

Neural representation of olfactory mixtures in the honeybee antennal lobe

Nina Deisig,¹ Martin Giurfa,¹ Harald Lachnit² and Jean-Christophe Sandoz¹

¹Research Centre for Animal Cognition, CNRS – Université Paul Sabatier (UMR 5169), 31062 Toulouse cedex 04, France

²Department of Psychology, Philipps-University of Marburg, Marburg, Germany

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Abstract

Natural olfactory stimuli occur as mixtures of many single odors. We studied whether the representation of a mixture in the brain retains single-odor information and how much mixture-specific information it includes. To understand mixture representation in the honeybee brain, we used *in vivo* calcium imaging at the level of the antennal lobe, and systematically measured odor-evoked activity in 24 identified glomeruli in response to four single odorants and all their possible binary, ternary and quaternary mixtures. Qualitatively, mixture-induced activity patterns always contained glomeruli belonging to the pattern of at least one of the components, suggesting a high conservation of component information in olfactory mixtures. Quantitatively, glomerular activity saturated quickly and increasing the number of components resulted in an increase of cases in which the response of a glomerulus to the mixture was lower than that to the strongest component ('suppression'). This shows global inhibition in the antennal lobe, probably acting as overall gain control. Single components were not equally salient (in terms of number of active glomeruli) and mixture activity patterns were always more similar to the more salient components, in a way that could be predicted linearly. Thus, although a gain control system in the honeybee antennal lobe prevents saturation of the olfactory system, mixture representation follows essentially elemental rules.

Introduction

Most natural sensory stimuli occur in combination with other stimuli. Therefore, the question of whether animals perceive the elements of a stimulus compound (elemental processing, e.g. Rescorla & Wagner, 1972; Rescorla, 1972, 1973) or treat a compound as an entity different from its elements (configural processing, e.g. Pearce, 1987, 1994) has been the subject of intensive debate both within and across sensory modalities. Within modality, the difficulty in deciding between these alternatives is well illustrated by the case of olfactory mixtures, which may allow the recognition of certain components (i.e. single odors) but which can also be perceived as distinct entities.

Olfactory mixture processing can be addressed using the honeybee *Apis mellifera*, which is a useful model for studies on olfaction (e.g. Deisig *et al.*, 2001, 2002, 2003; Guerrieri *et al.*, 2005) as intracolony life and recognition of profitable floral food sources depend to a great extent on olfactory cues (Menzel *et al.*, 1993). Honeybees detect odors with ~60 000 olfactory receptor neurons (ORNs) on each antenna. ORNs have broad and overlapping response profiles (Vareschi, 1971; Akers & Getz, 1993; Getz & Akers, 1994) such that odor identity has to be extracted from the combination of multiple parallel receptor inputs, i.e. across-fiber. ORNs project into the antennal lobe (AL), the first olfactory neuropile, analogous to the vertebrate olfactory bulb. The AL consists of 160 identified glomeruli (compared with >1000 non-identified in mammals), which are interconnected by local, inhibitory interneurons (~4000) (Fonta *et al.*, 1993). Processed

olfactory information is further conveyed by ~800 projection neurons (PNs) to higher-order brain centers (mushroom bodies or lateral protocerebrum, Abel *et al.*, 2001). Because of its numerical simplicity and the possibility to identify single glomeruli reliably across individuals, the honeybee AL is especially suited for the study of olfactory mixture processing.

Previous electrophysiological studies on mixture interactions in different species compared responses of individual ORNs to mixtures and their components (e.g. Ache *et al.*, 1988; Caprio *et al.*, 1989; Akers & Getz, 1993; Kang & Caprio, 1997; Steullet & Derby, 1997; Duchamp-Viret *et al.*, 2003) and revealed complex interactions with both antagonistic and synergistic phenomena. As the neural representation of an odor encompasses the whole combinatorial pattern of receptor activation, the analysis of mixture processing should consider as much of the actual information conveyed to the AL or olfactory bulb as possible. Optophysiological measures of AL or olfactory bulb activity are particularly valuable to this goal. In the honeybee, calcium imaging of AL activity confirmed that odor quality is a distributed code, represented by combinatorial activity patterns across glomeruli (Joerges *et al.*, 1997; Faber *et al.*, 1999; Galizia *et al.*, 1999a). The first of these studies (Joerges *et al.*, 1997) included a few recordings in which activity patterns for binary or ternary olfactory mixtures and their components were recorded. Joerges *et al.* (1997) observed that calcium signals to the mixtures included the glomeruli responding to the single elements. Moreover, they also showed that mixture responses were not pure sums of component responses, a fact which suggested inhibitory interactions. Since then, intensive behavioral work has allowed clear predictions to be made concerning how mixtures are processed in the honeybee brain (Deisig *et al.*, 2001,

Correspondence: Dr Nina Deisig, as above.

E-mail: deisig@cict.fr

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2002, 2003; Lachnit *et al.*, 2004). Based on our behavioral work and on the work of Joerges *et al.* (1997), we initiated a systematic study of mixture processing in the bee brain, starting at the level of the AL. In particular, we asked how the neural representation of a mixture relates to the representations of its components. We thus performed calcium imaging in the AL and measured odor-evoked activity in 24 identified glomeruli in response to four single odorants and all their possible binary, ternary and quaternary mixtures. Based on a staining method that is thought to emphasize ORN activity, we show that similarity between a mixture and its components can be linearly predicted based solely on the magnitude of the responses to each component (i.e. number of glomeruli) despite the occurrence of overall inhibitory phenomena. Thus, elemental processing predominates at the periphery of the honeybee olfactory pathway.

Materials and methods

Preparation and staining

Adult worker honeybees (*A. mellifera*) were caught at the entrance of outdoor hives in the morning of each experimental day. They were cooled on ice and mounted individually in a Plexiglas chamber. The head was fixed with low-temperature-melting wax (Deiberit 502, Böhme & Schöps Dental GmbH, Goslar, Germany) and a small pool of Ringer solution for imaging was created on top of the head by fixing additional walls on top of the chamber (see Sandoz *et al.*, 2003). The antennae were oriented to the front using very small insect needles and their base was fixed with two-component silicon (KwikSil; World Precision Instruments, FL, USA) providing a seal between the flagellae (remaining in the air so that they could be stimulated with odors) and the brain region (under Ringer solution). At the beginning of the preparation, a small window was cut in the head cuticle to expose the brain. All glands, membranes and trachea covering the brain were carefully removed to reveal the two ALs. To restrict possible movements of the ALs during optical recordings, the esophagus was removed. Throughout the preparation, the brain was regularly washed with Ringer (in mM: NaCl, 130; KCl, 6; MgCl₂, 4; CaCl₂, 5; sucrose, 160; glucose, 25; HEPES, 10; pH 6.7, 500 mOsmol; all chemicals from Sigma-Aldrich, Lyon, France). Before staining, illumination was reduced and switched to red light. Ringer was completely removed with a small piece of paper tissue and the brain was bathed with 50 μ L dye solution [50 μ g calcium green 2-AM dissolved with 50 μ L pluronic F-127 (20% in dimethylsulfoxide) in 800 μ L Ringer (calcium green and pluronic from Molecular Probes, Eugene, OR, USA)]. The piece of head cuticle was then replaced in its original position and the bee was left for 90 min on an ice bed. After staining, the brain was thoroughly washed with Ringer to remove the remaining dye solution, the residual membrane tissues near the ALs were removed and the abdomen immobilized with low-temperature-melting wax, paying attention to leave the spiracles free.

Optical recordings of odor-evoked activity

In vivo calcium imaging recordings were carried out using a T.I.L.L. Photonics imaging system (Martinsried, Germany). Bees were placed individually under an epifluorescent microscope (Axioscop, Zeiss, Jena, Germany) with a 20 \times water-immersion objective (NA 0.5, Olympus, Hamburg, Germany) and the brain region was covered with Ringer. One AL was recorded in each bee. Images were taken using a 640 \times 480-pixel 12-bit monochrome CCD camera (T.I.L.L. Imago) cooled to -12 °C. Each measurement consisted of 100 frames at a rate

of 5 frames/s (integration time for each frame: 10–15 ms). Odor stimuli were given at the 15th frame for 1 s. Pixel image size corresponded to $\sim 5 \times 5$ μ m after 4×4 binning on chip. Monochromatic excitation light at 475 nm was applied using a monochromator (T.I.L.L. Polychrom IV). The filter set on the microscope was composed of a 505-nm dichroic filter and a long pass 515-nm emission filter. Before recording, a computer-driven odor-supplying device (for detailed description see Galizia *et al.*, 1997) was prepared by mounting 1-mL plastic syringes in six valve positions, either containing filter paper (1 cm²) without any odor substance (for a pure, constant air-stream) or containing filter paper loaded with 4 μ L of pure odor substance. This concentration was chosen because it was used in all our previous behavioral experiments on olfactory mixture processing (Deisig *et al.*, 2001, 2002, 2003) and thus allows our physiological measurements to be compared to our behavioral data. Once a bee was placed under the microscope, the odor-supplying device was positioned 2 cm in front of the bee's antennae. Most of the time, the bee received a pure and constant air-stream. Odor stimulations were controlled directly from the optical imaging software (T.I.L.L. Vision) by switching the airflow from a syringe containing a blank filter paper to odor-loaded syringes. In the case of mixture stimulation, the airflow was directed into the syringes containing the odor components, mixing them during stimulation, such that single odors added up in the mixture. We used 1-hexanol, 2-octanol, (+/-) linalool and limonene (all from Sigma-Aldrich) as these single odors were previously used in behavioral experiments investigating mixture learning in bees (Deisig *et al.*, 2001, 2002, 2003). We included 1-nonanol (as it elicits conspicuous glomerular responses) and air (as it does not elicit responses) as controls. Each bee was subjected to a maximum of three runs of 17 stimulations each: the four single odors (A, B, C and D), their binary (AB, AC, AD, BC, BD and CD), ternary (ABC, ACD, BCD and ABD) and quaternary (ABCD) mixtures as well as air and 1-nonanol. As preparations did not last equally long, not all three runs could be performed in all bees. For analysis, only complete runs were kept. The intertrial interval between stimulations was 80 s. The order of odor presentations was randomized between bees.

