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RESEARCH ARTICLE

Learning about natural variation of odor mixtures enhances categorization in early olfactory processing

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ABSTRACT

Natural odors are typically mixtures of several chemical components. Mixtures vary in composition among odor objects that have the same meaning. Therefore a central 'categorization' problem for an animal as it makes decisions about odors in natural contexts is to correctly identify odor variants that have the same meaning and avoid variants that have a different meaning. We propose that identified mechanisms of associative and non-associative plasticity in early sensory processing in the insect antennal lobe and mammalian olfactory bulb are central to solving this problem. Accordingly, this plasticity should work to improve categorization of odors that have the opposite meanings in relation to important events. Using synthetic mixtures designed to mimic natural odor variation among flowers, we studied how honey bees learn about and generalize among floral odors associated with food. We behaviorally conditioned honey bees on a difficult odor discrimination problem using synthetic mixtures that mimic natural variation among snapdragon flowers. We then used calcium imaging to measure responses of projection neurons of the antennal lobe, which is the first synaptic relay of olfactory sensory information in the brain, to study how ensembles of projection neurons change as a result of behavioral conditioning. We show how these ensembles become 'tuned' through plasticity to improve categorization of odors that have the different meanings. We argue that this tuning allows more efficient use of the immense coding space of the antennal lobe and olfactory bulb to solve the categorization problem. Our data point to the need for a better understanding of the 'statistics' of the odor space.

KEY WORDS: Categorization, Natural odors, Olfaction, Plasticity, Variability

INTRODUCTION

Natural odors are generally blends of several chemical components (Dudareva et al., 2004; Raguso, 2008; Raguso et al., 2005; Smith et al., 2006). Changes in the composition of the blend by changing the identity and/or ratios of components (Raguso, 2008), or by changing a background odor context (Lenochová et al., 2012), can have a significant impact on the blend's perceptual qualities. These changes can pose a problem for animals, because slight but perceptible differences might separate odors that have the same meaning but which must be correctly recognized as being the same. This variation from one odor object to the next means that animals cannot always search for exactly same odor that was recently associated with an important event (Smith et al., 2006). Instead, animals must have the capacity to 'generalize' experience with an odor blend to slightly different blends. An important problem is to establish whether an odor is similar enough to belong to the same category, or whether the odor is dissimilar enough to belong to a different category. Therefore, the central issue is to establish boundaries for categorizing odors based on their meaning (Shepard, 1987).

The use of floral odors by honey bees is an example of this problem. Floral odor blends can contain up to dozens of component odorants, which collectively give rise to the perfumes of flowers (Chittka and Raine, 2006; Raguso, 2008). Honey bees must learn about the associations of floral odor blends with carbohydrate (nectar) and protein (pollen) resources the colony needs for survival (Smith et al., 2006). Having learned this association, honey bees can discriminate odor blends that represent small differences in mixture composition (Wright et al., 2002). These changes in odor composition can represent important information. Once flowers are pollinated, and no longer contain nectar, flowers change the amount and/or ratios of odor components they produce (Dudareva and Pichersky, 2000; Raguso, 2008; Theis and Raguso, 2005). Intraspecific genetic variation among flowers also influences blend composition (Dudareva et al., 2004) and may be associated with differences in floral rewards (Knauer and Schiestl, 2015). In order to increase foraging efficiency (Shafir et al., 1999), honey bees must form rewarding and non-rewarding 'categories' by learning different floral blends. Moreover, the perceptual boundaries of these categories may need to be adjusted over time because of the changing associations of odors with food.

We propose that plasticity in early olfactory processing in the brain plays a significant role in establishing these categories. Neural networks in the insect antennal lobe (AL) are the functional analogs to those of the mammalian olfactory bulb (OB) (Hildebrand and Shepherd, 1997). Indeed, floral blends are frequently distinguishable to both humans and to honey bees, which probably reflects similarities in odor coding (Su et al., 2009). Both non-associative and associative plasticity have been identified in the AL (Fernandez et al., 2009; Locatelli et al., 2013) and OB (Wilson et al., 2004). We have shown that plasticity in the AL acts to increase separation of patterns evoked by odors that have been differentially associated with food (Fernandez et al., 2009). However, research on olfactory coding and plasticity has focused on pure odors or simpler mixtures (Daly et al., 2004; Fernandez et al., 2009; Stopfer et al., 2003; Deisig et al., 2006, 2010; Guerrieri et al., 2005; Linster and Smith, 1999) rather than on the more complex odor problems presented by flowers. Even when more complex odor mixtures were used, odor compositions were not systematically varied as odors would vary from flower to flower of

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the same species in natural scenes. The work with monomolecular odors or simpler mixtures has revealed a lot about olfactory coding. However, systematic manipulation of natural variation will be crucial to show that plasticity acts to separate natural odors in such a way that it could help solve the categorization problem.

Therefore, we evaluate here more realistic odor mixtures. We have continued previous work with volatiles of varieties of snapdragon (*Antirrhinum majus*) (Wright et al., 2005a). Blends of these varieties differ in ratios of six to eight chemical components (Negre et al., 2003). Honey bees can detect and discriminate natural odors of *A. majus* in proportion to the chemical similarity of the blends (Wright et al., 2005a). We have now adopted the use of synthetic blends from two *A. majus* varieties. We made synthetic blends that mimic the statistical separation both within and between varieties. The goal was to analyze how these blends are represented by activity patterns in the AL (Fernandez et al., 2009). We also test whether patterns are changed by experience with the association of reinforcement with natural variability, which is an important prediction of the categorization hypothesis outlined above.

MATERIALS AND METHODS

Animals

Pollen forager (female) honey bees (*Apis mellifera* Linnaeus 1758) were collected in the morning at the entrance of the hive, briefly cooled and restrained in individual harnesses suitable for conditioning and optical recordings. After recovering from cooling, bees were fed 2 µl of a 1.0 mol l^{-1} sucrose solution and allowed to remain undisturbed for 2 h at room temperature until experiments took place.

