrich & Korsching, 1997; Joerges *et al.*, 1997; Galizia *et al.*, 1998, 1999a; Rubin & Katz, 1999; Sachse *et al.*, 1999; Uchida *et al.*, 2000; Carlsson *et al.*, 2002; Sachse & Galizia, 2002). Such neural code is dynamic and may undergo changes in its spatial and/or temporal components after associative learning events (Faber *et al.*, 1999; Sandoz *et al.*, 2003; Daly *et al.*, 2004; Yu *et al.*, 2004).

Although it has been shown that olfactory conditioning in adult bees can alter the glomerular activity patterns of conditioned odors (Faber *et al.*, 1999; SaSandoz *et al.*, 2003), the neural mechanisms by which precocious olfactory experiences during days 5–8 affect later adult performances remain unknown. It is known, however, that in the first days post-emergence, the olfactory system of a bee is still

subjected to remodeling and that the sensory environment can alter this process (Masson & Arnold, 1984, 1987) as well as learning and memory consolidation (Fuch *et al.*, 1989; Sandoz *et al.*, 2000; Ichikawa & Sasaki, 2003; Arenas & Farina, 2008). Moreover, calcium-imaging studies of AL activity showed that, in young bees, odors evoke weak but specific spatiotemporal activation patterns that become stronger and involve more glomeruli with increasing age (Wang *et al.*, 2005). Whether precocious olfactory learning during specific periods primes the olfactory system making it more responsive to the experienced odorant remains to be determined. Furthermore, whether such an effect generalizes to other odorants is also unknown.

To answer these questions we first studied the effect of early olfactory experiences at the behavioral level by offering sugar solution scented with 1-Nonanol (1-NON) to 5–8-day-old bees. The same bees were then tested for olfactory retention at the age of 17 days, when most of them initiate foraging (Rösch, 1925; Lindauer, 1952; Seeley, 1982). To this end, their proboscis extension responses (PER; Takeda, 1961; Bitterman *et al.*, 1983) were evaluated upon olfactory stimulation with the learned odor (1-NON) and with three novel odors presenting different degrees of similarity to 1-NON: Nonanal (NONA), 1-Hexanol (1-HEX) and Hexanal (HEXA). For each odorant, two concentrations were used. *In vivo* calcium imaging allowed measuring of odor-evoked responses to the same stimuli at the level of 19 identified glomeruli of the AL in adult honeybees. We determined whether the glomerular activity pattern of 1-NON is modified when retrieved 9–12 days later and whether changes are generalized to other odorants depending on their similarity to the trained odor.

Materials and methods

Animals

Emerging workers were obtained from sealed brood frames from European honeybee colonies (*Apis mellifera carnica*) located at the University of Toulouse. Frames were placed in an incubator at 36°C, 55% relative humidity and darkness. Zero- to one-day-old workers were collected in groups of ∼60 individuals and caged in wooden boxes (10 × 10 × 10 cm). Cages offered 1.8 M sugar solution, water and pollen *ad libitum.* Caged bees were kept in a different incubator (32°C, 55% relative humidity and darkness) until the age of 17 days when they were tested for their odor responses.

Early olfactory experiences

In order to generate precocious olfactory experiences we provided scented sugar solution within the wooden boxes for a defined period after emergence. Young bees learn in this way to associate the scent with the sugar reward so that precocious olfactory conditioning takes place. This method has repeatedly proved to be successful in studying olfactory learning within the hive (Farina *et al.*, 2005; Grüter *et al.*, 2006; Arenas *et al.*, 2007, 2008; Arenas & Farina, 2008). Sucrose solution, 1.8 M, was offered in a 10-mL feeder, which presented an orifice of 1 mm diameter that allowed the access of one bee at a time and simultaneously reduced possible volatile contamination of the the box. A control group of bees was fed with an unscented solution throughout the whole experimental period. Experimental groups had the same unscented solution from days 0 to 4 and from days 9 to 17. From days 5–8, they received 1-NON-scented sugar solution (50 μL of pure odorant in 1 L of 1.8 M sucrose solution). Olfactory learning during this period induces better retention performance at the adult stage (Arenas & Farina, 2008).

After day 17, bees were either tested for behavioral responses to odors in the PER protocol or they were used for optophysiological recordings of AL neural activity upon olfactory stimulation. In both conditions, four different odors were presented at two different concentrations: pure (1/1) and ten times diluted in distilled water (1/10); 1-NON, the familiar odor, and three novel odors, NONA, HEXA and 1-HEX. The odors tested were chosen based on perceptual similarity relationships established for odors in the honeybee (Guerrieri *et al.*, 2005). Generalization responses to odors in bees depend on carbon-chain length and functional group (Guerrieri *et al.*, 2005; Table 1) so that we chose odors that varied in these dimensions (functional group: aldehydes NONA and HEXA, primary alcohols 1-NON and 1-HEX; carbon-chain length: C6 1-HEX and HEXA, and C9 1-NON and NONA).

Behavioral responses

Conditioned responses after early olfactory experience were evaluated on day 17 using the PER protocol (Takeda, 1961; Bitterman *et al.*, 1983). Adult worker honeybees from the experimental cages were cooled on ice and harnessed in individual metal holders so that they could only move their mouthparts and antennae freely. Harnessed bees were kept in an incubator (30°C, 55% relative humidity, darkness) for ∼2–3 h before the test. Only bees that showed the unconditioned response (the reflexive extension of the proboscis to 1.8 M sucrose solution applied to the antennae) and that did not respond to the mechanical stimulus (airflow) were used (i.e., > 80% of the experimental bees). An olfactometer that delivered a continuous airflow of 50 mL/s into which controlled odorant pulses could be introduced was used to stimulate individual bees (Guerrieri *et al.*, 2005). To this end, 4 μL of pure odorant were applied to a piece of filter paper (30 × 3 mm), which was placed inside a syringe. Syringes were placed in the olfactometer and connected to a glass tube (1 cm in diameter), placed 2 cm in front of the bees' head. Odors were delivered through a secondary air stream (2.5 mL/s) injected into the main airflow through a system of valves controlled by a computer. Each test lasted 40 s. During the first 15 s, the bee received the clean airflow so that possible responses to the mechanical stimulation could be assessed. Then, the odorant was delivered for 6 s and a response

TABLE 1. Perceptual similarity among odorants in the honeybee

	1-NON	NONA	1-HEX	HEXA
(a) Training/Testing				
1-NON	100	70.37	37.5	32
NONA	54.17	82.61	25	65.22
1-HEX	14.81	29.17	79.17	66.67
HEXA	11.11	17.24	14.81	78.26
(b) Similarity to 1-NON				
NONA	62.27			
1-HEX	26.16			
HEXA	21.56			

Perceptual similarity among odors used in our study (data from Guerrieri *et al.*, 2005). (a) Generalization matrix representing the percentage of proboscis extension responses (PER) observed in nonrewarded tests (columns) after three-trial conditioning to each of four odors (lines). Only bees that actually learned the CS during training, i.e. that responded to the CS at the third conditioning trial, are shown. (b) Behavioral odor similarity between 1-nonanol (the treatment odor) and the three test odors, calculated as the average between generalization scores for a pair of odors. Similarity varied according to differences in both the functional group and the carbon-chain length between the learned and tested odors. Nonanal is thus more perceptually similar to 1-nonanol than to 1-hexanol or hexanal.

was recorded if the bee showed a PER to the odorant within that period. The bee remained in the experimental site exposed to the clean airflow for the next 19 s until completing 40 s. Each bee received the four odors in a randomized order, with an intertrial interval of 15 min. An extraction fan placed behind the bee was used to avoid odor contaminations.

Chi-squared tests were used to compare PER to each odor between treated and control bees (Sokal & Rohlf, 1995). To reduce the risk of type 1 errors due to the multiple use of the same data, we corrected the significance thresholds using the Bonferroni method ($\alpha' = \alpha/k$), with $\alpha = 0.05$ and $k = 4$. Thus, our significance threshold was $\alpha' = 0.0125$.