Mapping of glomeruli

To better visualize the glomerular structure of the AL after functional optical imaging, a mixture (125 : 1, v/v) of a protease solution (from *Bacillus licheniformis* in propylene glycol; Sigma-Aldrich), to digest the brain sheath, and of the dye RH795 (dissolved in absolute ethanol), to stain cell membranes (Molecular Probes), was applied to the brain for 1 h. The brain was then carefully washed with Ringer, and fluorescence photographs were taken at 10–20 different focal planes, using the polychromator at 530-nm excitation light, with a filter set composed of a 570-nm dichroic filter and long pass 590-nm emission filter. Photographs were then contrast-enhanced and subjected to an unsharp filter in Adobe PHOTOSHOP, in order to make the glomerular borderlines more visible. Individual glomeruli were then identified using the standardized AL atlas (Fig. 1A, Galizia *et al.*, 1999b). A total of 24 glomeruli, 23 innervated by the T1 tract and 1 by the T3 tract could be identified in all AL preparations (Fig. 1B). In this work, all glomeruli belonging to the T1 tract will be referred to by their number only (e.g. glomerulus 28 will correspond to T1-28). For each bee, the reconstructed glomerular mask allowed determination of the coordinates of the different glomeruli in the calcium imaging data (Fig. 1C). A few additional glomeruli were recognized in only a few preparations and were therefore not included in the analysis.

Signal calculation

Calcium-imaging data were analysed using custom-made software written in IDL (Research Systems Inc., CO, USA). Each odor stimulation recording corresponded to a three-dimensional matrix with two spatial dimensions (x, y pixels of the area of interest) and a temporal dimension (100 frames). Three steps were carried out to calculate the signals. First, to reduce photon (shot) noise, the raw data were filtered in both the spatial and temporal dimensions using a median filter with a size of 3 pixels. Second, relative fluorescence changes (called $\Delta F/F$) were calculated as $(F - F_0)/F_0$, taking as reference background F_0 the average of three frames before any odor stimulation, in this case from frame 5 to frame 7. Third, 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. This correction allows stabilization of the baseline of the recordings but does not remove pertinent signals. Odor-evoked signals were the typical stereotyped biphasic signals usually obtained with bath application of calcium green, with first a fast fluorescence increase and afterwards a slow fluorescence decrease below baseline (Galizia *et al.*, 1997; Stetter *et al.*, 2001; Sandoz *et al.*, 2003). The maximum signal was obtained about 1.4 s after odor onset (around frame 23) and the minimum about 9 s after odor onset (around frame 60). For visual observation of the repartition of the signals in the different glomeruli (Figs 1 and 2), we chose to show activity maps with the best possible spatial definition of odor-induced signals. We thus represented the amplitude of the signal from the maximum around frame 23 to the minimum around frame 60. Thus, each pixel represents the mean of its values at frames 22–24 minus the mean of its values at frames 59–61. Examples of activity maps showing the amplitude of odor responses are presented in Figs 1 and 2 in a false-color code, from dark blue (no signal) to red (maximum signal).

For quantitative analysis of the data (Figs 3–7), we focused our analysis on the fast (positive) signal component evoked by odor stimulation, which is related to an intracellular calcium increase from the extracellular medium, thought to reflect mostly pre-synaptic neuronal activity from ORNs (Galizia *et al.*, 1998; Sachse & Galizia, 2003; see also Discussion). For each glomerulus recognized after the reconstruction of AL structure, the time course of relative fluorescence changes was calculated by averaging 25 pixels (5×5) at the center of each glomerulus and well within its borders. The amplitude of odor-induced responses was calculated as the mean of three frames at the signal's maximum (frames 22–24) minus the mean of three frames before the stimulus (frames 12–14). This value was then used in all computations.

Analysis rationale

We used a range of different analyses in five main steps to study the relationships between mixture- and component-induced signals, both at the level of individual glomeruli and at the level of the whole pattern of 24 recorded glomeruli accessible to us.

Calcium signals to single odors and mixtures

We first asked whether mixture activity patterns include the glomeruli activated by their single odors. We did this in two ways.

- (i) At the level of individual bees, we compared the activity patterns obtained for the different mixtures, with patterns obtained after calculating arithmetic sums of the signals to the single odors (Fig. 2A and B). For the original data (Fig. 2A), the one to three measurements obtained with each stimulus were averaged pixel-

wise and activity maps were then scaled to a common minimum and maximum. For the calculated data (Fig. 2B), the map of a mixture ABC was obtained by adding pixel-wise the amplitudes of the signals obtained for the three odors A, B and C when presented alone. The maps for the binary, ternary and quaternary mixtures were each scaled independently to allow activated glomeruli to be clearly visible.

- (ii) Overall activity maps. As we could recognize the same set of 24 glomeruli in 10 different bees, we could build a physiological atlas of mixture activity, as was done for many odors by Galizia *et al.* (1999a). For this, the response amplitudes of each glomerulus (5×5 pixels, see above) for each odor were averaged within bees (one to three runs). We then considered the maximum response of each bee as 100% and normalized all its responses accordingly. After averaging data over all 10 individuals, we represented the response of each glomerulus in typical activity maps (Fig. 3A and B), using a color code and a glomerular map layout similar to that introduced by Galizia *et al.* (1999a).

Response magnitude (number of active glomeruli)

We asked how the magnitude of AL activity changes with added mixture components. We thus evaluated the number of activated glomeruli with increasing numbers of mixture components (Fig. 4A and B). A glomerulus was considered as activated when the amplitude of its response was higher than 20% of this bee's maximum, which was above noise level (see below). For the different odor types, we also represented the distribution of glomerular response in 20% classes, i.e. the number of glomeruli showing 20–40, 40–60, 60–80 or 80–100% response. Numbers of active glomeruli were compared between response classes and odor types (primary odor, binary, ternary and quaternary mixtures) using repeated-measure ANOVAs whenever possible (equal observation numbers).

Mixture interactions: suppression, hypoadditivity and synergy

We wanted to unravel possible inhibitory interactions between components by comparing within each glomerulus how the response to a mixture relates to the response to its components. A previous study (Duchamp-Viret *et al.*, 2003) applied a response classification from psycho-physical studies, comparing the responses to a mixture and to the strongest component (the single odor inducing the highest response in this glomerulus). For each mixture, we thus classified each glomerulus according to three categories: the response to the mixture could be higher than ('synergy'), equal to ('hypoadditivity') or lower than ('suppression') the response to the strongest component. As, according to such a classification, glomeruli that do not respond to any of the components or to the mixture would fall erroneously in the hypoadditivity class, we only classified glomeruli which showed heterogeneity in the responses to the components and mixture. Thus, a fourth category ('no difference') contained non-coding glomeruli, i.e. glomeruli in which responses to the mixture and to all of its components were equal. We defined a threshold to decide whether the response of a given glomerulus to stimulus A was different from that to stimulus B. To obtain a common measure of variability of glomerular responses, we computed the average SD of glomerular responses to each of the single odorants. We found values of 19, 11, 15 and 12% for 1-hexanol, limonene, 2-octanol and linalool, respectively (mean 14%). Thus, for categorizing responses as explained above, we used a minimum difference of 15% (of the maximal responses in the animal considered) between mixture and component responses. Repartitions in the different classes were tested using repeated-measure ANOVAs whenever possible (equal observation numbers).

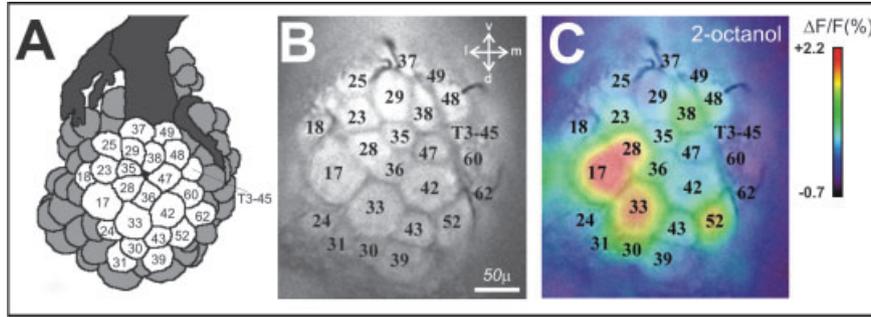


FIG. 1.