Artificial odor blends

Twelve artificial odor blends were designed based on flowers from two varieties of snapdragon – *Antirrhinum majus* Potomac pink (PP) and Pale hybrid (PH) (Wright et al., 2005a). The artificial floral blends were done by mixing six of the eight components of the natural blends. The blends were prepared in mineral oil in concentrations that reproduced the proportions and variability found in the natural populations of two varieties of snapdragon flowers (Fig. 2A). The blends PH1 and PP1 represented flowers that were the average of each variety and were made by using the average concentration of each component measured in the samples of the natural population. PP2 and PH2 were made by combining concentrations that corresponded to the 25th percentile of the concentration of each component in the respective variety. PP3 and PH3 were made combining concentrations that corresponded to the 75th percentile. PP4, PH4, PP5 and PH5 were random combinations of the components, each one in concentrations that represented the mean, the 25th or the 75th percentile of the concentration of the components in the respective variety. Finally, PP6 and PH6 were copied from natural examples and represented extreme cases within the natural distribution (see Fig. 2B).

Odor stimulation

The output of the odor delivery device was positioned 2 cm in front of the bee's head pointed toward the antennae. A continuous charcoal-filtered air stream (500 ml min^{-1}) ventilated the antennae. Behind the honey bee, an exhaust continuously removed air from the arena keeping the area clean of the olfactory stimuli. The odor cartridges consisted of a 1 ml glass syringe containing a filter paper strip (0.5×4 cm) loaded with 10 µl odor solution. A three-way valve (LFAA1200118H; The LEE Company) controlled the onset of the airflow through the odor cartridge. Opening of the valve was synchronized with the optical recordings by the acquisition software TILLVisION (TILL Photonics). Stimulation lasted 4 s. When the valve opened, the odor-laden air from the cartridges was pushed (1 ml s^{-1}) into the continuous air stream in a mixing chamber 2 cm before the output of the odor delivery device. When the valve switched to inject the odor into the main airstream, it switched off the injection of a similar amount of clean air, thus avoiding changes in total airflow perceived by the bee. The odor delivery device had 15 identical channels, each composed of a valve and an odor cartridge. The distribution of odors and channels was randomized between animals to counterbalance any difference between channels.

Olfactory conditioning

Behavioral conditioning of the proboscis extension reflex (PER) followed standard protocols (Smith and Burden, 2014). Two groups

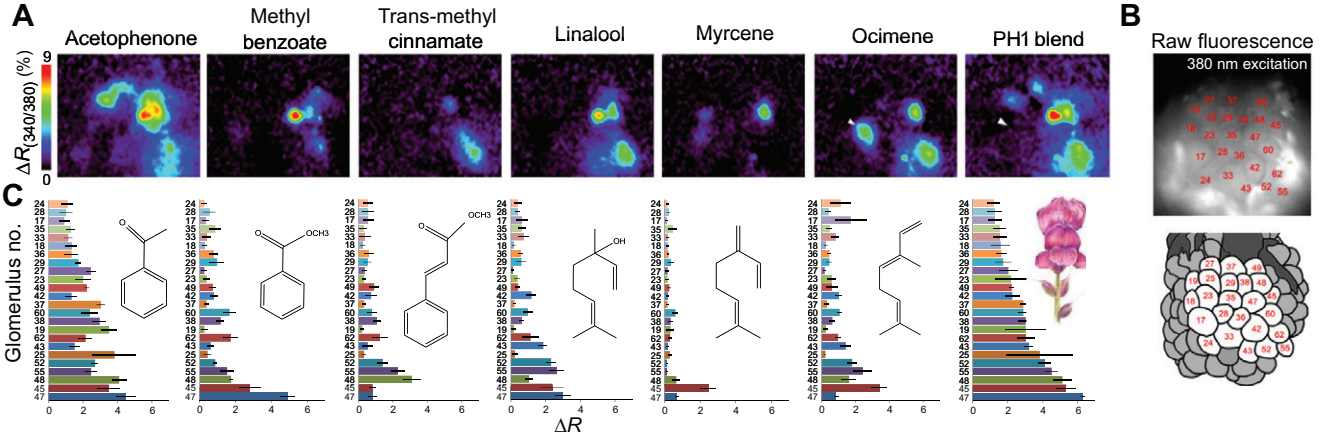

Fig. 1. Each component and the complete PH1 blend elicits unique patterns of activity across glomeruli using calcium imaging of projection neurons in the antennal lobe. (A) Pictures represent false color-coded images of activation patterns ($\Delta R_{340/380}$) induced by the six components and the blend in a representative bee. The activity shown for each component corresponds to the concentration of that component in the PH1 blend. Each picture is the average of the activity measured between 375 and 625 ms after odor onset. The white arrowhead points to glomerulus 17, which was activated by ocimene but suppressed in the blend in this example bee. (B) Raw fluorescence of the AL after staining the PNs with Fura-dextran and identification of 24 glomeruli on the dorsal surface. (C) Mean±s.e.m. activation across glomeruli elicited by each component and the PH1 blend (n=6 naïve animals). Glomeruli are ordered top to bottom from least to most activated during stimulation with the blend. The same ordering was used for each component to highlight the differences in the activation patterns elicited by different odors.

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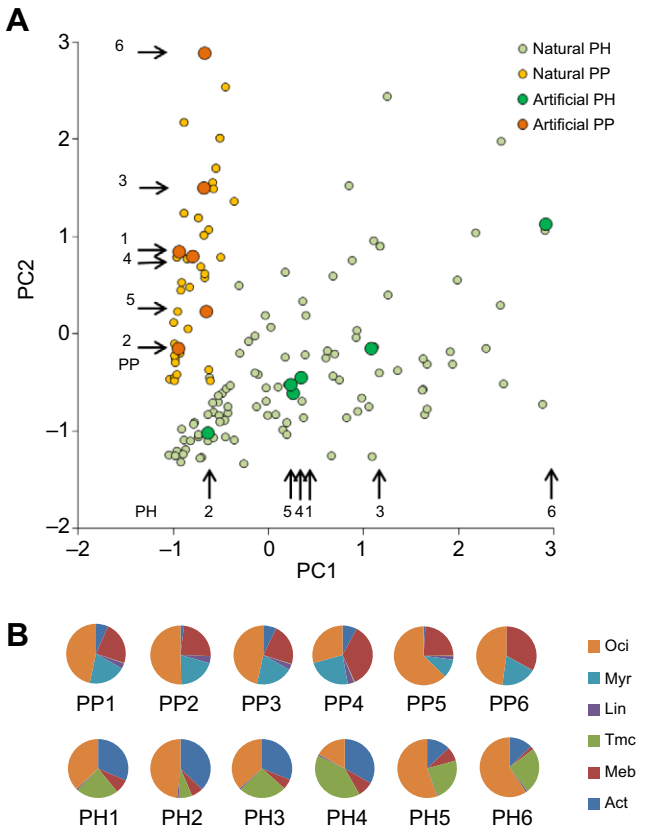