Optophysiological recordings

Honeybee preparation and staining

Seventeen-day-old bees were caught from the experimental cages, cooled on ice and mounted individually in a recording chamber. The head was fixed with low temperature-melting wax (Deiberit 502, Böhme & Schöps Dental GmbH, Goslar, Germany) and the antennae, oriented to the front, were fixed with a very thin wire inserted into the wax, and which gently pushed down the antennae without damaging them. A small pool containing saline solution was formed around the head by waxing small pieces of plastic foil to the front and sides of the chamber. To complete the seal of the pool, the bases of the flagella were glued with two-component silicon (KwikSil; World Precision Instruments, FL, USA) separating the brain region (under saline solution) from the antennae. The antennae remained in the air and could therefore be stimulated with odors. A small piece of cuticle was removed from the front of the head capsule. All glands, membranes and trachea covering the brain were carefully removed to expose both ALs. Systematically the preparation was bathed with saline solution (in mM: NaCl, 130; KCl, 6; $MgCl_2$, 4; $CaCl_2$ 5; sucrose, 160; glucose, 25; and HEPES, 10; pH 6.7, 500 mOsmol; all chemicals from Sigma-Aldrich, Lyon, France). For staining, saline solution was completely removed and the brain was bathed with 50 μL dye solution (50 μg Calcium-Green-2AM dissolved with 50 μL Pluronic F-127, 20% in dimethylsufoxide) in 800 μL saline (Calcium-Green and Pluronic from Molecular Probes, Oregon, USA). After a 1-h incubation (in darkness and cooled on ice) the brain was repeatedly washed to eliminate the remaining dye solution. When necessary, the abdomen was immobilized with low temperature-melting wax to reduce movements of the preparation.

Optical recording

In vivo calcium imaging recordings were carried out using a TILL Photonics imaging system (Martinsried, Germany). Stained bees were placed under an epifluorescent microscope (Olympus BX51WI; Olympus, Hamburg, Germany) with a 10 × water-immersion objective (NA 0.3; Olympus), and the brain region was covered with saline. A series of 100 frames, at a rate of 5 frames/s, was recorded with a 640 × 480 pixel 12-bit monochrome CCD-camera (TILL Imago). Odor stimuli, given at the 15th frame, lasted 1 s. The monochromatic excitation light (475 nm) was provided by a monochromator (TILL Polychrom IV). The filter set on the microscope was composed of a 505-nm dichroic filter and an LP 515-nm emission filter. Throughout the recording, a constant clean airflow of 50 mL/s was delivered through a tube (1 cm in diameter), placed 2 cm in front of the bee's head. Four microlitres of odorant (pure or diluted 1/10) was applied to a filter paper 30 × 3 mm, which was placed inside a pipette. The odorant was delivered through a secondary air stream (2.5 mL/s) injected into the main airflow through a system of valves controlled by a computer. The intertrial interval between stimulations was ~80 s. The order of odor presentations was randomized between bees but started with the odors at the lowest concentration. All experimental bees were tested three times with each odor at a given concentration.

Mapping of glomeruli

To reveal the glomerular structure of the AL after optical imaging, we used a mixture 125:1 (vol/vol) of a protease solution (from *Bacillus licheniformis* in propylene glycol; Sigma Aldrich) and of the dye RH795. The former was used to digest brain sheaths (dissolved in absolute ethanol) and the latter to stain cell membranes (Molecular Probes). One hour later, the brain was washed with saline. Fluorescence photographs were taken at 5–10 different focal planes, using the monochromator at 530 nm excitation light, with a filter set composed of a 570-nm dichroic filter and a 590-nm emission filter. After editing the photographs (Adobe Photoshop) to make the glomerular borderlines more visible, individual glomeruli were identified using the standardized AL atlas (Galizia *et al.*, 1999b). A total of 19 glomeruli were identified in each individual bee. A reconstructed glomerular mask allowed determination the coordinates of the different glomeruli in the calcium imaging data. Additional glomeruli that were recognized in only some preparations were not included in the analysis.

Data processing

Calcium-imaging data were analyzed using software written in IDL (Research Systems Inc., Colorado, USA). Each recording performed upon odor stimulation corresponded to a three-dimensional array with two spatial dimensions (x, y pixels of the area of interest) and a temporal dimension (100 frames). Raw data were filtered in both the spatial and in the temporal dimensions using a median filter with a size of 3 pixels in order to reduce photon (shot) noise. To correct for bleaching and for possible irregularities of lamp illumination in the temporal dimension, we subtracted from each pixel in each frame the median value of all the pixels of that frame, thus stabilizing the baseline of the recordings. Finally, relative fluorescence changes ($\Delta F/F$) were calculated as $(F–F_0)/F_0$. Background fluorescence (F_0) was defined as an average of three frames before any odor stimulation (frames 5–7). The signals evoked by the odors were the typical stereotyped signals obtained with bath-applied Calcium Green. They exhibit two components: a first one corresponding to a fast fluorescence increase and a second one corresponding to a slow fluorescence decrease below baseline (Galizia *et al.*, 1997; Stetter *et al.*, 2001; Sandoz *et al.*, 2003). We focused on the fast (positive) signal evoked by odor stimulation, which is thought to correspond mostly to intracellular calcium entry from the extracellular medium due to neuronal activity in olfactory-receptor neurons (Galizia *et al.*, 1998; Sachse & Galizia, 2003). For each identified glomerulus, the time course of relative fluorescence changes was calculated by averaging 25 pixels (5 × 5) at the centre of each glomerulus, and well within its borders. Response amplitude was calculated as the mean of frames at the signal's maximum (frames 22–24) minus the mean of three frames before the stimulus (frames 12–14). The median of the three stimuli with each stimulus was calculated within one animal. In a few cases (< 20%) we eliminated a whole run of stimulations which was too noisy and instead computed the median of only two stimulations.

To ask whether early odor–rewarded experiences change the intensity of odor-evoked responses in the AL, we quantified the global intensity of activation, calculated as the sum of the glomerular response (ΔF/F) in all 19 identified glomeruli within each animal. We also compared the number of active glomeruli that responded to each odor between treated and untreated bees. We considered that a

glomerulus was activated when the amplitude of the response was above a noise level defined as 2 SD of an average signal obtained before the stimulus (frames 1–14). To determine whether early olfactory experiences change the quality of odor representations, we analyzed whether similarity between the activity pattern of the experienced odor and those of the three test odors was modified. To quantify similarity between patterns, we calculated Pearson's correlation coefficients between responses in the 19 glomeruli.

Moreover, as the neural representation of an odor can be regarded as a vector in a multidimensional space in which each dimension is represented by a particular glomerulus, evaluation of similarity between the neural representations of odors was calculated using a Euclidian metric (Deisig *et al.*, 2006). As it is not possible to visualize vectors in an *n*-dimensional space, principal component analysis (PCA) was used to project the data into a lower-dimensionality space formed of a subset of highest-variance components.

Statistics

The effect of early olfactory experience on the three variables (overall activation, global intensity and number of active glomeruli) was evaluated using repeated-measures ANOVA, with one factor being treatment (two levels) and the other odor (four levels, except for neural odor similarity with three levels). Simple effects were used for describing interactions detected in the ANOVA. Scheffé tests were performed for *post hoc* comparisons. Odor concentration was not included as a third factor as treated groups did not present equal numbers of observations. Therefore data were analyzed separately for the two concentrations.

Results

Behavioral responses

Figure 1 shows the PER (%) of 17-day-old bees to each odor tested. Bees that experienced 1-NON-scented sugar solution between days 5 and 8 after emergence responded significantly more to two out of the four tested odors (Fig. 1). One of them was 1-NON, the odor associated with sucrose at a precocious stage. In this case, significant differences between treated and untreated bees were found at both concentrations used ($\chi^2_{\text{1-NON 1/1}} = 28.74$; $P < 0.001$, df = 1; $\chi^2_{\text{1-NON 1/10}} = 35.10$; $P < 0.001$, df = 1; Fig. 1A and B). The other odor for which an increase in responses was found was NONA, a novel odor that presents a high perceptual similarity to 1-NON (see Table 1). This increase was again found at both concentrations used ($\chi^2_{\text{NONA 1/1}} = 11.68$; $P < 0.001$, df = 1; $\chi^2_{\text{NONA 1/10}} = 8.83$; $P = 0.003$; df = 1). Responses to 1-HEX and HEXA did not differ between treated and control bees at either concentration. We conclude that bees, which experienced 1-NON associated with sucrose solution at the age of 5–8 days, remembered this odor and generalized their responses to the similar aldehyde NONA.

Optophysiological responses

Calcium responses to four different odors were monitored in the AL of early-experienced bees as well as of untreated bees, at the age of 17 days (Fig. 2). In both treated and untreated bees, different odorants elicited different glomerular activity patterns (Fig. 2). The same odorant elicited similar activation patterns in treated and untreated bees at both concentrations used. To further compare odor-evoked responses in the AL of treated and untreated bees, we evaluated responses according to three main variables: global intensity, number of activated glomeruli, and odor similarity.