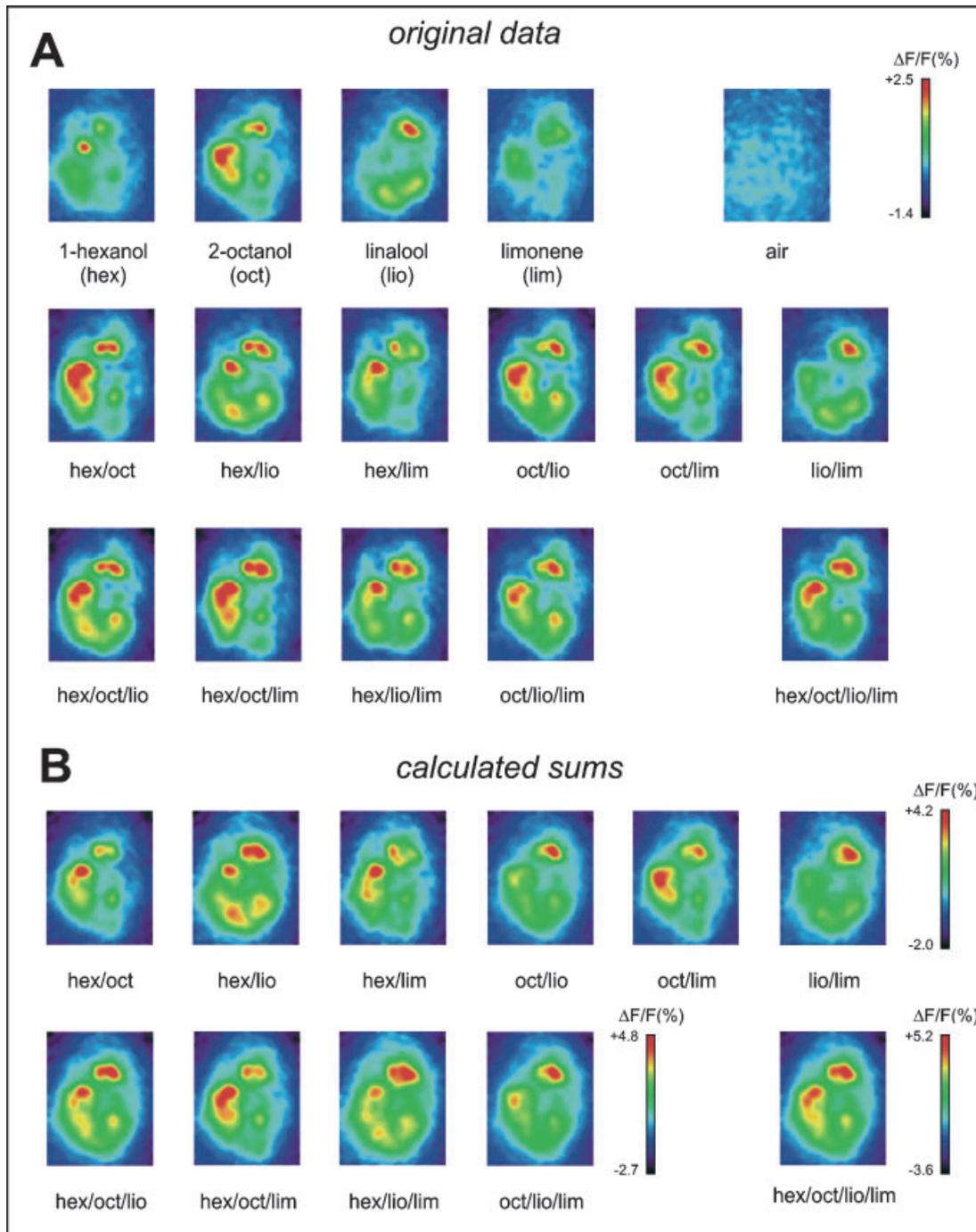


FIG. 2.

Similarity between mixture and single odor representations

We then focused on the whole odor representation, and attempted to classify the different mixtures and components according to their proximity in a putative neural olfactory space, using multivariate analyses (Fig. 5). 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 (thus, in our case a 24-dimensional space). As it is not possible to visualize vectors in a 24-dimensional space, we used a principal component analysis which identifies orthogonal axes (factors) of maximum variance in the data, and thus projects the data into a lower-dimensionality space formed of a subset of the highest-variance components. A varimax rotation, which maximizes the variance of the calculated factors while minimizing the variance around them, was applied. The first six factors represented 52.5, 14.6, 10.2, 7.1, 6.1 and 2.5% overall variance, respectively. We chose the first four factors (together representing 84.4%) for representing each odorant in a figure (Fig. 5A).

To evaluate the relationships between the representations of each mixture and the representation of its components, we calculated how close they were to each other in the putative olfactory space by calculating their Euclidian distance, d , in the 24-dimensional space as:

$$d_{ij} = \sqrt{\sum_{k=1}^p (X_{ik} - X_{jk})^2}$$

with i and j indicating odors, p the number of dimensions, i.e. glomeruli, and X_{ik} the response in glomerulus k to odor i .

To give a comprehensive view of these relationships between odors, we performed cluster analyses to group single odors and the different mixtures according to their distance in the olfactory space, using Ward's classification method.

Linearity of mixture representation

Lastly, we tested the hypothesis that mixture representation in the AL is elemental, i.e. whether the individual characteristics of the mixture components determine their relative similarity to the mixture. Our prediction was that if the mixture representation is elemental, then information about each component should be present in the mixture representation relative to the physiological salience of this component, i.e. the more strongly a single odor activated the AL, the more information about this odor should be present in the mixture's representation. Conversely, a configural representation would confer mixture-specific properties to a mixture and would be observed as a departure from such a relationship. We thus evaluated the similarity between the neural representation of the mixtures and of their components, using a geometrical analysis of Euclidian distances. We

thus compared the relative Euclidian distance between the representation of a mixture and that of each of its components with the relative 'weight' of the components in the mixture.

The relative distance between a mixture and one of its components was calculated as:

$$D_{i,mix} = \frac{d_{i,mix}}{\sum_{k=1}^p d_{k,mix}}$$

with i indicating a component, mix the mixture, p the number of components in the mixture and $d_{k,mix}$ the Euclidian distance between a component k and the mixture in the putative olfactory space (for a calculation example, see Fig. 7A).

Similarly, the relative 'weight' of a component in the mixture is represented by the magnitude of the response elicited by this component, relative to the sum of response magnitudes of all components of the same mixture.

The response magnitude of a component was defined as the number of glomeruli activated by this component when presented alone (see Fig. 3C). Thus, the relative 'weight' of a component in a mixture was:

$$W_{i,mix} = \frac{N_i}{\sum_{k=1}^p N_k}$$

with i indicating a component, mix the mixture, p the number of components in the mixture and N_k the number of active glomeruli when presenting odor k alone (for a calculation example, see Fig. 7A). For each type of mixture (binary, ternary and quaternary), we evaluated possible linear relationships between $D_{i,mix}$ and $W_{i,mix}$. All linear correlations were tested for significance by calculating Pearson's r and using Student's t -test (Zar, 1999).

Results

Calcium signals to single odors and mixtures

The four single odors (1-hexanol, 2-octanol, linalool and limonene) and all binary, ternary and quaternary combinations of these odors induced calcium signals in the AL (Fig. 2A). These signals were highly reproducible within and between individuals (data not shown) as observed in previous studies on olfactory coding in the honeybee AL that have used the same imaging procedure (Joerges *et al.*, 1997; Faber *et al.*, 1999; Galizia *et al.*, 1999a; Sachse *et al.*, 1999; Sandoz *et al.*, 2003). All single odors evoked activity in different glomerular combinations. However, some glomeruli could be activated by several odors (see for instance activity for 2-octanol and linalool on the upper right part of the AL). Observation of the responses to binary mixtures clearly showed that the glomeruli activated by the mixture were also activated by one or both single components such that glomerular

FIG. 1. Mapping odor-evoked calcium signals onto a glomerular atlas. (A) Schematic view of the anatomical layout of the 24 glomeruli identified in this study (left antennal lobe; adapted from Galizia *et al.*, 1999a). (B) Example of an anatomical staining of the frontal part of a left antennal lobe using the dye RH795, with the 24 identified glomeruli studied (d, dorsal; l, lateral; m, medial; v, ventral). (C) Superimposed activity map in response to the odor 2-octanol, showing which glomeruli were activated.

FIG. 2. Odor-induced calcium activity in the antennal lobe. (A) Activity signals obtained in one bee to four individual odorants and all the binary, ternary and quaternary mixtures. The maps are represented according to a common intensity scale (upper right) and represent averages of three presentations of each stimulus. In all cases (except the air control), combinations of glomeruli were activated to the individual odorants and their mixtures. Mixture signals always included glomeruli that were activated by at least one of their single odor components. (B) Virtual activity maps to the mixtures obtained by making the arithmetic sum of the signals recorded with the single odors in A. Qualitatively, the measured (A) and calculated (B) maps show good agreement, suggesting that they contain the same set of glomeruli. However, there is no quantitative agreement, the scales of the calculated maps (on the right of each mixture type) being much higher than the measured data in A (see text for explanations). hex, 1-hexanol; oct, 2-octanol; lio, linalool; lim, limonene.

signals for mixtures were combinations of those of the single odors. The same can be observed for all binary, ternary and quaternary mixtures (Fig. 2A). To document this fact visually, we calculated virtual activity maps for all possible mixtures, using the arithmetic sum of the signals obtained with the single odors (Fig. 2B). When comparing the activated glomerular patterns in the actual data (Fig. 2A) and in the calculated data (Fig. 2B), a very good agreement appears; for instance, presentation of the hexanol/linalool binary mixture induces clear activity in five glomeruli (Fig. 2A). The same pattern of five activated glomeruli is obtained in the map based on the single odor signals (Fig. 2B). However, even if qualitatively we observe a good agreement between measured and calculated data regarding which glomeruli are activated, quantitatively no such agreement is found; arithmetic sums based on single odor signals have much higher amplitudes than the original measured data (see scales on the right side of each mixture type). This effect is due (i) to the fact that the odor intensity–glomerulus response relationship is not linear and (ii) to inhibitory effects which are studied in detail below. These observations thus suggest that much information about each component is preserved in mixture representations, although the signals are not arithmetic sums of the single odor signals.