Fig. 2. Natural variation in composition of blends from *Antirrhinum majus* varieties. (A) The variation projected onto the first two principal components, which extract over 70% of the natural variation. The small green and orange circles represent volatiles collected from individual flowers of Pale hybrid (PH) and Potomac pink (PP), respectively (Wright et al., 2005a). Factor loadings of each of the six original components into the first two PCs are shown in Table S1. (B) Relative proportion of the six components in each one of the artificial blends (large darker circles in A). Each blend was made by mixing components in proportions that mimicked the variability found in the natural samples. The concentrations of the components in the PH1 and PP1 blends corresponded to the average proportion of each component measured in natural samples. The PH2 and PP2 blends were made with concentrations that corresponded to the 25th percentile of the concentrations measured in the natural varieties. PP3 and PH3 were composed of concentrations that correspond to the 75th percentile. Blends 4 and 5 were random combinations of the average, 25th or 75th percentile of each component. PP6 and PH6 were selected from the natural samples as extreme examples. Act, acetophenone; Meb, methyl benzoate; Tmc, trans-methyl cinnamate; Lin, linalool; Myr, myrcene; Oci, ocimene.

of bees treated in parallel were subjected to different olfactory experiences (Fig. 3A shows a scheme of the experimental design). One group of bees was trained using PP blends and PH blends. The PP blends were never rewarded and the PH blends were always rewarded. This group of bees is called PH+/PP− throughout this work (N=45). The second group of bees received experience only with blends from the PH variety and was stimulated with examples of the PP variety only during the transfer test trials and for calcium imaging. During unrewarded trials, this group of bees received the same handling but the olfactory stimulation was minimized using cartridges that contained only mineral oil (MO), the solvent in which odors were prepared. This group of bees is called PH+/MO− (n=33). The training protocol was divided into two sessions for both groups (1) pre-exposure and (2) discrimination conditioning. The first session consisted of 40 unrewarded trials. The bees from the PH+/PP− group were stimulated with all examples of the PP variety with the exception of the PP1 blend. This first phase was performed to mimic a situation in which a bee is seeking food sources and lands on different flowers of the same variety that have no nectar. The bees from the PH+/MO− group were manipulated in the same way but received only blank trials, i.e. no reward and no odor. The inter-trial interval during this session was 1 min. After this 40 min session, the bees were subjected to the second session, discrimination conditioning, in which unrewarded trials were intermingled with rewarded trials using PH blends. The discrimination conditioning protocol consisted of 16 trials in pseudorandom order of 8 rewarded and 8 unrewarded trials. For the PH+/PP− bees the sequence was: PHn+, PPn, PPn, PHn+, PPn, PHn+, PHn+, PPn, PHn+, PPn, PHn+, PPn, PPn, PHn+, PPn, PHn+, where PHn+ is an artificial blend selected from PH2 to PH6 and is paired with sucrose. PPn is an artificial blend selected from PP2 to PP6 presented without any reward. The bees from the PH+/MO− group followed a similar conditioning protocol but only mineral oil was loaded into the odor cartridges used in unrewarded trials. The PP1 and the PH1 blends were not used during conditioning. The inter-trial interval during discrimination conditioning was 5 min. In rewarded trials, the odor was paired with the unconditioned stimulus (US), which consisted of first touching the antennae with a 2.0 mol l^{-1} sucrose solution to elicit proboscis extension and immediately followed by feeding the bee with 0.4 µl of the same solution. Odor stimulation lasted for 4 s in all cases and the reward was applied 3 s after odor onset. During discrimination conditioning trials, the response of each subject was counted as a positive if the bee extended its proboscis during the first 3 s with odor stimulation. Twenty minutes after that, a transfer test session was performed to measure if bees would respond to odors that had not been used during training (Fig. 3A). The conditioned response was evaluated using 2-octanone as control odor and the two blends PP1 and PH1 in random order and separated by 10 min intervals. The odor stimulation lasted 4 s and the reward was omitted.

The transfer test trials were videorecorded for offline analysis of latency and duration of the proboscis extension. These two parameters of the proboscis extension have been shown to be more sensitive in identifying differences in the strength of the conditioned response (Fernandez et al., 2009; Smith, 1997). Duration was defined as the elapsed time that the proboscis is extended beyond the line connecting the tips of the opened mandibles. Latency is defined as the elapsed time between odor onset and the start of proboscis extension.

Projection neuron staining

Approximately 8 h after the transfer test, projection neurons (PNs) were stained by backfilling with the calcium sensor dye Fura-dextran (potassium salt, 10,000 MW, ThermoFisher Scientific). A window was cut in the head capsule dorsal to the joints of the antennae and rostral to the medial ocellus. The glands were carefully moved aside until the vertical-lobes (Rybak and Menzel, 1993) in the brain were visible and served as spatial reference for the staining (Sachse and Galizia, 2002). The tip of a glass electrode coated with Fura-dextran was inserted into both sides of the protocerebrum dorsolateral to the vertical lobes, aiming for the lateral antennal lobe tract (l-ALT) that contains the axons of uniglomerular PNs (Galizia and Rossler, 2010). The dye bolus dissolved into the tissue in 3–5 s. The window in the headcapsule was closed using the same piece of cuticle that was previously removed. Eicosane (Sigma-Aldrich) was used to seal the cuticle. The dye was left to travel along the tracts for 12–16 h. Calcium

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A

Group	Pre-exposure	Discrimination conditioning (16 trials)							Transfer test			~8 h	PN staining	~8 h	Calcium imaging
	40 trials	1	2	3	4	…	15	16	1	2	3				
PH+/PP–	PP*n*–	PH*n*+	PP*n*–	PP*n*–	PH*n*+	…	PP*n*–	PH*n*+	PH1–	PP1–	Oct–				PH1–PH6 PP1–PP6
PH+/MO–	MO–	PH*n*+	MO–	MO–	PH*n*+	…	MO–	PH*n*+	PH1–	PP1–	Oct–				

(Pseudo-random order; *n* = any blend from 2 to 6)