We found that early experience with sucrose solution scented with 1-NON led to an increase in the global intensity of the response to 1-NON, but also to the three other odors (Fig. 3A and B). For both concentrations, global intensity appeared higher in experienced than in untreated bees but differences only reached statistical significance when odors were presented at the 1/1 concentration (Repeated-measures two-way $\text{ANOVA}_{\text{Treatment-1/1}}$, $F = 4.97$, $P = 0.047$, df = 1; $\text{ANOVA}_{\text{Treatment-1/10}}$, $F = 0.502$, $P = 0.494$, df = 1). Neither the odor

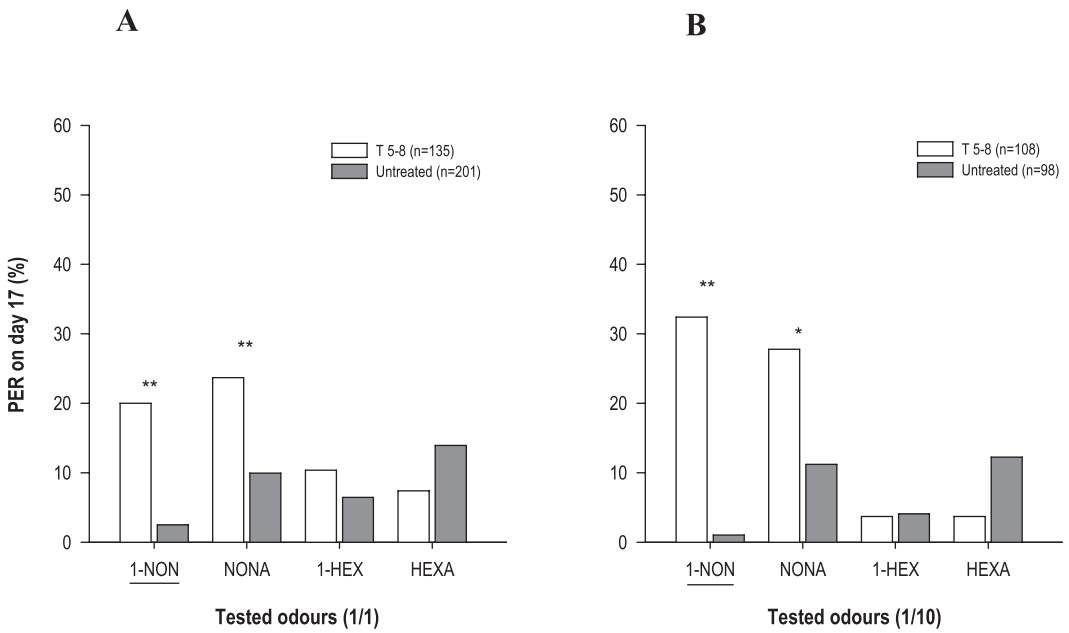

FIG. 1. Proboscis extension responses (PER) of 17-day-old bees to the experienced odor (1-NON) and to three novel odors (NONA, 1-HEX and HEXA). (A) Test odors presented at 1/1 concentration. (B) Tested odors presented at 1/10 concentration. Asterisks indicate statistical differences in χ^2 after Bonferroni correction (*$P < 0.0125$, **$P < 0.001$; NS, not significant; see Results for details). Sample sizes are given in the key.

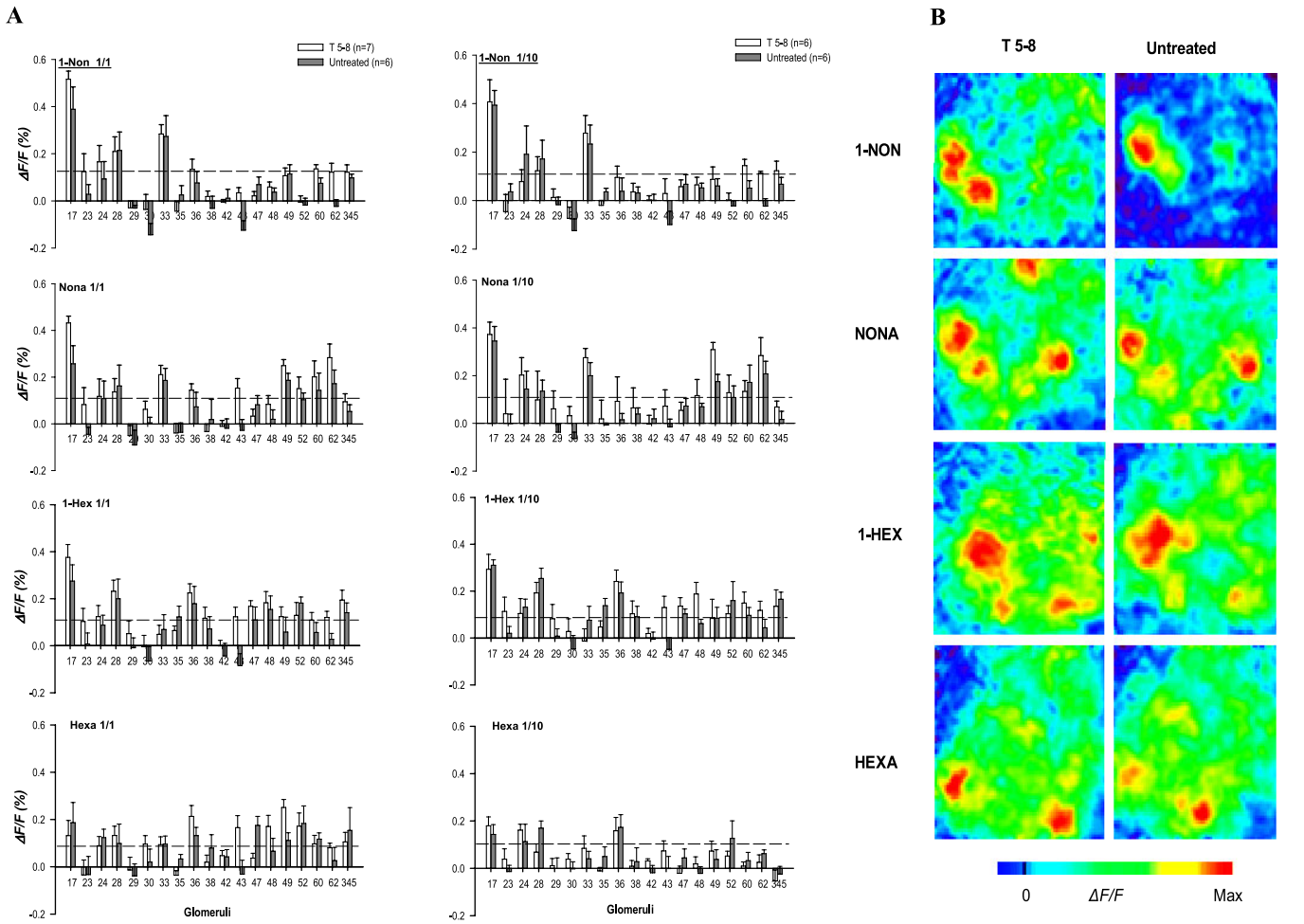

FIG. 2. Glomerular responses in the antennal lobe of 17-day-old bees. (A) Response of 19 identified glomeruli to the experienced odor (1-NON) and to three novel odors (NONA, 1-HEX and HEXA) presented at 1/1 or 1/10 concentrations. Intensities are expressed as relative fluorescence change during odor presentation ($\Delta F/F$) ± SEM. The hatched lines show average noise level (2 SD of signal before odor presentation). (B) Example of false-color-coded activity patterns in an early conditioned bee (T5-8) and an untreated bee. Quantitative changes in odor-evoked activity patterns were observed in T5-8 for both the rewarded (1-NON) and the non-rewarded (NONA) similar odor, but not for HEXA, the most dissimilar one.

effect nor the treatment × odor interaction were significant (repeated-measures two-way $ANOVA_{Treatment\text{-}odor\text{-}1/1}$, $F = 1.244$, $P = 0.309$, df = 3; $ANOVA_{odor\text{-}1/1}$ $F = 2.057$, $P = 0.124$, df = 3), which suggests that the increase in global intensity observed at day 17 after precocious learning of 1-NON generalized to all odors. Thus, early olfactory experience quantitatively modified the glomerular pattern of the learned odor but also of the other test odors (Fig. 3A). At the 1/10 concentration only an odor effect was found (repeated-measures two-way $ANOVA_{Odor\text{-}1/10}$, $F = 7.342$, $P < 0.001$, df = 3), meaning that different odors activate the lobe differently. No effect of interaction was found ($ANOVA_{Treatment \times Odor\text{-}1/10}$, $F = 0.776$, $P = 0.516$, df = 3).