As the glomerular layout is highly conserved between individual bees, an anatomical atlas of the honeybee AL is available (Flanagan & Mercer, 1989a; Galizia *et al.*, 1999b), which can be used to reconstruct the glomerular structure of all imaged bees and to represent the responses to the single odors and all possible mixtures in a common map (Fig. 3A and B). The maps of bee's responses to the individual odors ($n = 10$, Fig. 3A; here each map is scaled to its own minimum and maximum) showed good agreement with previously published maps for the same odors (Galizia *et al.*, 1999a; Sachse *et al.*, 1999; <http://www.neuro.uni-konstanz.de>). The best fit was obtained for 1-hexanol and 2-octanol, two odors that have been recorded in numerous animals in previous work (Sachse *et al.*, 1999). When response maps were scaled according to a unique common scale (Fig. 3B, upper row), differences in response intensity between single odors appeared; whereas 2-octanol and 1-hexanol induced high responses (80–100%) in some glomeruli, linalool and limonene induced overall lower responses (< 60%). This difference can be clearly seen when representing the number of active glomeruli ('response magnitude') for each odor (Fig. 3C), i.e. the number of glomeruli showing $\Delta F/F$ responses above 20% of the maximum. On average, 2-octanol and 1-hexanol induced responses in 9.1 ± 0.9 and 9.1 ± 1.7 glomeruli, whereas limonene and linalool induced responses in 5.8 ± 0.7 and 5.4 ± 1.0 glomeruli, respectively (mean \pm SEM). These values were significantly different (repeated-measure one-factor ANOVA, $F_{3,27} = 4.1$, $P < 0.05$). Thus, single odors differed in their response magnitude in the imaged AL region. We refer to this fact later in the text as a difference in the physiological salience of the single odors (i.e. 'strong' and 'weak' odors).

As depicted for a single individual in Fig. 2, activity maps over 10 individuals clearly revealed that responses to mixtures were a combination of glomerular responses to each component. However, activity maps of mixtures including both strong and weak single odorants (e.g. the mixtures 1-hexanol/linalool and 2-octanol/limonene) showed a high similarity to the map of the strongest component. This observation is analysed in detail below. Overall, binary and ternary mixtures activated the highest number of glomeruli. The quaternary mixture showed fewer active glomeruli and fewer glomeruli which were highly activated (no glomerulus showed activity between 80 and 100% of the maximum; see scale on Fig. 3B). Also, whereas activity maps of all individual components were quite distinct (Fig. 3A and B), mixtures with three or four components sometimes

showed very similar maps (see e.g. the maps for the ternary mixture 1-hexanol/limonene/linalool and the quaternary mixture in Fig. 3B).

Response magnitude (number of active glomeruli)

To confirm our observations based on the activity maps, we represented the number of glomeruli activated by the air control, the single odors and all possible mixtures (Fig. 4A). Although the air control induced almost no activity, the single odors induced activity in 7.4 ± 0.6 glomeruli. The number of active glomeruli increased to 9.8 ± 0.5 , 10.3 ± 0.6 and 10.4 ± 1.0 for binary, ternary and quaternary mixtures, respectively. There was an overall significant heterogeneity in the number of activated glomeruli when the air control was included in the analysis (one-factor ANOVA, $F_{4,155} = 17.3$, $P < 0.001$). Such heterogeneity remained when the analysis was restricted to the odor stimuli ($F_{3,146} = 5.0$, $P < 0.01$). The number of activated glomeruli differed between all odor types and the air control but also between the quaternary mixture and the single odors (Tukey test, $P < 0.05$). Thus, increasing the number of elements in the mixture from one to four significantly increased the number of activated glomeruli. However, a clear saturation was observed as almost the same number of activated glomeruli was found from the binary to the quaternary mixtures.

We then analysed the distribution of activated glomeruli into different activity classes (20–40, 40–60, 60–80 and 80–100% activity) for each odor type (Fig. 4B). As air stimulations did not induce measurable responses, these data were not included in further analyses. We found that all odor types evoked mostly low-activity responses (20–40%). Indeed, a two-factor repeated-measure ANOVA (component number \times activity class) showed a very strong activity class effect ($F_{3,438} = 222.5$, $P < 0.001$). This analysis also indicated a significant component number effect ($F_{3,146} = 5.0$, $P < 0.01$), which corresponds to the difference in overall active glomeruli found above. Most interestingly, the interaction between the two factors was marginally non-significant, suggesting that the distribution in activity classes may be different for mixtures with increasing numbers of components ($F_{9,438} = 1.9$, $P = 0.054$).

When analysing the effect of the number of mixture components within each activity class, we found that the number of low-activity glomeruli significantly increased with the number of mixture components (one-factor ANOVA, $F_{3,146} = 4.2$, $P < 0.01$), data for single odors differing from ternary and quaternary mixtures (Tukey test, $P < 0.05$). This was not the case for glomeruli exhibiting higher activity, whose number remained either constant or decreased slightly (see ternary and quaternary mixtures, 60–80 and 80–100% response classes in Fig. 4B). In none of these cases did the analysis reveal any significant effect. Thus, stimulation with more complex mixtures increased the number of active glomeruli, namely those exhibiting low activity. A quick saturation of the number of activated glomeruli with the number of mixture components was observed.

Mixture interactions: suppression, hypoadditivity and synergy

This analysis was designed to unravel the types of mixture interactions that take place at the glomerular level. Duchamp-Viret *et al.* (2003) classified the response of individual ORNs to olfactory mixtures in three categories developed for psycho-physical studies: (i) suppression, when the response to the mixture is lower than the response to the most effective component; (ii) hypoadditivity, when the response to the mixture is equivalent to that induced by the most effective component and (iii) synergy, when the response to the mixture is higher than that to the most effective component. We thus represented,

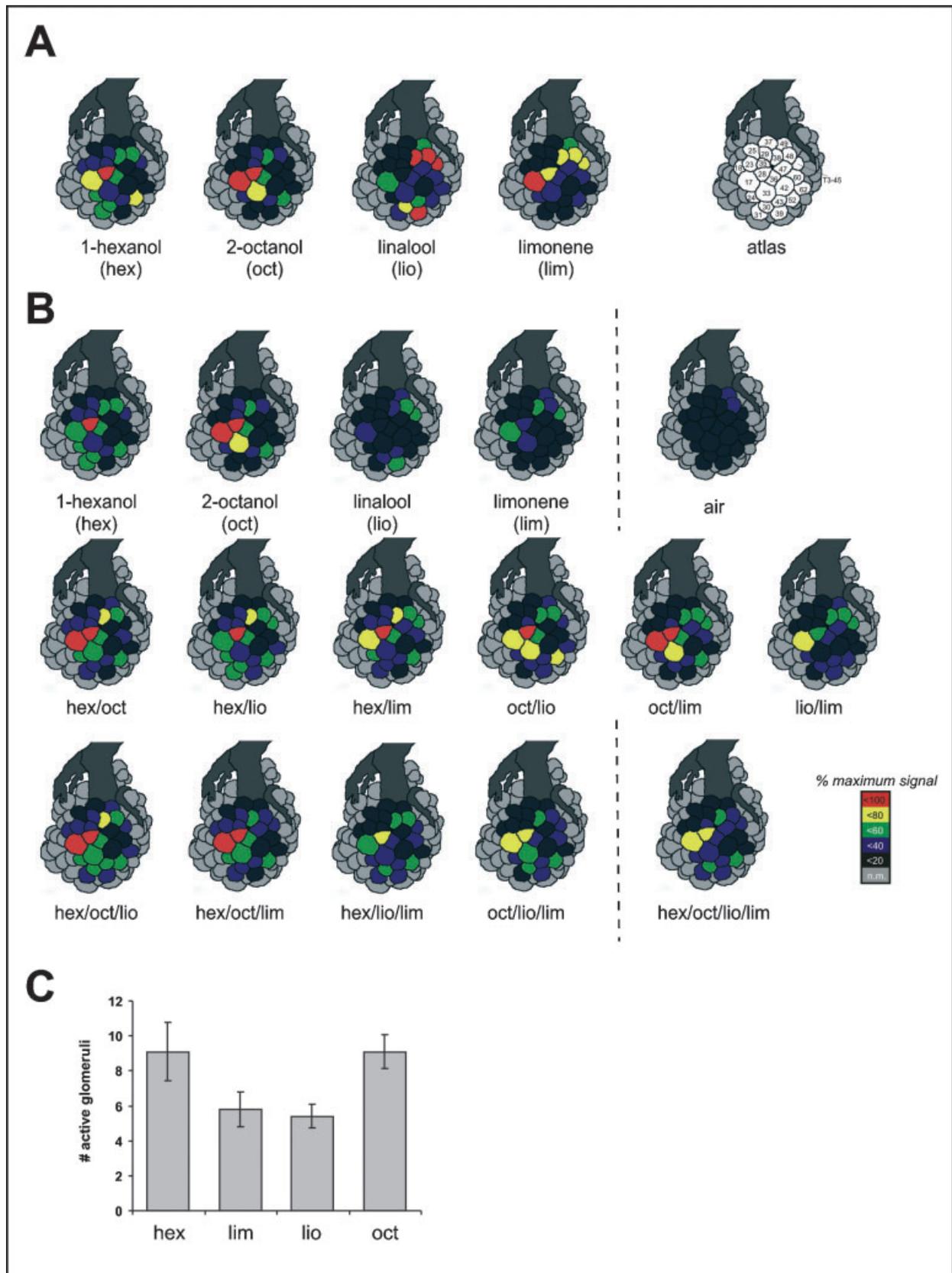


FIG. 3. Schematic representation of activity patterns averaged over 10 bees. (A) Activity patterns for the four individual odors, each map being scaled to its own maximum as in Sachse *et al.* (1999). Color codes indicate relative response intensity in five categories as indicated on the side. (B) Activity patterns for the four individual odors, all possible mixtures and the air control, each map being scaled to a common maximum. Activity maps to the mixtures involve the glomeruli activated by their components. (C) Count of the number of glomeruli activated by each individual odor. A glomerulus was considered activated when its response was above 20% of this bee's maximum response. Two odors showed stronger activity than the other two: hex, 1-hexanol; oct, 2-octanol; lio, linalool; lim, limonene.