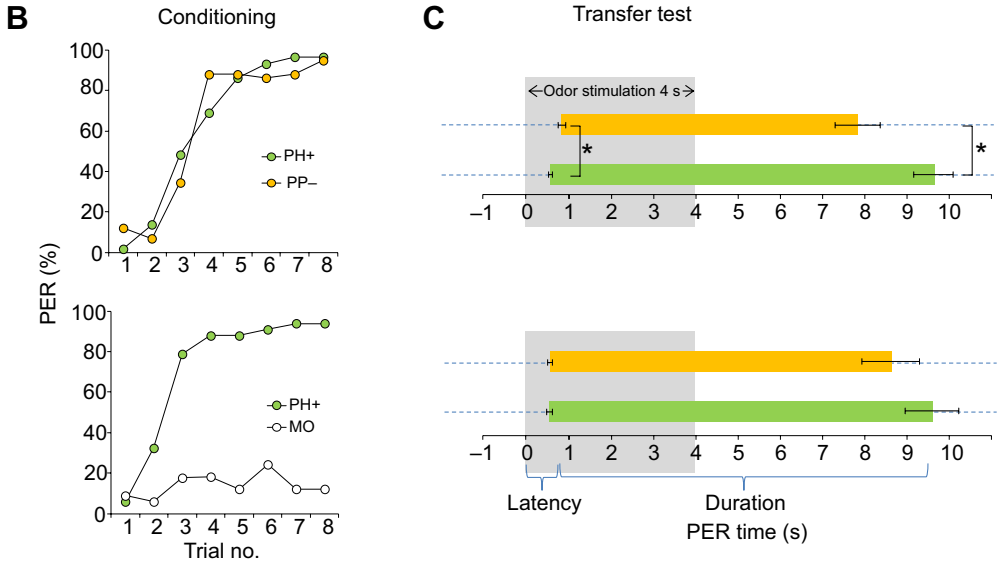

Fig. 3. Behavioral conditioning. (A) Two groups of bees were conditioned. Pre-exposure: 40 unrewarded trials with mineral oil (MO–) or pseudorandomized presentations of artificial blends of PP (PP2–PP6). Then, bees were differentially conditioned along 8 rewarded trials using the PH blends (PH2–PH6) intermingled with 8 unrewarded trials. Groups differed on unrewarded trials: the PH+/MO– group received mineral oil trials while the PH+/PP– group received trials with PP2–PP6 in randomized order. After training, all bees were subjected to a transfer test with PH1 and PP1, which had not been used during training, as well as with 2-octanone. (B) Percentage of proboscis extension across sequential acquisition trials in PH+/PP– (top; *n*=45 bees) and PH+/MO– (bottom; *n*=33 bees). (C) Transfer test results showing timelines representing latency and duration of proboscis extension (mean±s.e.) in the same bees as in B. Latency two-way repeated-measures ANOVA: training protocol $F_{1,77}$=6.233, P=0.01; test blend PH1 vs PP1 (repeated factor) $F_{1,77}$=6.121, P=0.01; interaction $F_{1,77}$=0.418, *P<0.05. Tukey's HSD test revealed significant differences in response latency to PP1 from group PH+/PP– in comparison with PH1 from the same group (P<0.01) and PH1 (P<0.01) and PP1 (P<0.01) from the PH+/MO– group. Duration two-way repeated measures ANOVA: training protocol $F_{1,77}$=0.270, NS; test blend PH1 vs PP1 (repeated factor) $F_{1,77}$=14.308, P<0.001; interaction $F_{1,77}$=0.267, NS. Tukey's HSD test, revealed significant differences in response duration to PH1 and PP1 in the differentially conditioned bees PH+/PP– (*P<0.01), whereas responses were not significantly different in PH+/MO– bees.

imaging was performed on the next day. Before imaging, the antennae were fixed pointing toward the front using Eicosane. Body movements were prevented by gently compressing the abdomen and thorax with a piece of foam. The brain was rinsed with Ringer solution (130 mmol l^{-1} NaCl, 6 mmol l^{-1} KCl, 4 mmol l^{-1} $MgCl_2$, 5 mmol l^{-1} $CaCl_2$, 160 mmol l^{-1} sucrose, 25 mmol l^{-1} glucose, 10 mmol l^{-1} HEPES, pH 6.7, 500 mOsmol; all Sigma-Aldrich), and glands and trachea covering the ALs were removed. A second hole was cut ventrally to the antennae and the compact structure of muscles, esophagus and supporting chitin was lifted and put under slight tension to prevent movements of the brain (Mauelshagen, 1993). The ALs were examined for staining and the side that presented more homogenous staining was selected for the measurements. After preparation and imaging of the bee, it was allowed to recover for 20 min before starting measurements.

Calcium imaging

Calcium imaging was done using a CCD camera (SensiCamQE, TILL Photonics) mounted on an upright fluorescence microscope (Olympus BX-50WI, Japan) equipped with a 20× objective, numerical aperture 0.95 (Olympus), 505 DRLPXR dichroic mirror and 515 nm LP filter (TILL Photonics). Monochromatic excitation light provided by a PolichromeV (TILL Photonics) alternated between 340 and 380 nm. Fluorescence was detected at a sampling rate of 8 Hz. Even though it is too slow to define fast temporal patterning or to analyze neural activity at spike time resolution, the sampling rate used here for calcium signals allows precise spatial resolution of the glomeruli that are recruited by the different odors and represent an indirect measure of spiking rate in each glomerulus (Galizia and Kimmerle, 2004). Spatial resolution was 172×130 pixels, after a binning of 8×8 on a chip of 1376×1040 pixels, resulting in a spatial sampling of 2.6 µm per pixel side. Exposure times were 8 and 2 ms for 340 and 380 nm, respectively. The entire image analyses were done using custom software written in Interactive Data Language (IDL; Research Systems) and routines created by Giovanni Galizia (University of Konstanz, Konstanz, Germany). Each data set of measurements consisted in a double sequence of 80 fluorescence images, obtained at 340 and 380 nm excitation ($F_{i,340\ nm}$, $F_{i,380\ nm}$, where i is the number of images from 1 to 80). Calcium signals were subsequently calculated using a ratiometric method: for each pair of images, we calculated the ratio R_i=($F_{i,340\ nm}$/$F_{i,380\ nm}$)×100 and subtracted the background R_b, obtained by averaging the R_i values 1 s immediately before the odor onset [R_b=1/8 (R_{16}+ …+ R_{23})]. Resulting values (ΔR) represent percentage of change from the reference window (R_{16}–R_{23}) and are proportional to the changes in the intracellular calcium concentration. The analysis was based on the calcium signals in glomeruli identified on the basis of their morphology and relative position using the digital atlas of the honeybee AL as reference