Figure 4 shows the number of active glomeruli as measured on day 17 for the four odorants tested in treated and untreated bees. As for global intensity, we found a significant effect of early olfactory experience on the number of active glomeruli only when odors were presented at the 1/1 concentration (repeated-measures two-way $ANOVA_{Treatment\text{-}1/1}$, $F = 5.419$, $P = 0.040$, df = 1, $ANOVA_{Treatment\text{-}1/10}$, $F = 0.001$, $P = 0.968$, df = 1). Again, the increase in the number of active glomeruli was observed for the experienced odor 1-NON but also for novel odors, and accordingly the treatment × odor interaction was not significant (repeated-measures two-way $ANOVA_{Treatment\text{-}odor\text{-}1/1}$, $F = 0.317$, $P = 0.805$, df = 3. The number of active glomeruli was different between the tested odors at both concentrations (repeated-measures two-way $ANOVA_{Odor\text{-}1/1}$, $F = 3.257$, $P = 0.033$, df = 3; repeated-measures two-way $ANOVA_{Odor\text{-}1/10}$, $F = 6.984$, $P = 0.001$, df = 3). Thus, the observed increase in general activity may be explained by an increase in the number of active glomeruli upon olfactory stimulation in experienced bees.

We then asked whether early olfactory experience changed odor-evoked activity patterns in such a way that the neural similarity among odors was modified, in particular between the experienced odor and the novel odors. To compare neural odor similarity for the different odorants and concentrations, we calculated Pearson correlation coefficients (*r*-values) between the neural responses to the experienced odor (1-NON) and those to the novel odors (NONA, 1-HEX and HEXA). A perfect match between glomerular activity patterns elicited by two odors corresponds to an *r*-value of 1. Figure 5 presents the r-values obtained for correlations between 1-NON and each of the three novel odors. For treated bees, 1-NON was qualitatively more similar to NONA than to 1-HEX, than to HEXA. Accordingly, an odor effect was found for both concentrations (repeated-measures two-way $ANOVA_{Odor\text{-}1/1}$, $F = 10.750$, $P < 0.001$, df = 2; repeated-measures two-way $ANOVA_{Odor\text{-}1/10}$, $F = 8.929$, $P = 0.002$, df = 2). The overall odor effect was explained by the differences in r-values between

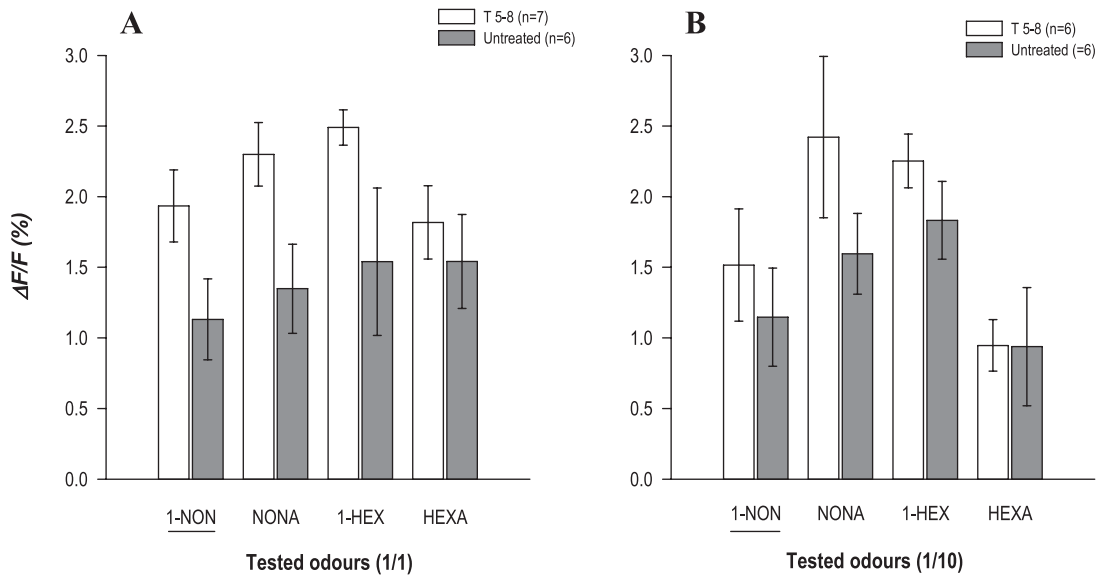

FIG. 3. Global intensity, obtained as the sum of relative fluorescence changes ($\Delta F/F$) in the 19 identified glomeruli of the AL of 17-day-old bees. (A) Global intensity elicited by 1-NON as experienced odor and NONA, 1-HEX and HEXA as novel odors, presented at 1/1 concentration. A two-way ANOVA shows a significant difference in global intensity between treated and untreated bees (treatment factor, $P < 0.05$). (B) Global intensity elicited by the same odors presented at 1/10 concentration. The ANOVA shows no difference due to treatment but a significant effect of test odor (odor factor, $P < 0.05$). Sample sizes are given in the key.

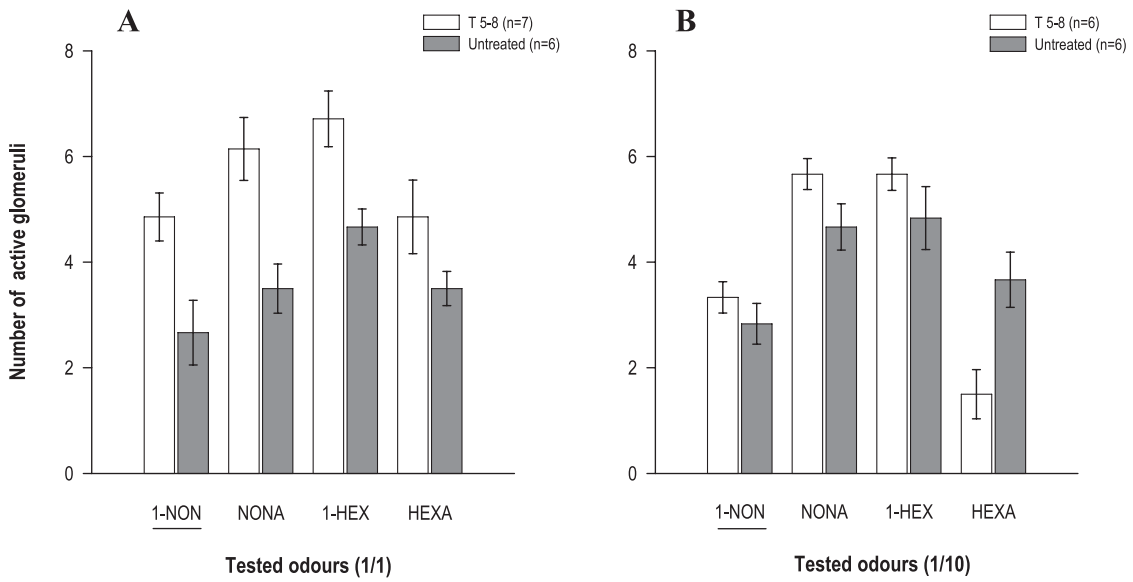

FIG. 4. Number of active glomeruli in the AL of 17-day-old bees. (A) Number of active glomeruli obtained for 1-NON as treated odor and NONA, 1-HEX and HEXA as novel odors, presented at 1/1 concentration. A two-way ANOVA shows a significant difference between treated and untreated bees (treatment factor, $P < 0.05$) but also for the different test odors (odor factor, $P < 0.05$). (B) Number of active glomeruli recruited by the same odors presented at 1/10 concentration. A two-way ANOVA shows that there is a significant difference between the number of active glomeruli elicited by the test odors (odor factor, $P < 0.05$) when presented at this concentration. The number of active glomeruli was calculated as the number of glomeruli that showed response amplitude above noise level. Sample sizes are given in the key.

1-NON/NONA and 1-NON/HEXA (Scheffé *post hoc* comparison$_{1/1}$, $P = 0.0009$; Scheffé *post hoc* comparison$_{1/10}$, $P = 0.0024$) and 1-NON/1-HEX and 1-NON/HEXA (Scheffé *post hoc* comparison$_{1/1}$, $P = 0.0074$; Scheffé *post hoc* comparison$_{1/10}$, $P = 0.0217$). While 1-NON/NONA and 1-NON/1-HEX pairs gave highly positive correlations as many of the most active glomeruli were present in both patterns, 1-NON/HEXA showed a lower correlation index. This similarity order based on glomerular activity patterns matches perfectly the bees' generalization behavior quantified in terms of PER responses to a conditioned odor (see Table 1 and Guerrieri *et al.*, 2005). It also agrees with our behavioral results showing high generalization to NONA after early experience to 1-NON. Comparison of this neural similarity between treated and untreated bees yielded nonsignificant results for both concentrations (repeated-measures two-way ANOVA$_{\text{Treatment-1/1}}$, $F = 0.384$, $P = 0.547$, df = 1; repeated-measures two-way ANOVA$_{\text{Treatment-1/10}}$, $F = 1.830$, $P = 0.205$, df = 1).