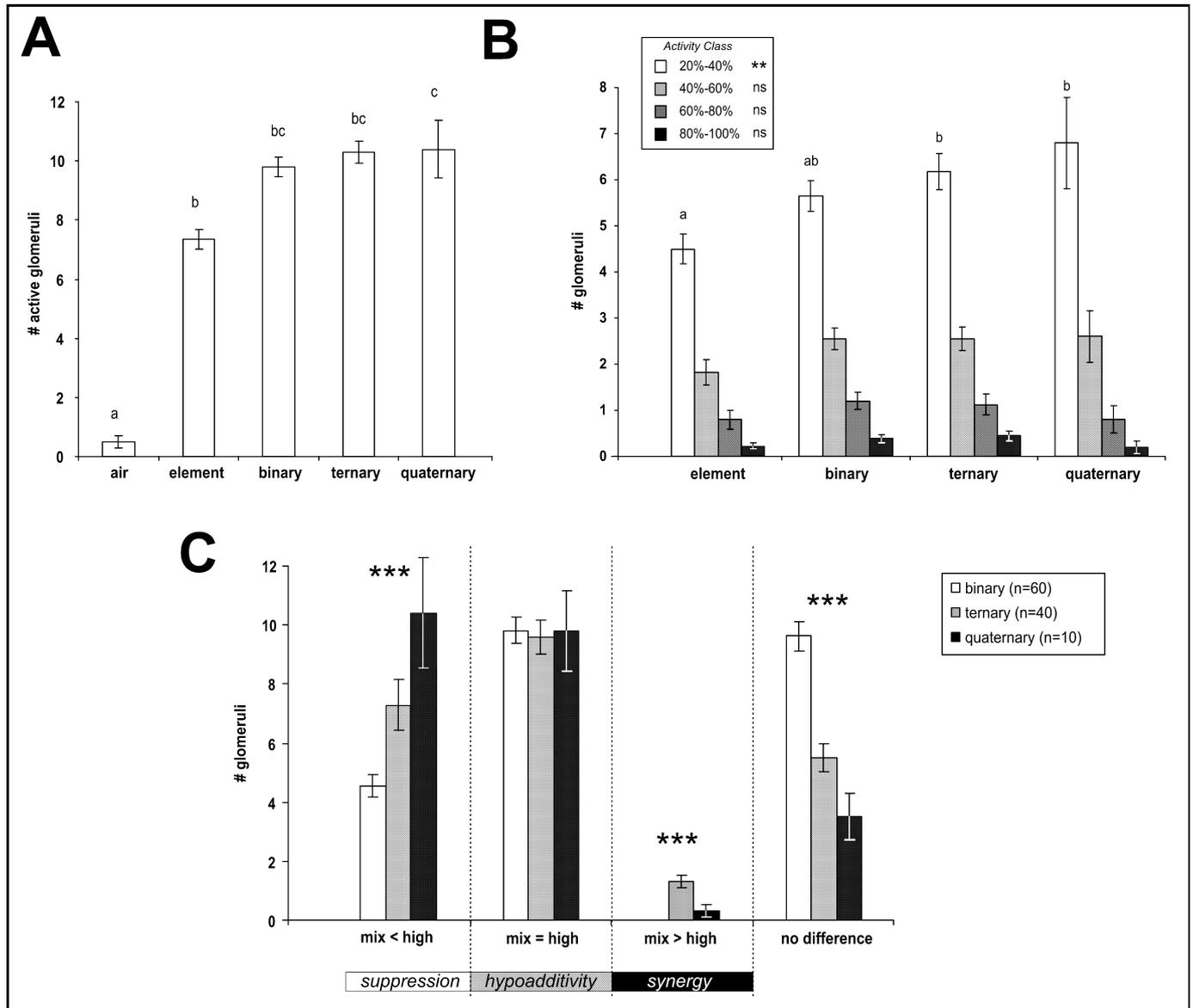


FIG. 4. Effect of the number of mixture components on glomerular activation. (A) Number of glomeruli activated for the air control, individual odors (elements), and binary, ternary and quaternary mixtures. A quick saturation of the number of activated glomeruli is observed. (B) Number of glomeruli represented in four activity classes, relative to each bee's maximum response. Although the number of low-activity glomeruli increases with the number of mixture components, the number of highly active glomeruli stagnates or tends to decrease. (C) Repartition of glomerular responses in classes depending on how the response to a mixture in each glomerulus relates to the highest response to individual components. The different cases relate in psycho-physical terms to phenomena of 'suppression' (response to the mixture is below the response to the strongest component), 'hypoadditivity' (response to the mixture is equal to that to the strongest component) or 'synergy' (response to the mixture is higher than that to the strongest component). A difference in glomerular response of 15% from the maximum was considered as significant (see text for explanations). Most glomerular responses were classified as hypoadditive or characteristic of suppression. Suppression increased significantly with the number of mixture components. Very few cases of synergy were recorded (** $P < 0.01$, *** $P < 0.001$; different letters indicate significant differences in post-hoc tests). ns, not significant.

for each glomerulus and for all mixtures, how the responses to a mixture related to the responses to the strongest component (Fig. 4C). We observed significant changes in the distribution of glomeruli according to these classes and to the number of mixture components (repeated-measure component number \times class ANOVA, component number effect $F_{2,107} = 23.4$, $P < 0.001$, class effect $F_{2,214} = 109.8$, $P < 0.001$; interaction $F_{4,214} = 4.9$, $P < 0.001$). For binary mixtures, most glomeruli exhibited hypoadditivity, with a few glomeruli showing suppression. For ternary and quaternary mixtures, we observed the same proportion of glomeruli in the hypoadditivity class as for binary mixtures (ANOVA, $F_{2,107} = 0.04$, not significant) but we

found a significant increase in the number of suppression cases (ANOVA, $F_{2,107} = 12.0$, $P < 0.001$). Overall, only a few cases of synergy were found. These appeared for ternary mixtures, with a significant heterogeneity in this class (ANOVA, $F_{2,107} = 27.9$, $P < 0.001$). This analysis thus shows that increasing the number of elements in an olfactory mixture leads to dramatic changes in glomerular response profiles and that suppression is increased in more complex mixtures. A process of active inhibition thus seems to occur upon mixture stimulation, which increases with more components in the mixture. We next studied the effect of inhibition on the whole odor representation.