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(Flanagan and Mercer, 1989; Galizia et al., 1999). The visualization of glomeruli is possible by observing the raw fluorescence images obtained at 380 nm excitation. An additional tool written and provided by Mathias Ditzen (Freie Universitaet Berlin, Germany) was used to confirm glomerular identification. This tool calculates images representing the degree of correlation between neighboring pixels. Since glomeruli respond as functional units, pixels stemming from the same glomerulus are highly correlated over time. In contrast, pixels from different glomeruli are uncorrelated. This provides images in which glomeruli are clearly separated by contrasting boundaries. Twenty-four glomeruli were identified in all animals and were used in the present analysis (Fig. 1). All glomeruli were located in the dorsorostral side of the AL and correspond to a subset of glomeruli innervated by the antennal nerve tract 1, with exception of glomeruli 45, which belongs to tract III (Kirschner et al., 2006). We calculated activity in glomeruli 17–19, 23–25, 27–29, 33, 35–38, 42, 43, 45, 47–49, 52, 55, 60 and 62 according to previously established nomenclature (Flanagan and Mercer, 1989; Galizia et al., 1999). Glomerular activation was calculated by averaging activity in a square area of 9×9 pixels that correspond to 23.4×23.4 µm and fits well within the boundaries of the glomeruli. Glomerular activity in the present study refers to the activity of the uniglomerular PNs in each glomerulus because only these neurons were stained, and therefore, only this particular population of cells was measured in each glomerulus.

Pattern similarity assessment

The analysis was designed to determine how both training regimes (PH+/PP− and PH+/MO−) affect the neural representation of PH and PP flowers. It was based on Pearson correlation coefficients and Euclidean distances as a measure of the degree of similarity between the neural representations of the odors. For the analysis shown in Figs 1, 4 and 5, we collapsed the temporal detail of the measurements and averaged the values of ΔR measured from 375 ms to 625 ms after odor onset. Thus, the activation pattern elicited by each odor was reduced to a single vector with 24 elements that correspond to the activity measured in 24 glomeruli (see Fig. 4). This time interval was considered because it includes the time point at which odor representation reaches the maximal distance after odor onset (see Fig. 6) and because it includes the time point at which the conditioned response is elicited after odor onset (see Fig. 3). The similarity between any two odor patterns was calculated as the Pearson's correlation coefficients between the respective 24-dimensional vectors followed by Fisher's *Z*-transformation of the correlation value. We calculated the similarity between all possible combinations of PH blends (i.e. PH1 vs PH2, PH1 vs PH3,…, total=15 pairings), all possible combinations of PP blends (total=15 pairings) and all possible combination of PH and PP blends (total=36 pairings). In addition, since each odor was measured twice per animal, we could calculate the similarity between replicates (6 values for PH blends and