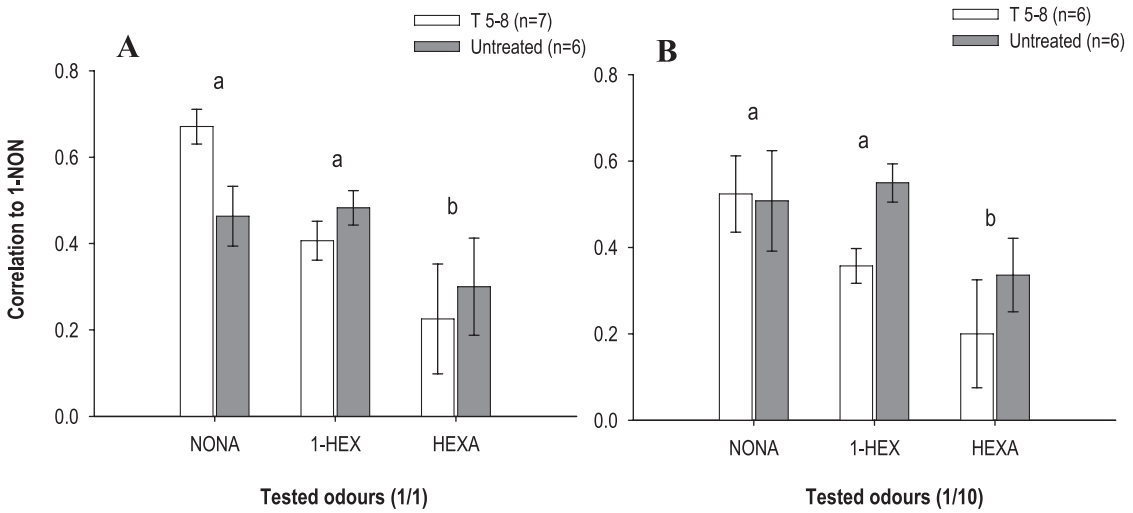

FIG. 5. Physiological odor similarity in the AL of 17-day-old bees. (A) Correlation index (*r*-values) for the experienced odor (1-NON) versus each novel odor (NONA, 1-HEX and HEXA) presented at 1/1 concentration (see text). (B) Correlation index (*r*-values) as a function of the same pairs of odors at 1/10 concentration. No significant differences appeared according to treatment, but different odor pairs show different similarities (ANOVA, odor factor, $P < 0.05$). Different letters indicate significant differences in *post hoc* comparisons. Sample sizes are given in the key.

To understand the relationships between neural representations of odors in both treated and untreated bees, we represented odor responses in a putative olfactory space in which activity recorded in each glomerulus constitutes a dimension of the neural space (Fig. 6). We then performed a PCA on individual data to extract the factors accounting for the variance observed. The first three factors extracted represented 36.8, 12.3 and 8.9% of overall variance, respectively; i.e. a total of 58%. In Fig. 6A, we present the median average values of the coordinates derived from the PCA analysis for the first three factors. In order to better analyze the contributions of these factors, we represented our data for both treated and untreated bees in two-dimensional graphs showing Factor 1 vs. Factor 2 (Fig. 6B) and Factor 2 vs. Factor 3 (Fig. 6C), respectively The SEM was provided for the mean value of each group. Figure 6B shows that Factor 1, which provided the major contribution to variance, segregated responses mainly according to carbon chain length. Indeed, 9-carbon stimuli appeared clearly separated from 6-carbon ones according to this factor. Factor 2 (Fig. 6B) clearly segregated the data in terms of the functional group, primary alcohols and aldehydes, aldehydes being placed in the upper part of the graph with the alcohols being placed in the lower part. Interestingly, this factor also segregated data in terms of precocious experience. Indeed, treated-bee data mostly appeared above untreated-bee data (Fig. 6B). Segregation of the neural representations of treated animals relative to control animals is particularly clear in the case of 1-NON but can also be seen for NONA and HEXA, whereas odor representation remained almost unchanged for 1-HEX. This suggests that early experience introduced a general modification of odor representation, encompassing at least three out of the four tested odors. Thus, Factor 2, the second most important contribution to variance in our dataset, consisted of an interaction between two variables, functional group and precocious olfactory learning. Figure 6C shows Factor 3, in which no clear correlation with the potential variables could be detected. The contribution to overall variance of this factor was, however, low (8.9%).

Discussion

Our work shows that early olfactory learning during young adulthood, i.e. 5–8 days after emergence, affects neural activity in the honeybee AL on a long-term scale, i.e. at the age of 17 days, when bees commonly initiate foraging activities (Rösch, 1925; Lindauer, 1952; Seeley, 1982). Conditioned odors evoke enhanced glomerular activity and modified spatiotemporal response patterns, changes that might underlie a long-term olfactory memory for the early-experienced odor. In that sense, our study constitutes the first post-conditioning recordings of antennal lobe activity performed in a temporal window that corresponds to the so-called late long-term memory (l-LTM; see Menzel, 1999), a memory phase characterized by the presence of a robust and stable memory trace that can be retrieved from 72 h after conditioning and that is protein synthesis-dependent. In contrast to studies on olfactory long-term memory in bees (see Menzel, 1999 for review), we did not control olfactory acquisition at the individual level given that the odorant was delivered in the sucrose solution placed within the cages where bees were reared. A previous study using the same experimental conditions (Arenas & Farina, 2008) ensures nevertheless that food–odor associations are acquired by bees at the age of 5–8 days and that such associations can be retrieved several days later in a long-term memory test. Arenas & Farina (2008) showed that experiencing scented food at the age of 5–8 days induces better memory retention when bees are 17 days old than experiencing the same scented food at the age of 9–12 days. Due to this nonlinear effect of early olfactory experience, it was proposed that consolidation of early olfactory memories established at 5–8 days induces a rapid structural–functional change in the AL that accompanies the high plasticity of behavior, especially because the olfactory system finishes its maturation at that stage (Masson & Arnold, 1987; Winnington *et al.*, 1996).

As we did not test the effect of olfactory experience at other time periods (for instance 9–12 days of age), we cannot yet establish the specificity of the observed effects with respect to the time window of sensory experience. We show, nevertheless, that early experience can alter odor-induced activity in the primary olfactory center of bees. It has been proposed that experience-dependent plasticity in primary olfactory structures is more robust in developing physiological systems than in mature ones (Woo & Leon, 1987; Hamrick *et al.*, 1993). This relates to the idea that peripheral brain regions are more likely to be modified by earlier experience, while more central regions are prone to be modified by later experience (Wilson & Sullivan,