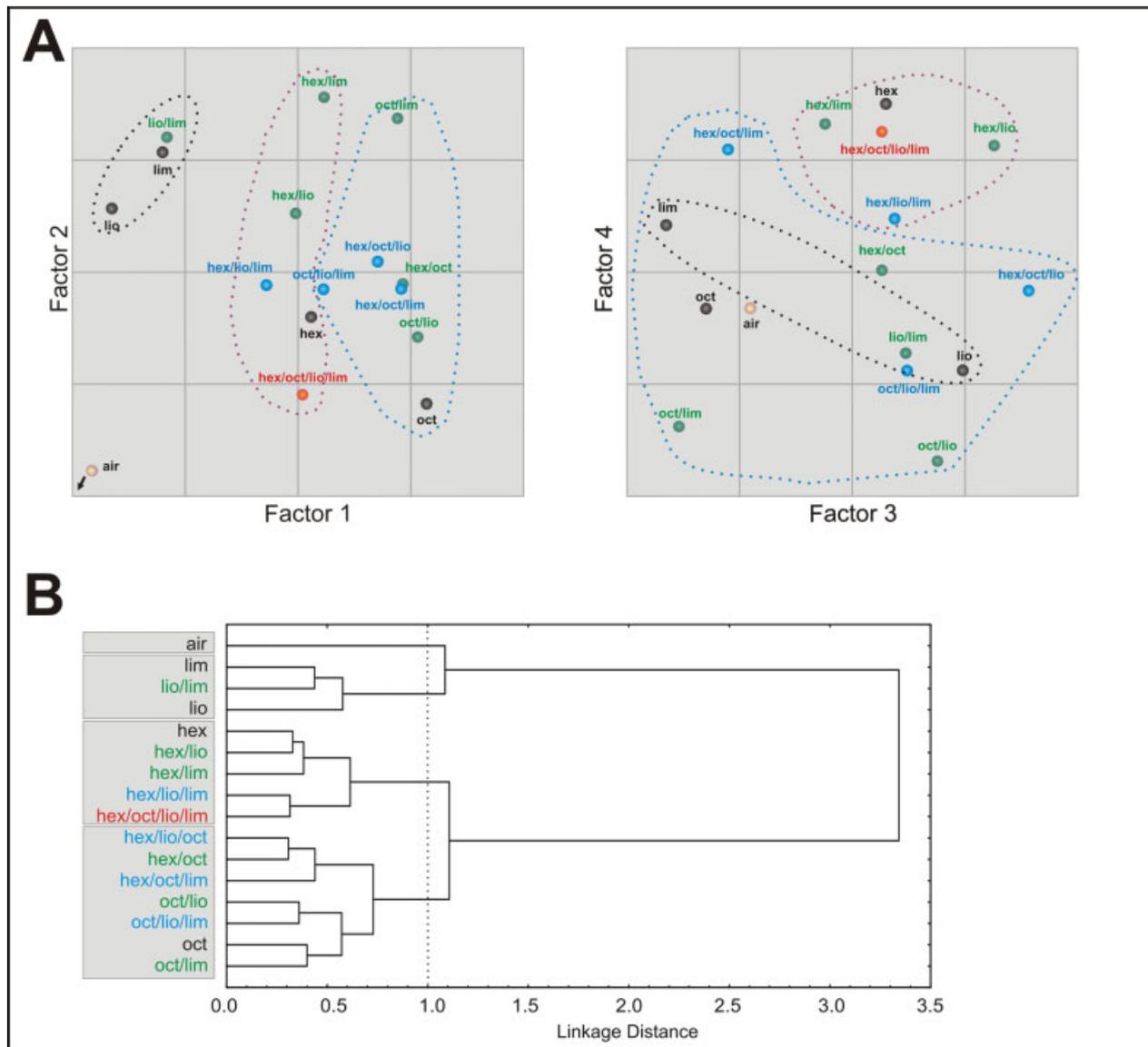


FIG. 5. Multidimensional analysis of odor representation in a putative olfactory space in which each glomerulus represents one dimension. (A) Representation of the relative position of odor patterns according to major factors extracted using a principal component analysis (four factors explaining 84.4% of total variance). Factor 1 was the most representative (52% of total variance) and separated linalool and limonene from 1-hexanol and 2-octanol. (B) Cluster analysis using Ward's classification method. From this classification three main odor clusters emerged (outlined in A): (i) linalool, limonene and their binary mixture; (ii) 1-hexanol and mixtures of this odor with linalool and/or limonene and (iii) 2-octanol and all mixtures with this odors except the quaternary mixture. hex, 1-hexanol; oct, 2-octanol; lio, linalool; lim, limonene.

Similarity between mixture and single odor representations

To understand the relationships between neural representations of single odors and mixtures, we represented odor responses in a putative olfactory space in which activity recorded in each glomerulus represents a dimension. The first four factors extracted by the principal component analysis represented 52.5, 14.6, 10.2 and 7.1%, respectively, of overall variance for a total of 84.4%. We represented all odors in two graphs, each with two factors (Fig. 5A). When looking at the single odors, the first two factors mostly separated the two weaker odors, linalool and limonene, from the two stronger odors, 2-octanol and 1-hexanol. According to Factor 1, most mixtures appear on the side of the strong components, except for the binary mixture including the two weaker odors (linalool/limonene). In general, mixtures containing 1-octanol were rather clustered on the right part of Factor 1, whereas mixtures containing 1-hexanol were grouped in the

middle (Fig. 5A left). When looking at the repartition of the different types of mixtures, it clearly appears that ternary mixtures lie close to each other whereas binary mixtures and single odors are more dispersed. This again reflects the fact that mixtures become increasingly similar with increasing number of components. The right-hand part of Fig. 5A shows that Factor 3 separates linalool from limonene. Mixtures containing limonene lie on the left side whereas mixtures containing linalool lie on the right side. Mixtures containing both odors lie in the middle. Factor 4 separates mixtures containing 1-hexanol from all other mixtures.

To further unravel the relationships between odors, we also used a cluster analysis grouping odors together depending on their Euclidean distances in the 24-dimensional space represented by the different glomeruli (Fig. 5B). A similar analysis using different metrics (i.e. City Block metric) yielded similar results (data not shown). Four main

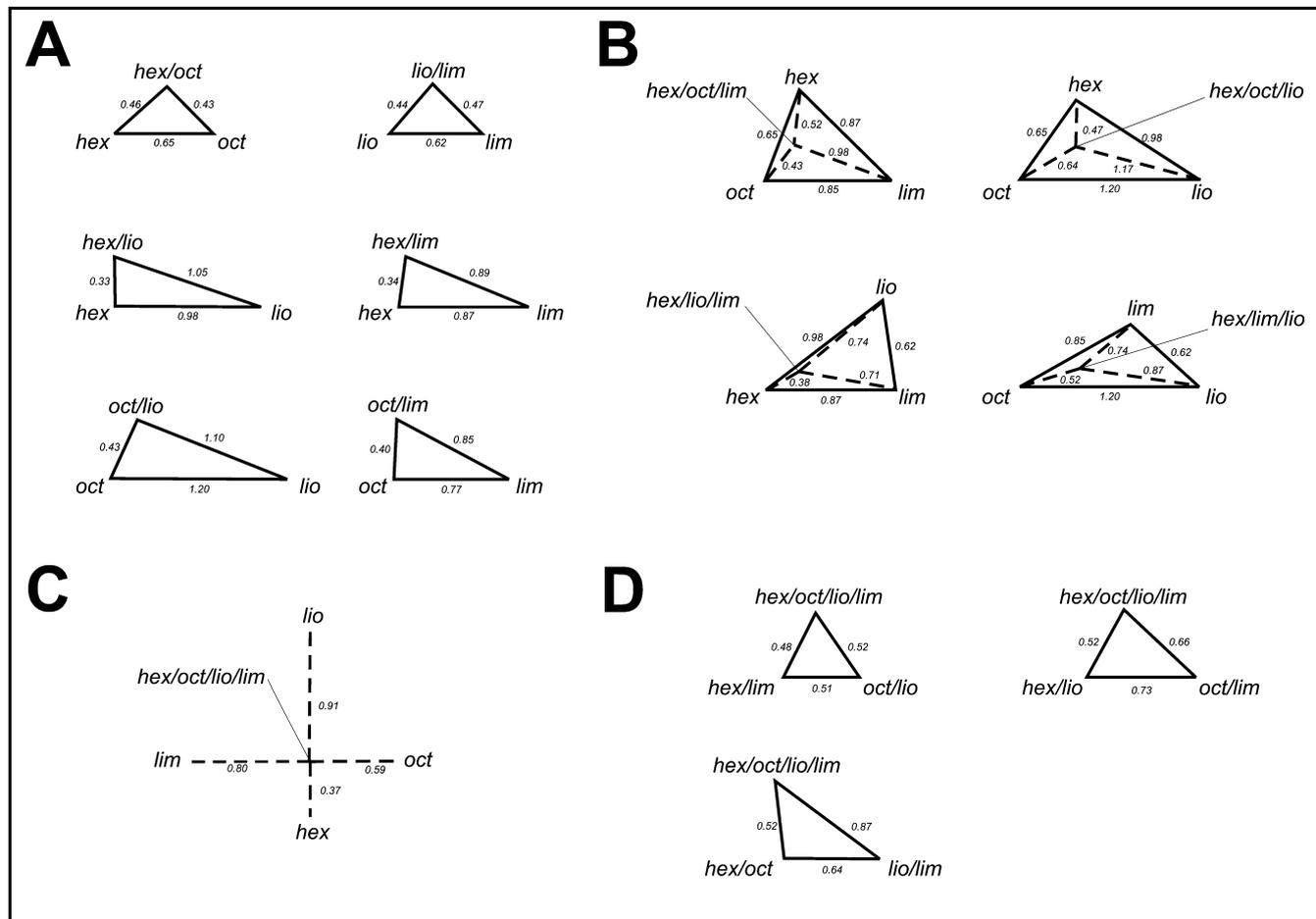


FIG. 6. Physiological similarity between mixture representations and their components, measured as the Euclidian distance between representations in the putative 24-dimensional olfactory space. (A) Distances between binary mixtures and each of their components (corresponding to triangles in the olfactory space). Relationships were either symmetrical (upper row) or asymmetrical (two lower rows). (B) Similar representation between ternary mixtures and their components (tetrahedrons in the olfactory space). (C) Schematic representation of the quaternary mixture and each of its components (in theory forming a pentahedron in four dimensions of the olfactory space). (D) Distances between the quaternary mixture and each pair of binary mixtures that it corresponds to (triangles in the olfactory space). All of these cases indicate that two odors, 1-hexanol and 2-octanol, had more weight on mixture representation, the mixture always being more similar to these odors than to linalool or limonene. hex, 1-hexanol; oct, 2-octanol; lio, linalool; lim, limonene.

clusters were formed, in which the air control was clearly separated from three odor groups: first, the weaker odors limonene and linalool and their binary mixture; second, a cluster with 1-hexanol and mixtures with the two weaker odors; and third, a cluster containing 2-octanol and all mixtures with this odor, except for the quaternary mixture. Note also that mixtures containing both 2-octanol and 1-hexanol are grouped in a subcluster. Thus, it appears that odor representations are clustered according to their composition, the stronger single odors attracting more mixture representations than the weaker odors.

Linearity of mixture representation

Our analysis suggests that single odors are differently represented in a mixture, depending on their physiological salience in the AL. Generally, mixtures appeared more similar to strong than to weak single odors. To depict this fact more clearly, we calculated the Euclidian distances between the representations of each mixture and its components in the 24-dimensional space (Fig. 6). In this space, the three points representing two single odors and their binary mixture build a triangle in a two-dimensional plane. Thus, Fig. 6A represents

the relationships between each binary mixture and its components (note that each triangle would correspond to a different plane in the 24-dimensional space). Distances between the components and mixture were either symmetrical (first row) when the mixture included two odors having the same weight or strongly asymmetrical (second and third rows), in which case the mixture representation was much closer to the strongest odor component 1-hexanol (second row) or 2-octanol (third row).