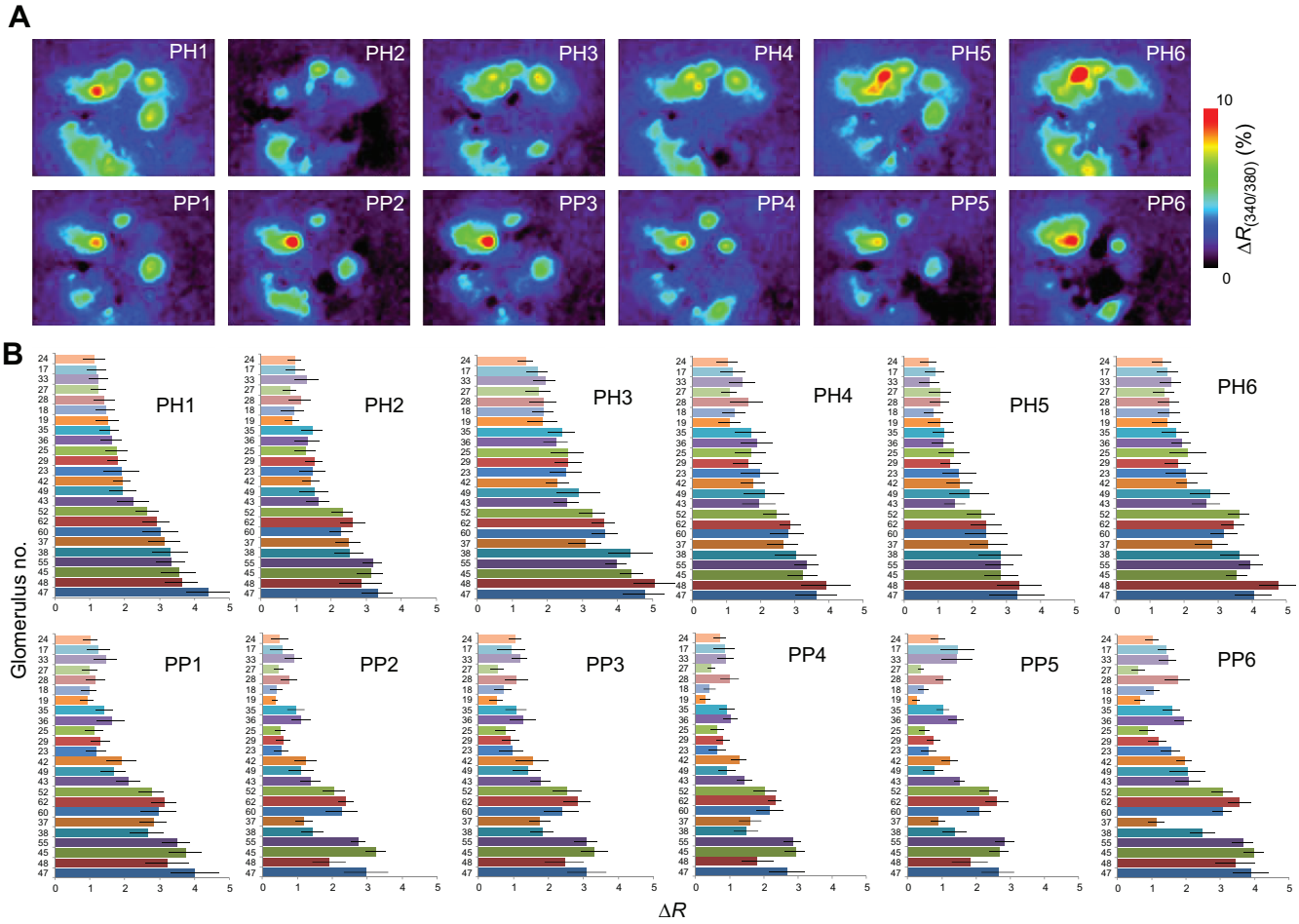

Fig. 4. Projection neuron responses to artificial blends. (A) Calcium imaging responses ($\Delta R_{340/380}$) elicited by artificial blends of PH and PP in a representative animal. (B) Graphs show the mean±s.e. activity measured in 24 identified glomeruli between 325 and 625 ms after stimulus onset in animals trained to PH+/MO− (*n*=10 bees). The glomeruli were ordered from lowest to highest response according to the blend PH1 and the same ordering was repeated for the remaining blends.

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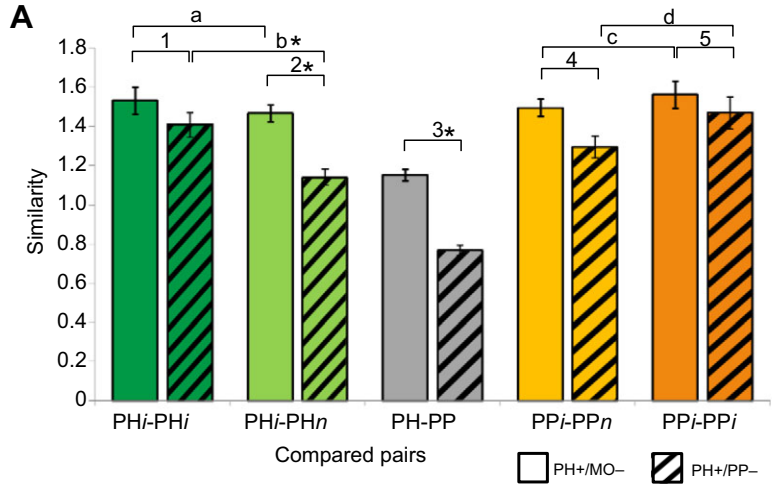

Two-factor ANOVA			
Factor	df	*F*	*p*
Compared pair	4:1221	61,51	<0.001
Training	1:1221	39,53	<0.001
Interaction	4:1221	3,45	0.008

	Newman–Keuls comparisons		*p*
1	PH*i*-PH*i* (PH+/MO–)	PH*i*-PH*i* (PH+/PP–)	0,535
2	PH*i*-PH*n* (PH+/MO–)	PH*i*-PH*n* (PH+/PP–)	<0.001
3	PH-PP (PH+/MO–)	PH-PP (PH+/PP–)	<0.001
4	PP*i*-PP*n* (PH+/MO–)	PP*i*-PP*n* (PH+/PP–)	0.097
5	PP*i*-PP*i* (PH+/MO–)	PP*i*-PP*i* (PH+/PP–)	0.649
a	PH*i*-PH*i* (PH+/MO–)	PH*i*-PH*n* (PH+/MO–)	0.854
b	PH*i*-PH*i* (PH+/PP–)	PP*i*-PH*n* (PH+/PP–)	0.004
c	PP*i*-PP*n* (PH+/MO–)	PP*i*-PP*i* (PH+/MO–)	0.683
d	PP*i*-PP*n* (PH+/PP–)	PP*i*-PP*i* (PH+/PP–)	0.141

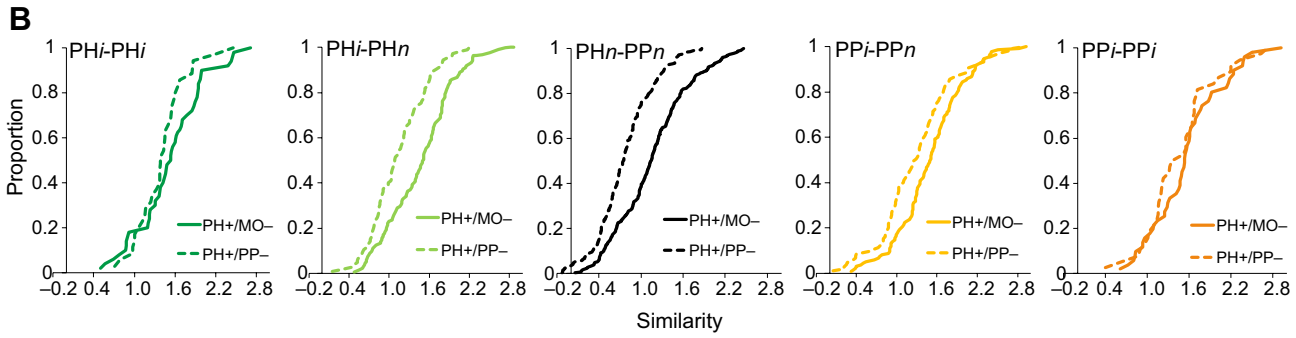

Fig. 5. Differential conditioning increases separation between the representations of floral blends. (A) To quantify similarity among neural representations of odors within and between varieties, we calculated correlations between all possible pairs of patterns elicited by the 12 synthetic floral blends as well as between replicate measurements of the same blend for both groups of bees (Table S2). The correlation values obtained were further Fisher's *Z*-transformed to be used in graphs and in statistical analyses. The obtained values were grouped according to: replicate measurements of the same blend (dark green and dark orange); different blends from the same cultivar (light green and light orange); and any two blends from different cultivars (gray bars). The graph shows mean±s.e.m. correlation values for each of these categories for PH+/MO– trained bees (solid, n=10) and PH+/PP– trained bees (hatched, n=7). Tables on the right show results of two-factor ANOVA with training condition (PH+/MO– or PH+/PP–) as one factor and correlated pair (5 categories indicated in the abscissa and explained above) as the second factor. *P*-values in the lower table correspond to the *post hoc* contrasts that are indicated with brackets and corresponding numbers or letters on the figure. Only statistically significant differences are indicated with an asterisk in the graph. (B) Same data as in A shown as cumulative proportion of correlations with specific values (x-axis) for each of the test conditions. Data represent accumulated numbers of values that were used to calculate the means in each cell in a color code (green, orange or gray) of Table S2.