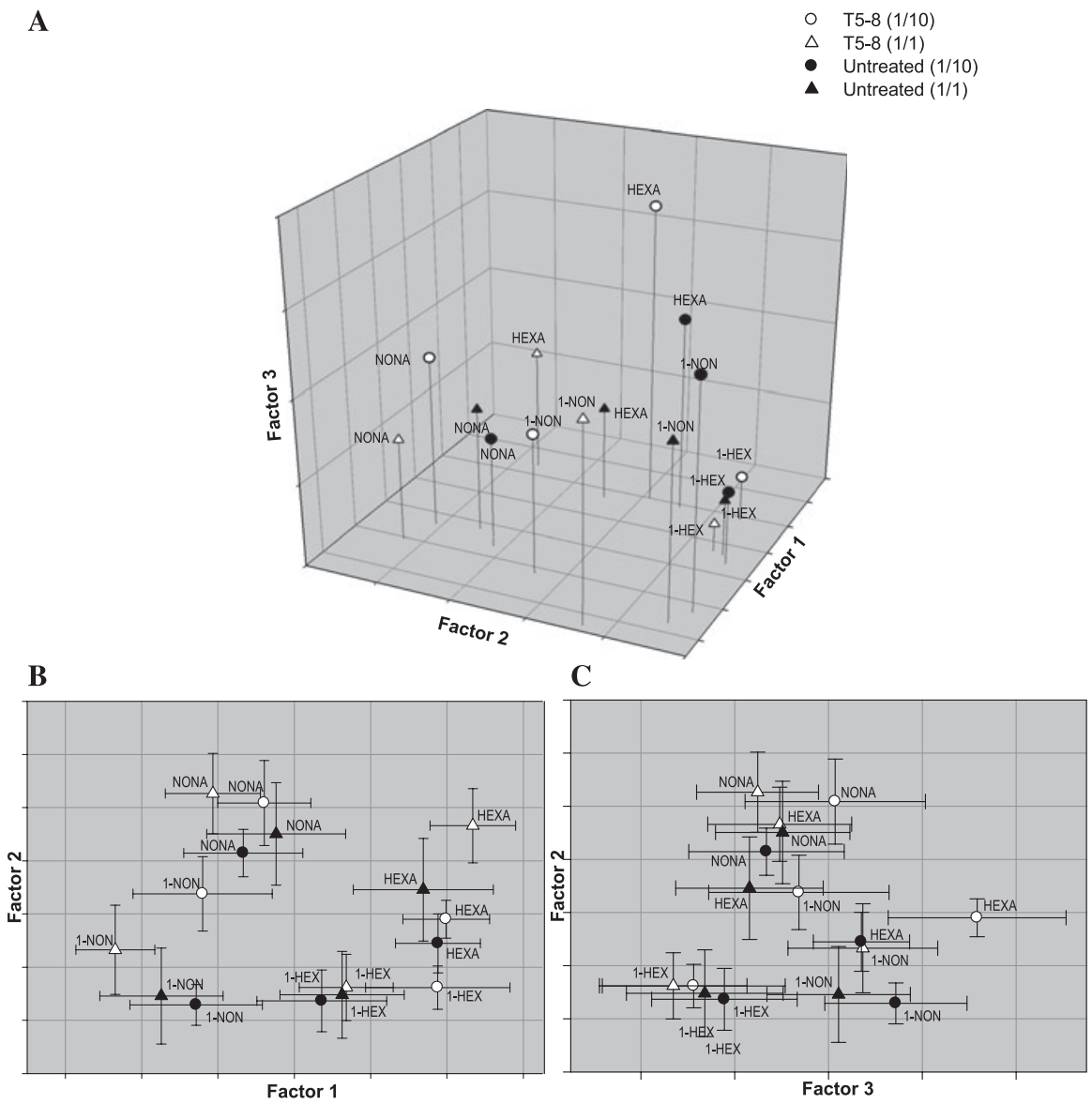

FIG. 6. Odor representations in the AL of 17-day-old bees. (A) Representation of the relative position of odor response patterns according to three principal factors after a PCA that accounted for 57.94% of overall data variance. (B) Odor representation according to Factors 1 and 2; and (C) according to Factors 2 and 3. For a better illustration of the neural representation of each group we only show the median values of the coordinates obtained from the PCA outcome. In B and C we provided these mean values ± SEM for each group. Positions of olfactory response patterns of precocious treated bees are indicated in white while those of untreated appear in black. Different concentrations are presented with distinctive symbols (circles for odors presented at the lower concentration during testing and triangles for those presented at the higher one). Odors with the same chain length are grouped according to Factor 1 (36.8% of total variance). Chemical functional group and precocious experience are set by Factor 2 and represent 12.3% of total variance. Factor 3 accounts for 8.9% of variance.

1994). The enduring odor–response changes observed in treated bees support this hypothesis and open the question of whether early odor experience at 5–8 days of age is indeed critical for the normal development and maturation of the olfactory system (Gascuel & Masson, 1991; Masson *et al.*, 1993).

Experience with odors, both food odors and pheromones, is supposed to have an impact on the normal development of the olfactory system. A prominent example is the case of the queen mandibular pheromone (QMP). Exposure to QMP at a young age has been shown to alter dopamine levels in the brain of workers (Beggs *et al.*, 2007) and to have a strong impact on aversive learning abilities of young bees (Vergoz *et al.*, 2007). Thus, changes in AL neural activity evinced at the adult ages after precocious olfactory conditioning may result from a normal maturation process that may take place in the bee colony.

Olfactory associative learning has been shown to induce functional modifications at the neurophysiological level in mammals (Coopersmith & Leon, 1984; Sullivan & Leon, 1986; Wilson & Leon, 1988). In insects, the establishment of odor–reward associations leads to changes in neural activity in the principal structures of the olfactory circuit, the antennal lobes and the mushroom bodies. Such changes are thought to constitute the neural correlate of memory traces that can be visualized from the short-term to the long-term post conditioning (e.g. flies, Yu *et al.*, 2004; moths, Daly *et al.*, 2004; locusts, Bazhenov *et al.*, 2005). In honeybees, whether olfactory learning leads to modification of neural activity in the antennal lobes has been the

subject of debate. While Faber *et al.* (1999) and Sandoz *et al.* (2003) reported changes in glomerular activation after olfactory conditioning, Peele *et al.* (2006) did not find any change in antennal lobe activity after different conditioning protocols. It is difficult, however, deciding between these results given that they involve different conditioning protocols (elemental vs. nonelemental) and different recording times after conditioning.

In *Drosophila*, Yu *et al.* (2004) used a protein-based reporter of synaptic activity in the ALs and showed that, after pairing an odor and an electric shock, certain glomeruli acquired enhanced synaptic activity for ~5 min after training. This suggests that the AL hosts an olfactory memory trace for only a few minutes after conditioning. Electrophysiological data in locusts (Bazhenov *et al.*, 2005) and moths (Daly *et al.*, 2004) also support a role for the ALs in short-term olfactory memory. Recently, it was shown that projection neurons in *Drosophila* host a long-term memory as olfactory learning induces glomerular patterns of protein synthesis that are specific to the learned odor (Ashraf *et al.*, 2006). These patterns of local protein synthesis were detected as early as 3 h after training and maintained for at least 24 h post-training. Although our findings are in line with findings in *Drosophila*, caution is required in interpreting our data due to potential limitations of our recording technique. Firstly, due to the bath staining procedure used in which the entire brain was stained, the recorded fluorescence signals could represent the combined activity of several neuronal populations of the AL including olfactory receptor neurons, local interneurons, projection neurons and even glial cells (Heil *et al.*, 2007). From these candidates, receptor neurons seem to provide the most important contribution to the signals recorded (Galizia *et al.*, 1998; Galizia & Vetter, 2005). Thus, the observed changes in glomerular responses would represent changes in odor-evoked responses of afferent olfactory neurons. Given the results obtained in *Drosophila* (see above), it would be worth performing new optophysiological experiments using our experimental protocols but focusing on the activity of projection neurons which can be specifically stained for calcium imaging experiments (Sachse & Galizia, 2002). Secondly, optophysiological measurements as done here only concern the ventral part of the AL, which is the region accessible when the head capsule is opened for recordings (~12% of the ~160 glomeruli of the bee AL). Although the responses of glomeruli imaged have proved to be enough to account for behavioral perceptual assessments of odor similarity in bees (Guerrieri *et al.*, 2005), we cannot exclude the possibility that early olfactory experience affects differently other regions of the AL. Moreover, it must be kept in mind that other brain regions such as the mushroom bodies are likely to be involved and affected by early olfactory conditioning as performed in our work.

On the other hand, early olfactory experiences may modify not only functional but also structural properties of the AL, potentially changing the sizes of certain glomeruli, as has been reported in *Drosophila* (Devaud *et al.*, 2001, 2003). These experiments showed that exposing flies to a single odor for several days causes a stimulus-dependent decrease in glomerular volume. Structural changes of the *Drosophila* AL could be induced and even reversed within a critical period early in the adult life following CO_2 exposure (Sachse *et al.*, 2007). It should be kept in mind that our early exposure cannot be directly compared with those performed in *Drosophila* (Devaud *et al.*, 2001, 2003; Sachse *et al.*, 2007) as, in our case, bees experienced food together with the odor. For this reason, we do not necessarily expect a decrease in glomerular volume; on the contrary, one could argue that simultaneous activation of the sucrose reinforcing system and the olfactory system may lead to increases in glomerular size. As our procedure leads to long-term memory formation, which is protein synthesis-dependent, one could expect that such a process is accompanied by an increase in the synaptic branching within glomeruli, thus resulting in larger glomerular sizes. This possibility will be addressed in future work.