In the case of ternary mixtures, the mixture and the components' representations build a tetrahedron in a three-dimensional space. We thus represented, as seen from the top, the four tetrahedrons corresponding to the four ternary mixtures (Fig. 6B). As for binary mixtures, the mixture representation was in each case closer to that of the strongest component(s). When both 1-hexanol and 2-octanol were present (first row), the mixture was closer to these two odors than to the weaker odors limonene or linalool. When only one stronger odor was present (second row), the mixture representation was closest to this odor. Very similar rules were observed when looking at the relationships between the quaternary mixture and each single odor (Fig. 6C) or between the quaternary mixture and all possible pairs of binary mixtures (Fig. 6D). Thus, in the pentahedron formed by the ternary mixture and all its components in a four-dimensional space

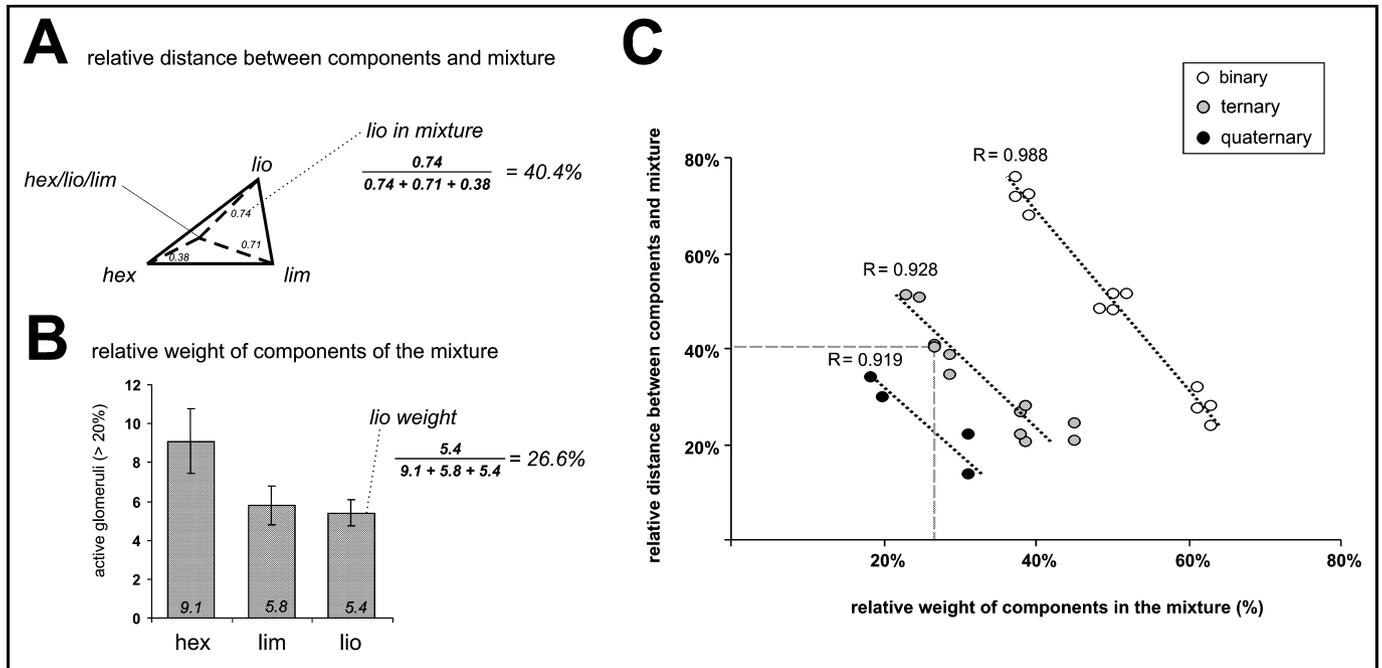


FIG. 7. Relationship between the relative weight of a component in a mixture and the similarity of the mixture to this component. (A) Example of the calculation of the relative distance between components and mixture. The Euclidian distance between this component and the mixture in the putative olfactory space is divided by the sum of distances of all the components to the mixture. (B) Example of the calculation of the relative weight of a component in a mixture. The number of glomeruli activated by this odorant when presented alone is divided by the sum of the glomeruli activated by each of the components. (C) Correlation for binary, ternary and quaternary mixtures between the relative distance between each component and the mixture (calculated in A) and the relative weight of the component in the mixture (calculated in B). In all cases clear linear relationships are observed. hex, 1-hexanol; lio, linalool; lim, limonene.

(the figure is only indicative of the distances between the mixture and elements), the quaternary mixture was closest to 1-hexanol, then to 2-octanol and further away from the two weaker odors (Fig. 6C).

When looking at the relationships between the quaternary mixture and the possible binary mixtures composing it (Fig. 6D), we found that two binary mixtures with similar weights (one stronger and one weaker odor, as in 1-hexanol/limonene and 2-octanol/linalool) were at the same distance from the quaternary mixture (first row). By contrast, the quaternary mixture was much closer to the mixture of the two stronger components (1-hexanol/2-octanol) than to the binary mixture of the two weaker components (linalool/limonene).

Taken together, our results show rather elemental within-mixture interactions, each mixture being more similar to strong than to weak components. To confirm this fact, we performed correlation analyses relating for each mixture the relative distance of each component to the mixture (for calculation example see Fig. 7A) with the relative 'weight' (number of active glomeruli) of the different single odors in the mixture (for calculation example see Fig. 7B). Figure 7C presents the three data sets corresponding to binary, ternary and quaternary mixtures. In all three cases, we found a very clear linear relationship between the similarity of each component to the mixture and the relative weight of this component compared with the other components of the mixture. The correlation coefficients were $R = 0.988$, $R = 0.928$ and $R = 0.919$ for binary, ternary and quaternary mixtures, respectively. These coefficients were significant for binary and ternary mixtures ($P < 0.001$), and nearly significant for the quaternary mixture due to the lower number of points ($P < 0.10$). Thus, odor representations for binary, ternary and quaternary mixtures, as measured using bath application of calcium green on the AL, follow linear relationships such that the higher the relative number of glomeruli activated by a single odor (i.e. the more physiologically

salient the odor is), the smaller the relative distance between this odor and its mixture (i.e. the more physiologically similar the odor is to the mixture).

Discussion

Previous studies on olfactory mixture processing mainly focused on electrophysiological recordings of single unidentified ORNs (e.g. rats, Duchamp-Viret *et al.*, 2003; fish, Caprio *et al.*, 1989; Kang & Caprio, 1991, 1995, 1997; spiny lobsters, Cromarty & Derby, 1997; Steullet & Derby, 1997; Gentilcore & Derby, 1998; bees, Akers & Getz, 1993; Getz & Akers, 1994) and showed that mixture-specific interactions occur already at the peripheral level. However, as the neural representation of an odor encompasses the whole combinatorial pattern of receptor activation reaching the brain, a comprehensive analysis of mixture processing has to be performed at the level of the primary olfactory centers (olfactory bulb or AL) as each of their functional units, the glomeruli, is believed to represent a different receptor channel (mice, Mombaerts *et al.*, 1996; *Drosophila*, Vosshall, 2000). Using such an approach, we studied how much of the component information is present in mixture representations. An important advantage of the present study is that we could analyse activity from clearly identified neuronal structures over different animals, which requires the reliable identification of individual glomeruli across individuals. Although individual glomeruli have been labeled in mice (Vassar *et al.*, 1994; Mombaerts *et al.*, 1996), complete mapping and identification of the approx. 2000 glomeruli has not been achieved so far. In the honeybee, however, the 160 glomeruli of the AL have each been identified, mapped and presented in a standard atlas (Galizia *et al.*, 1999b) so that they can be

recognized in different individuals. Thanks to this advantage, we established a physiological atlas of mixture-evoked activity for four single odors, their binary, ternary and quaternary mixtures over 10 bees. Our results indicate that increasing the number of mixture components results in an increase of the number of active glomeruli, which quickly saturates. Component information was highly conserved within the activity patterns of the olfactory mixtures, and similarity relationships between the different mixtures and their components could be predicted based solely on the magnitude (number of activated glomeruli) of the components' responses. This is an important result as it demonstrates the existence of a strongly elemental mixture representation in the AL.