6 values for PP blends). All these similarity values were determined for each animal; 10 bees for PH+/MO– and 7 bees for PH+/PP– (see Table S2). For statistical analysis, all the obtained similarity values were grouped in five classes according to the criteria: (a) similarity values between replicates of PH blends; (b) similarity values between different PH blends; (c) similarity values between PH and PP blends; (d) similarity values between different PP blends; (e) similarity values between replicates of PP blends. Then, we performed a two-factor ANOVA with these five categories as one factor and the kind of training protocol (PH+/MO– and PH+/PP–) as the second factor, to determine whether training modifies the degree of similarity within and between cultivars.

The analyses shown in Fig. 6 and Fig. S1 were performed taking into consideration the entire temporal detail of the measurements. For visualization of the odor trajectories, we reduced the 24 dimensions (glomeruli) using a principal component analysis (PCA) that identifies orthogonal axes (factors) that explain maximum variance in the data, and project the data into a lower-dimensionality space. The first two factors that explain most of the observed variance were further subjected to Varimax rotation (SPSS). Such rotational strategy maximizes the variance accounted by the calculated factors, while minimizing the variance around them. To quantify the separation between PH and PP blends that is visible in the PCA, we calculated the Euclidean distances (EDs) between all combinations of PP-PH blends. The EDs were calculated in the 24-dimensional space in which each dimension represents the activation of one glomerulus. The ED between any two odors X and Y was calculated as : $ED_{x-y}= [(R_{17,X}-R_{17,Y})^2 +\ldots+ (R_{62,X} -R_{62,Y})^2]^{1/2}$, where for example, $R_{17,X}$ means the response of glomerulus 17 during stimulation with odor X, and $R_{17,Y}$ means the response of glomerulus 17 during stimulation with odor Y. The 36 Euclidean distances obtained from 36 possible pairing between PH and PP blends, were averaged within an animal to obtain a single value representative of the separation PP-PH per animal. Once we had one value per animal, we calculated the mean±s.e.m. across animals of the distance between PH and PP for both training protocols (Fig. 6B). The same determination was also made to calculate the Pearson correlation coefficients instead of the EDs (Fig. S1).

RESULTS

Variation in natural floral blends separates varieties of flowers

We used six major chemical components of *A. majus* blends from Potomac pink (PP) and Pale hybrid (PH) (Wright et al., 2005a)

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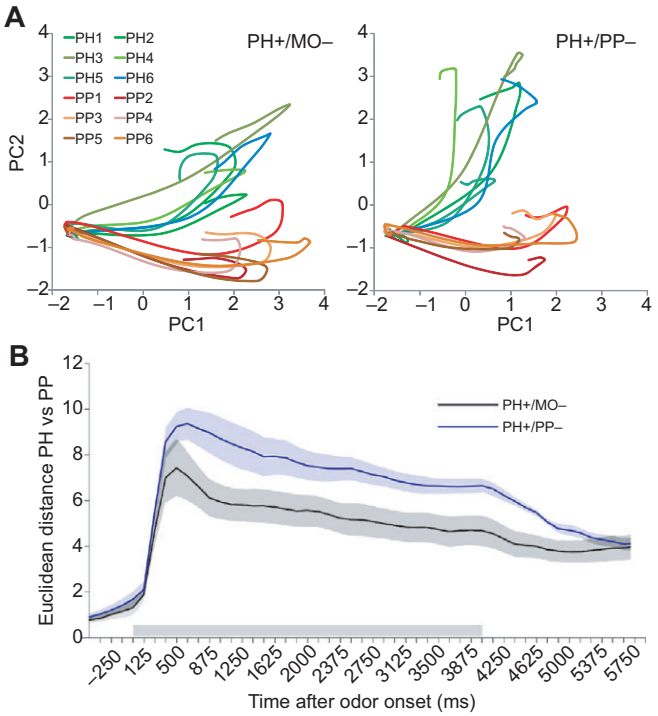

Fig. 6. Differential conditioning separates the spatiotemporal activity patterns of rewarded and non-rewarded blends. (A) 10 PH+/MO– bees and 7 PH+/PP– bees were combined by group to create two 'average' bees. The 12 spatiotemporal trajectories in each graph correspond to each of the synthetic blends. Each trajectory represents the activity of 24 glomeruli during the evolution of odor elicited activation pattern. Thus, the whole dataset of both average bees (24 activation patterns) were aligned by glomeruli and subjected to principal component analysis. The trajectories correspond to each blend shown from 3 frames before odor onset to 1 s after odor onset (12 frames out of a total of 80) plotted using the first two principal components (89% variance explained). PH+/MO– and PH+/PP– bees were plotted in separate graphs, but both graphs were obtained from a common PCA to make them comparable. Red/orange colors correspond to PP blends and green/blue colors to PH blends. (B) Euclidean distance between PH and PP blends is higher for differentially trained bees for the duration of the stimulus. ED was calculated in the original 24-dimensional space, frame by frame, and for all possible pairs of PH and PP flowers (36 combinations). The 36 distances were averaged to obtain a unique value of PH-PP distance per honey bee. The traces represent the mean±s.e. of 10 PH+/MO– bees (black) and 7 PH+/PP– bees (blue). Gray bar along *x*-axis represents odor stimulation.

(Fig. 1). The blends contain the same components, thus the varieties cannot be easily differentiated simply by the presence or absence of components. The first step was to evaluate whether the AL responds to each of the components, or whether the response to a mixture of all six might be dominated by a subset of components (Riffell et al., 2013).

We used calcium imaging of projection neurons to evaluate the activation patterns of the AL by each pure component and a six-component mixture. This method has been used to describe spatiotemporal activity in the insect AL (Fernandez et al., 2009; Locatelli et al., 2013; Sachse and Galizia, 2002). Each of the components elicited a distinct pattern of activation across the glomeruli on the dorsal surface of the AL (Fig. 1). Furthermore, the activity pattern for each of the components was distinct from the mixture of all six. A visual inspection of the example shown in Fig. 1 shows that the glomeruli excited in the PH1 blend were excited by the components found in highest proportion in this blend (i.e. acetophenone, ocimene and trans-methyl-cinnamate). There was also evidence of inhibitory interactions that shaped responses to blends. For example, in case of the bee shown in Fig. 1A, glomerulus 17 (arrow), which is excited by ocimene, vanishes in the blend. However, the suppression of glomerulus 17 was not consistent across all bees, which is evident in the quantification of the activity across all bees shown in Fig. 1C. The fact that the reduction in glomerulus 17 was not consistent across bees rules out the possibility that suppression is a consequence of physical interactions between the components in the mixture and suggests inter-individual differences with regard to how odors are encoded (Chen et al., 2015).