Olfactory similarity is known to influence behavioral discrimination in honeybees (e.g. Deisig *et al.*, 2002). One important finding of our study was that the plasticity of odor responses did not only affect responses to the experienced odor (1-NON) but tended to generalize to novel test odors such as NONA (Fig. 5A). At the AL level, a similar observation was previously made in the honeybee in which differential conditioning led to an increase in glomerular response intensity for the rewarded odor but also for a novel odor, while the response to the nonrewarded odor remained unchanged (Faber *et al.*, 1999). In our experiments, NONA and 1-HEX presentations induced increased general activity in treated bees (Fig. 3) and a higher number of activated glomeruli (Fig. 4). This effect was somewhat lower for HEXA, the odor least chemically and perceptually similar to the experienced odor, 1-NON (see Table 1). Thus, we found a general tendency towards an increase in activity for those odors exhibiting more similarity with the experienced odor. The similarity relationships between 1-NON and the three test odors shown in Table 1 (taken from the work by Guerrieri *et al.*, 2005) were generally confirmed in the present study, both at the behavioral level (bees exposed to 1-NON also responded to NONA) and at the neural level when measuring physiological similarities between neural response patterns (Figs 5 and 6). This last effect was clearer for treated bees, in which similarity with 1-NON decreased from NONA (the most similar) to HEXA (the least similar), following the same pattern found in behavioral experiments (Table 1; Guerrieri *et al.*, 2005). There, responses of untreated bees to HEXA were higher, albeit not significantly, than the responses elicited by the same odor in treated subjects. This finding may be influenced by olfactory memories that increased responses to the CS (and CS-similar odors), but at the same time would decrease responses to the most dissimilar odors such as HEXA. Figure 5 also provides some evidence for this: in treated bees, 1-NON/HEXA perceptual similarities were lower than within the untreated group, probably due to more specific olfactory responses after associative learning. For untreated bees, 1-HEX and NONA were equally dissimilar to 1-NON at both concentrations. Therefore, we conclude that early olfactory learning appears to affect both behavioral and neural responses not only to the experienced odor but also to unknown odors depending on their perceptual similarity to the experienced one.

This conclusion is strongly supported by our PCA analysis (see Fig. 6), which identified three main factors accounting for 58% of dataset variance. The two first factors clearly corresponded to the molecular structure of the odorants, i.e. carbon-chain length and functional group. This result confirms previous analyses underlining the importance of these dimensions for insect olfactory perception (e.g. Guerrieri *et al.*, 2005). However, Factor 2 also segregated data in terms of precocious olfactory learning as it separated treated from untreated bees, an effect that was clearer for 9-carbon odorants than for 6-carbon ones. It seems therefore that changes in the neural space of treated animals relative to control ones depend on the similarity between the molecular structure of the tested and experienced odors. This finding underlines the fact that precocious learning induces qualitative long-term changes in AL that can be easily tracked when odors which were very similar (NONA) or dissimilar (HEXA) to the rewarded one were tested.

At the neural level, precocious learning significantly affected general AL activity and the number of recruited glomeruli but only at the higher odor concentration (1/1). This effect is not due to differences in the overall intensity of calcium signals or the number of activated

glomeruli at the two concentrations, as these variables were found to be very similar at the two concentrations in untreated bees. It seems rather to be a more specific effect at one concentration. Increasing the intensity of an odor can qualitatively and quantitatively change its glomerular activation pattern (Sachse & Galizia, 2003). Indeed, higher concentrations induce activation in more glomeruli, thus promoting more overlapping with other odor-evoked patterns. Accordingly, odor concentration qualitatively affects odor perception in behavioral experiments (Ditzen *et al.*, 2003; : Wright *et al.*, 2005). In our experimental setup, we cannot directly compare the concentration of the odor experienced by bees at days 5–8 with that offered in the PER or imaging experiments. Therefore, we may interpret the fact that plasticity was clearer at the higher concentration as indicative that this higher concentration (1/1) was perceived for bees as more similar to that offered during the early odor experience than the lower one (1/10).

In conclusion, the retrieval of early olfactory memories is associated with an increase in odor-evoked neural activity in the primary olfactory center of the honeybee brain. Besides its role in reformatting odor representations, the antennal lobe has been suggested as a site for olfactory memory formation. Although neuropharmacological experiments (Hammer & Menzel, 1998) have revealed a short-lived memory trace residing in the antennal lobe shortly after conditioning, our results suggest that this structure may also take part in the storage of long-term memories acquired precociously during the first days of adulthood. An alternative hypothesis to consider is that the observed changes in antennal lobe activity in fact reflect retrograde modulation of glomerular activity patterns by the mushroom bodies, the structures that have been traditionally considered the main site hosting long-term memories. Further experiments focusing on feedback neurons from the mushroom bodies to the antennal lobes are necessary to elucidate this question.

It also seems that the odors experienced by a bee during days 5–8 after emergence will be prioritized afterwards when this bee becomes a forager. In other words, early olfactory experiences might bias foraging activities. Given the complex olfactory landscape existing within a hive and the fact that dominant odors may change periodically as a result of changing crops, the honeybee colony will have a pool of foragers with variable olfactory predispositions. This will enhance the chances of a colony of responding efficiently to a changing floral market.

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Abbreviations

1-HEX, 1-Hexanol; 1-NON, 1-Nonanol; AL, antennal lobe; HEXA, Hexanal; NONA, Nonanal; PCA, principal component analysis; PER, proboscis extension responses.

References

Arenas, A. & Farina, W.F. (2008) Age and rearing environment interact in the retention of early olfactory memories in honeybees. *J. Comp. Physiol. A.*, **194**, 629–640.

Arenas, A., Fernández, V.M. & Farina, W.M. (2007) Floral odor learning within the hive affects honeybees' foraging decisions. *Naturwissenschaften*, **94**, 218–222.

Arenas, A., Fernández, V.M. & Farina, W.M. (2008) Floral scents experienced within the colony affect long-term foraging preferences in honeybees. *Apidologie*, **39**, 714–722.

Ashraf, S.I., McLoon, A.L., Sclarsic, S.M. & Kunes, S.N. (2006) Synaptic protein synthesis associated with memory is regulated by the RISC pathway in Drosophila. *Cell*, **124**, 191–205.

Bazhenov, M., Stopfer, M., Sejnowski, T.J. & Laurent, G. (2005) Fast odor learning improves reliability of odor responses in the locust antennal lobe. *Neuron*, **46**, 483–492.

Beggs, K.T., Glendining, K.A., Marechal, N.M., Vergoz, V., Nakamura, I., Slessor, K.N. & Mercer, A.R. (2007) Queen pheromone modulates brain dopamine function in worker honey bees. *Proc. Natl. Acad. Sci. U S A*, **104**, 2460–2464.

Bitterman, M.E., Menzel, R., Fietz, A. & Schaefer, S. (1983) Classical conditioning of proboscis extension in honeybees. *J. Comp. Psychol.*, **97**, 107.

Carlsson, M.A., Galizia, C.G. & Hansson, B.S. (2002) Spatial representation of odours in the antennal lobe of the moth *Spodoptera littoralis* (Lepidoptera: Noctuidae). *Chem. Senses*, **27**, 231–244.

Caubet, Y., Jaisson, P. & Lenoir, A. (1992) Preimaginal induction of adult behaviour in insects. *Q. J. Exp. Psychol. B*, **44**, 165–178.

Coopersmith, R. & Leon, M. (1984) Enhanced neural response to familiar olfactory cues. *Science*, **4664**, 849–851.

Daly, K.C., Christensen, T.A., Lei, H., Smith, B.H. & Hildebrand, J.G. (2004) Learning modulates the ensemble representations for odors in primary olfactory networks. *PNAS*, **101**, 10476–10481.

Deisig, N., Lachnit, H. & Giurfa, M. (2002) The effect of similarity between elemental stimuli and compounds in olfactory patterning discriminations. *Learn. Mem.*, **9**, 112–121.

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.

Devaud, J.M., Acebes, A. & Ferrús, A. (2001) Odor exposure causes central adaptation and morphological changes in selected olfactory glomeruli in *Drosophila*. *J. Neurosci.*, **21**, 6274–6282.

Devaud, J.M., Acebes, A., Ramaswami, M. & Ferrús, A. (2003) Structural and functional changes in the olfactory pathway of adult *Drosophila* take place at a critical age. *J. Neurobiol.*, **56**, 13–23.

Ditzen, M., Evers, J.F. & Galizia, C.G. (2003) Odor similarity does not influence the time needed for odor processing. *Chem. Senses*, **28**, 781–789.

Faber, T., Joerges, J. & Menzel, R. (1999) Associative learning modifies neural representations of odors in the insect brain. *Nat. Neurosci.*, **2**, 74–78.

Farina, W.M., Grüter, C. & Diaz, P.C. (2005) Social learning of floral odours inside the honeybee hive. *Proc. R. Soc. B*, **272**, 1923–1928.

Friedrich, R.W. & Korsching, S.I. (1997) Combinatorial and chemotopic odorant coding in the zebrafish olfactory bulb visualized by optical imaging. *Neuron*, **18**, 737–752.

Fuch, E., Dustmann, H., Stadler, H. & Schürmann, F.W. (1989) Neuroactive compounds in the brain of the honeybee during imaginal life. *Comp. Biochem. Physiol.*, **92C**, 337.

Galizia, C.G. & Vetter, R. (2005) Optical methods for analyzing odor-evoked activity in the insect brain. In Christensen, T.A. (Ed.), *Advances in Insect Sensory Neuroscience*. CRC, Boca Raton, FL, pp. 349–392.