Global inhibition at the input to the antennal lobe

The glomerular activity patterns recorded for the different mixtures involved a combination of the glomeruli activated by each component, which confirms observations made previously in the honeybee (Joerges *et al.*, 1997) and zebra fish (Tabor *et al.*, 2004). In the bee, Joerges *et al.* (1997) reported that the amplitude of glomerular responses to a mixture was often lower than arithmetic sums of the components' responses, more so for the ternary than for the binary mixture. Indeed, we calculated in Fig. 2B arithmetic sums of responses to single components and observed that the spatial repartition of the calculated patterns coincided well with the recorded patterns of all mixtures but that the amplitudes of the calculated mixture patterns were strongly overestimated, more so with increasing component number. Rightly, Tabor *et al.* (2004) argued that a direct comparison between observed data and the arithmetic sum of responses to components is not valid because the relationship between odor intensity and glomerular response is not linear but follows a receptor–ligand relationship, i.e. a sigmoid curve over several log units of odor concentration. Thus, even if two components of a binary mixture activate the same olfactory receptor, the response of the respective glomerulus to the mixture will be less than the sum of the glomerular activations produced by the components. Tabor *et al.* (2004) provided an elegant model allowing the prediction of binary mixture responses based on recordings at two different concentrations for each mixture component. In our study, such an approach, based on testing several concentrations for each component, was not feasible because we prioritized a systematic study of the neural responses to all the possible mixtures of four odors, which constituted a significant amount of work. Testing in addition the responses to different concentrations for each stimulus used would represent an even greater effort and would constitute a subject of study *per se*. Our strategy was therefore to describe precisely the evolution of glomerular responses with increasing number of mixture components. We show here that the number of activated glomeruli saturates with very few components in the mixture (Fig. 4A and B). Moreover, the number of active glomeruli saturated at a value of 10 glomeruli, which is lower than the potential number of active glomeruli when counting glomeruli giving a significant response to at least one of the components (17 above noise level, 12 above 40% of maximum signal, Fig. 3A). Indeed, when comparing within-glomerulus responses to each mixture and its components, even if most cases concerned 'hypoadditivity' (equal response to the mixture and to the strongest component), we found that the number of 'suppression' cases (lower response to the mixture than to the strongest component) increased significantly with component number (Fig. 4C). Thus, not only was a saturation of glomerular responses due to the non-linearity of odor/receptor response curves (corresponding to 'hypoadditivity') revealed but also an inhibitory

phenomenon (corresponding to 'suppression' cases), which decreases glomerular activation by olfactory mixtures. We show that the strength of this phenomenon increases with the number of mixture components and was, in our conditions, maximal with four components. Both phenomena would lead to very limited patterns of activation for complex mixtures. In fact, recordings with very complex mixtures, such as clove (also called 'carnation') or orange essential oils, containing tens to hundreds of components, induce rather restricted glomerular patterns in the honeybee AL, which cannot be visually distinguished from those obtained for pure odorants (Joerges *et al.*, 1997; Galizia *et al.*, 1999a; Sandoz *et al.*, 2003).

The repartition of glomerular responses mostly in the hypoadditivity and suppression classes is very similar to that obtained for ORNs in the rat (Duchamp-Viret *et al.*, 2003). Hypoadditivity implies that each mixture contains a 'strongest' component, which dominates over the other(s). At a peripheral processing level, this could be due to its higher molecular concentration or its higher affinity to the receptor sites, and suggests an action of the different odor molecules on common receptors (Kajiya *et al.*, 2001; Touhara, 2002). This finding is coherent with the idea that, in honeybees, glomeruli are the projection sites of ORNs carrying the same receptor, as is the case in other species (Mombaerts *et al.*, 1996; Vosshall, 2000). In the case of suppression, odor molecules in a mixture would counteract each other through inhibitory binding mechanisms on the same olfactory receptor or by activating inhibitory intracellular cross-talk between transduction cascades. Such inhibition phenomena have been shown in both vertebrates and invertebrates (e.g. Ache *et al.*, 1988; Burgess *et al.*, 1994; Ache & Zhainazarov, 1995; Cromarty & Derby, 1997; Kang & Caprio, 1997; Steullet & Derby, 1997; Spehr *et al.*, 2002). In honeybees, peripheral inhibitory interactions between mixture components have also been suggested within the placode sensillae on the antennae, which house several tens of ORNs that may interact negatively with each other (Getz & Akers, 1994). This could, in part, explain the quick saturation of glomerular responses to mixtures. Alternatively, inhibition could arise centrally, at the level of the AL circuitry (see below). The fact that synergistic responses were very rare in our recordings could be related to the use of relatively high concentrations of the odors (Duchamp-Viret *et al.*, 2003). However, such concentrations are commonly used in behavioral experiments and yield standard learning performances. In our case, we chose to work explicitly under the same stimulation conditions in which mixture-processing rules were studied behaviorally (Deisig *et al.*, 2001, 2002, 2003; see below). Further experiments studying concentration effects should be performed to understand how odor concentration modifies the repartition of glomerular responses into the hypoadditivity, suppression and synergy classes. Lower component concentrations could induce slower saturation of the number of activated glomeruli with increasing numbers of mixture components (Fig. 4A), could yield less suppression cases and could reveal mixture representations that are more similar to the arithmetic sum of component representations than found in the present case.

Neural basis of global inhibition in the antennal lobe

Using bath application of calcium green 2-AM, we recorded a composite calcium signal which could potentially come from all cell populations of the AL: ORNs, local interneurons, PNs and glial cells (Joerges *et al.*, 1997). Because of the numerical preponderance of ORNs and because odor-induced signals have a very stereotypical time course and do not show spontaneous activity or inhibitory responses (the hallmark of local interneurons and PNs), these

recordings are thought to emphasize pre-synaptic calcium variations from ORNs (Galizia *et al.*, 1998; Sachse & Galizia, 2003), with a possibly significant contribution from glial cells (Galizia & Vetter, 2004). Thus, the bath-applied signals may correspond, for the main part, to input information to the AL. Although peripheral mixture interactions may occur at the receptor level (see above), global inhibition could also take place within the AL. In cockroaches (Distler & Boeckh, 1997), inhibitory interneurons can synapse back onto the pre-synaptic terminals of ORNs and such an inhibitory system effectively inhibits the electrophysiological responses of olfactory receptors in spiny lobsters (Wachowiak & Cohen, 1999) and rats (Aroniadou-Anderjaska *et al.*, 2000). In these species, global inhibition is considered as a gain control preventing saturation of odor-evoked responses, thus allowing encoding odors over a wide dynamic range (Wachowiak *et al.*, 2002). Indeed, pre-synaptic modulation of sensory input seems to be a general strategy of neural networks to carry out gain control (Watson, 1992). Our finding that glomerular responses are more intensively inhibited with an increasing number of components could reflect such a gain control mechanism. Local interneurons in the bee AL are mainly heterogeneous, branching unevenly within the AL (87%), or homogeneous, branching evenly among glomeruli (13%) (Flanagan & Mercer, 1989b; Fonta *et al.*, 1993). Recently, models of honeybee AL circuitry have been proposed based on local interneuron morphology and optical imaging recordings of PN activity (Sachse & Galizia, 2002; see also Lachnit *et al.*, 2004). In these models, homogeneous local interneurons, which are thought to be GABAergic, are involved in gain control, whereas heterogeneous local interneurons, possibly histaminergic or glutamatergic, accomplish focused lateral inhibition between physiologically neighboring glomeruli. Such an inhibitory network could underlie the suppression phenomena observed in our mixture experiments.

Linearity of mixture representation

An 'elemental' processing system allows the prediction of the properties of a stimulus compound (here an olfactory mixture) based on the properties of its elements (the components). Conversely, a 'configural' processing system attributes novel perceptual qualities, which are not present in the individual components, to the compound. To differentiate between the two possibilities in the case of the bee AL, we asked whether it is possible to predict how similar the neural representation of a mixture will be to that of each of its components without knowing anything about the mixture. We observed that the distance between an olfactory mixture and each of its components in a glomerular physiological-similarity space can be predicted based on the number of glomeruli activated by each component when presented alone (Fig. 7). This important result suggests that odor representation in the AL follows a rather elemental rule. This finding is reminiscent of a previous electrophysiological study on spiny lobster receptor neurons, in which blend ratios of binary mixtures evoked across-neuron response patterns that were always arranged between those elicited by the single components (Steullet & Derby, 1997). The more concentrated that each component was relative to the other, the more similar the cross-neuron pattern of the mixture was to that component's pattern. The present study leads us to conclude that, at the peripheral level of the olfactory pathway, odor mixture representation is elemental.

The odors tested in this work were used at the same concentrations used in previous behavioral experiments (e.g. Deisig *et al.*, 2001, 2002, 2003) in which bees had to differentiate olfactory mixtures from their components, in so-called negative and positive patterning

discriminations (respectively A+B+ vs. AB- and A-B- vs. AB+, with + and - indicating the presence and absence of sucrose reward, respectively). These particular odors allowed successful differentiation by bees in both tasks. We knew therefore that the necessary information for solving these discriminations was present in the neural representations of these odors. However, contrary to the findings of the present study, the behavioral experiments did not support pure elemental mixture processing but processing at the interface between elemental and configural strategies (Deisig *et al.*, 2001, 2002, 2003). In other words, olfactory mixtures were treated by bees as being the sum of their individual components plus an additional internal representation, the 'unique cue', resulting from the joint presentation of these components. Theoretically, the brain could use any difference existing between the mixture-induced pattern and a computed sum of the patterns of the single odors as a mixture-unique code for discriminating between mixture and odor components. For instance, glomeruli that would be active during presentation of the mixture but not during presentation of any of the single odors would qualify as a unique cue. Such a clear mixture-unique code was not observed in our study. We rather found elemental processing, such that the extraction of mixture-unique properties would certainly need further processing at the central level, either within the AL or in the mushroom bodies (Lachnit *et al.*, 2004). As it is thought that AL processing is responsible for contrast enhancement between odors through active inhibition from heterogeneous local interneurons (Linster *et al.*, 2005), mixture and component representations could be more distinct and show mixture-specific properties when recorded at the level of PNs (i.e. at the output from the AL). Future recordings at this level (Sachse & Galizia, 2002) or in the mushroom bodies (Szyzka *et al.*, 2005) could reveal the neural basis for the unique cue.

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Abbreviations

AL, antennal lobe; ORN, olfactory receptor neuron; PN, projection neuron.

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