Galizia, C.G., Joerges, J., Kuttner, A., Faber, T. & Menzel, R. (1997) A semi-in vivo preparation for optical recording of the insect brain. *J. Neurosci. Methods*, **76**, 61–69.

Galizia, C.G., Nägler, K., Hölldobler, B. & Menzel, R. (1998) Odour coding is bilaterally symmetrical in the AL of honeybees (*Apis mellifera*). *Eur. J. Neurosci.*, **10**, 2964–2974.

Galizia, C.G., McIlwrath, S.L. & Menzel, R. (1999a) A digital three-dimensional atlas of the honeybee AL based on optical sections acquired using confocal microscopy. *Cell Tissue Res.*, **295**, 383–394.

Galizia, C.G., Sachse, S., Rappert, A. & Menzel, R. (1999b) The glomerular code for odor representation is species specific in the honeybee *Apis mellifera*. *Nat. Neurosci.*, **2**, 473–478.

Gascuel, J. & Masson, C. (1991) Developmental study of afferented and deafferented bee antennal lobes. *J. Neurobiol.*, **22**, 795–810.

Giurfa, M. (2007) Behavioral and neural analysis of associative learning in the honeybee: a taste from the magic well. *J. Comp. Physiol. A.*, **193**, 801–824.

Grüter, C., Acosta, L.E. & Farina, W.M. (2006) Propagation of olfactory information within the honeybee hive. *Behav. Ecol. Sociobiol.*, **60**, 707–715.

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

Hammer, M. & Menzel, R. (1998) Multiple sites of associative odor learning as revealed by local brain microinjections of Octopamine in honeybees. *Learn. Mem.*, **5**, 146–156.
Hamrick, W.D., Wilson, D.A. & Sullivan, R.M. (1993) Neural correlates of memory for odor detection conditioning in adult rats. *Neurosci. Lett.*, **163**, 36–40.
Heil, J.E., Oland, L.A. & Lohr, C. (2007) Acetylcholine-mediated axon-glia signaling in the developing insect olfactory system. *Eur. J. Neurosci.*, **26**, 1227–1241.
Hildebrand, J.G. & Shepherd, G.M. (1997) Mechanisms of olfactory discrimination: converging evidence for common principles across phyla. *Annu. Rev. Neurosci.*, **20**, 595–631.
Ichikawa, N. & Sasaki, M. (2003) Importance of social stimuli for the development of learning capability in honeybee. *Appl. Entomol. Zool.*, **38**, 203–209.
Iso, H., Simoda, S. & Matsuyama, T. (2007) Environmental change during postnatal development alters behaviour, cognitions and neurogenesis of mice. *Behav. Brain Res.*, **179**, 90–98.
Joerges, J., Küttner, A., Galizia, C.G. & Menzel, R. (1997) Representations of odours and odour mixtures visualized in the honeybee brain. *Nature*, **387**, 285–288.
Lindauer, M. (1952) Ein Beitrag zur Frage der Arbeitsteilung im Bienenstaat. *Z. vergl. Physiol.*, **34**, 299–345.
Lorenz, K. (1935) Der Kumpan in der Umwelt des Vogels. *Zeitschrift für Ornithologie*, **83**, 137–213.
Masson, C. & Arnold, G. (1984) Ontogeny, maturation and plasticity of the olfactory system in the worker bee. *J. Insect Physiol.*, **30**, 7–14.
Masson, C. & Arnold, G. (1987) Organization and plasticity of the olfactory system of the honeybee, *Apis mellifera*. In Menzel, R. & Mercer, A. (Eds), *Neurobiology and Behavior of Honeybee*. Springer Verlag, Berlin, Heidelberg, p. 281.
Masson, C., Pham- Delègue, M.H., Fonta, C., Gascuel, J., Arnold, G., Nicolas, G. & Kerszberg, M. (1993) Recent advances in the concept of adaptation to natural odour signals in the honeybee *Apis mellifera* L. *Apidologie*, **24**, 169–194.
Menzel, R. (1999) Memory dynamics in the honeybee. *J. Comp. Physiol. A.*, **185**, 323–340.
Oitzl, M.S., Workel, J.O., Fluttert, M., Frosch, F. & De Kloet, E.R. (2000) Maternal deprivation affects behaviour from youth to senescence: Amplification of individual differences in spatial learning and memory in senescent Brown Norway rats. *Eur J Neurosci*, **12**, 3771–3780.
Peele, P., Ditzen, M., Menzel, R. & Galizia, C.G. (2006) Appetitive odor learning does not change olfactory coding in a subpopulation of honeybee antennal lobe neurons. *J. Comp. Physiol. A.*, **192**, 1083–1103.
Rösch, G.A. (1925) Untersuchungen über die Arbeitsteilung im Bienenstaat. *Z. vergl. Physiol.*, **2**, 571–631.
Rubin, B.D. & Katz, L.C. (1999) Optical imaging of odorant representations in the mammalian olfactory bulb. *Neuron*, **23**, 499–511.
Sachse, S. & Galizia, C.G. (2002) The role of inhibition for temporal and spatial odor representation in olfactory output neurons: a calcium imaging study. *J. Neurophysiol.*, **87**, 1106–1117.
Sachse, S. & Galizia, C.G. (2003) The coding of odour-intensity in the honeybee antennal lobe: local computation optimizes odor representation. *Eur. J. Neurosci.*, **18**, 2119–2132.
Sachse, S., Rappert, A. & Galizia, C.G. (1999) The spatial representation of chemical structures in the AL of honeybees: steps towards the olfactory code. *Eur. J. Neurosci.*, **11**, 3970–3982.
Sachse, S., Rueckert, E., Keller, A., Okada, R., Tanaka, N.K., Ito, K. & Vosshall, L.B. (2007) Activity-dependent plasticity in an olfactory circuit. *Neuron*, **56**, 838–850.
Sandoz, J.C., Laloi, D., Odoux, J.F. & Pham-Delègue, M.H. (2000) Olfactory information transfer in the honeybee: compared efficiency of classical conditioning and early exposure. *Anim. Behav.*, **59**, 1025–1034.
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.
Seeley, T.D. (1982) Adaptive significance of the age polytheism schedule in honeybee colonies. *Behav. Ecol. Sociobiol.*, **11**, 287–293.
Sokal, R.R. & Rohlf, F.J. (1995) *Biometry: The Principles and Practice of Statistics in Biological Research*, 3rd Edn. W. H. Freeman and Co., New York, pp. 715.
Stetter, M., Greve, H., Galizia, C.G. & Obermayer, K. (2001) Analysis of calcium imaging signals from the honeybee brain by nonlinear models. *Neuroimage*, **13**, 119–128.
Sullivan, R.M. & Leon, M. (1986) Early olfactory learning induces and enhanced olfactory bulb response in young rats. *Dev. Brain Res.*, **27**, 278–282.
Takeda, K. (1961) Classical conditioned response in the honey bee. *J. Insect Physiol.*, **6**, 168–179.
Uchida, N., Takahashi, Y.K., Tanifuji, M. & Mori, K. (2000) Odor maps in the mammalian olfactory bulb: domain organization and odorant structural features. *Nat. Neurosci.*, **3**, 1035–1043.
Vergoz, V., Schreurs, H.A. & Mercer, A.R. (2007) Queen pheromone blocks aversive learning in young worker bees. *Science*, **317**, 384–386.
Wang, W.S., Zhang, S., Sato, K. & Srinivasan, M.V. (2005) Maturation of odor representation in the honeybee antennal lobe. *J. Insect Physiol.*, **51**, 1244–1254.
Wilson, D.A. & Leon, M. (1988) Noradrenergic modulation of olfactory bulb excitability in the postnatal rat. *Dev. Brain Res.*, **42**, 69–75.
Wilson, D.A. & Sullivan, R.M. (1994) Neurobiology of associative learning in the neonate: Early olfactory learning. *Behav. Neural Biol.*, **61**, 1–18.
Winnington, A., Napper, R.M. & Mercer, A.R. (1996) Structural plasticity of identified glomeruli in the antennal lobes of the adult worker honey bee. *J. Comp. Neurol.*, **365**, 479–490.
Woo, C.C. & Leon, M. (1987) Sensitive period for neural and behavioral response development to learned odors. *Dev. Brain Res.*, **36**, 309–313.
Wright, G.A., Thomson, M.G.A. & Smith, B.H. (2005) Odour concentration affects odour identity in honeybees. *Proc. R. Soc. B*, **272**, 2417–2422.
Yu, D., Ponomarev, A. & Davis, R.L. (2004) Altered representation of the spatial code for odors after olfactory classical conditioning: memory trace formation by synaptic recruitment. *Neuron*, **42**, 437–449.