We then analyzed the statistical variation among odor blends emitted by individual *A. majus* flowers from published gas chromatographic data (Wright et al., 2005a) (Fig. 2A). We used PCA (see Materials and Methods and Table S1) to represent the variation within and between the varieties. Most (70%) of the variation was captured by the first two principal components. The two components each described correlated variation in two independent groups of three odors. Factor 1 contained strong (>0.75) factor loadings for acetophenone, T-methyl cinnamate and ocimene. Factor 2 contained strong loadings for linalool, methyl benzoate and myrcene. Therefore, all of the components contributed to the PCA. PP and PH flowers were each represented by a 'cluster' of points using these two axes. Individual samples of PP (Fig. 2A, small orange points) spread out within a cloud, and the most variation for this variety was described by PC2. In contrast, PH samples (Fig. 2A, small green points) fell within a different cloud that stretched along its longest dimension using a combination of PC1 and PC2.

There was very little overlap between clusters, even though the samples are all blends of the same six components. Therefore, the ratios of the blends statistically differentiated PP and PH. We used this information to create 12 different synthetic mixtures of the six components (Fig. 2B). Six of the mixtures fell within the PP cluster and the remaining six within the PH cluster (Fig. 2A, large circles). Moreover, we composed the synthetic blends such that they were distributed throughout the respective clusters of points. These synthetic mixtures therefore represent the range of variation that honey bees would encounter while foraging on these flowers.

Honey bees can behaviorally discriminate and categorize blends

We used PER conditioning (Smith and Burden, 2014) to evaluate whether honey bees can discriminate variation in *A. majus*. PER conditioning has been used to show that variation in molecular structures (Guerrieri et al., 2005; Smith and Menzel, 1989b), concentration (Wright et al., 2009) and blends (Fernandez et al., 2009; Wright et al., 2005b) define perceptual dimensions in the honey bee olfactory system.

We conditioned two groups of honey bees for behavioral tests prior to imaging in the AL (Fig. 3A). We adopted a procedure in which honey bees were conditioned using five of the six synthetic mixtures of each variety (PH and PP varieties 2 to 6; Fig. 2B). In the procedure we used differential conditioning across 16 trials, 8 of which were reinforced with sucrose pseudorandomly intermingled with 8 trials that were not. For the reinforced trials, both groups of honey bees experienced forward pairing of PH2–PH6 blends with sucrose in a way that produces conditioned responding. The blend differed from one trial to the next such that each of the five blends was presented at least one time and not more than twice over the sequence of 8 rewarded trials. For unreinforced trials, one group received presentations of the solvent mineral oil (MO) with no

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added odor (PH+/MO− group). The remaining group received presentations of PP2–PP6 blends (PH+/PP− group).

Our initial experiments revealed that discrimination between PH and PP was difficult and would require many trials. Thus, before conditioning, we added a pre-exposure phase (Fig. 3A) designed to reduce responses to the unreinforced odor (Chandra et al., 2010). The PH+/PP− group received pre-exposure to PP blends, and the PH+/MO− group was pre-exposed to mineral oil. Cumulatively, all of the trials added up to far fewer than the flower visits that a honey bee would experience during a single foraging trip in a field of flowers (Mattu et al., 2012). Additionally, this conditioning procedure involving many unreinforced exposures to odors provided a closer approximation of the experiences honey bees would have in a normal foraging context, when they can be exposed to flowers with and without reinforcement at different frequencies (Bertazzini and Forlani, 2016).

During the discrimination conditioning phase, honey bees quickly learned to respond to the odor blends (Fig. 3B). Honey bees in the PH+/MO− group could easily discriminate the PH odor from MO blank. They began to respond to PH odors by the second trial and reached peak response levels by the 4th to 8th trials. In contrast, honey bees had difficulty learning to respond specifically to PH when differentially conditioned against PP. As before, animals began to respond by the second trial and reached peak response levels by trials 4–8. However, in the PH+/PP− group, the acquisition curves failed to reveal discrimination of the PH and PP cultivars, indicating that discrimination is difficult.

Following the conditioning session, we performed a 'transfer test' during which all animals were identically tested in a randomized sequence without reinforcement with the two average blends – PH1 and PP1 – as well as with a completely different odor (2-octanone) as a control (Fig. 3C). None of the odors used in the transfer test were exposed during conditioning. During this transfer test, all of the bees in the two groups responded to PH1 and PP1. Neither group responded strongly to 2-octanone (13% and 14%, respectively), which shows that responses generalized among floral odors but not to any odor or some other aspect of the experimental context (data not shown).

Because of the high responses levels to PH1 and PP1, we analyzed response duration and latency. Both measures are sensitive to subtle differences in response topologies (Smith, 1997; Smith and Menzel, 1989a) and these measures frequently reveal differences in responses even when all animals respond with PER. Both measures revealed discrimination of PH1 from PP1 in differentially conditioned bees. Response durations were longer to PH1 relative to PP1 in the PH+/PP− group (Fig. 3C). The differences in the PH +/MO− group were in the same direction but failed to reach significance. Latency showed even more consistent differences. Animals responded to PP1 with longer latencies than to PH1 in the PH+/PP−, which indicates discrimination of the two odors. Latencies in the PH+/MO− group were equal, and they were also similar to the PH1 latency in the PH+/PP− group (Fig. 3C).

Synthetic blends can be distinguished using calcium imaging in the antennal lobe

The behavioral data imply that the blends are perceptually different to honey bees, but that they are difficult to discriminate relative to pure odors and simple binary mixtures (Chandra and Smith, 1998; Fernandez et al., 2009). We used calcium imaging to investigate how the AL separates the synthetic blends through differential activation of glomeruli. Eight hours after the transfer test, all the bees were stained for imaging AL activity on the following day. Ten learners from the PH+/MO− group and seven from the PH+/PP− group provided high-quality staining and reliable calcium signals that could be analyzed. We tested each animal twice with all PH1–PH6 and PP1–PP6 synthetic blends presented in a randomized

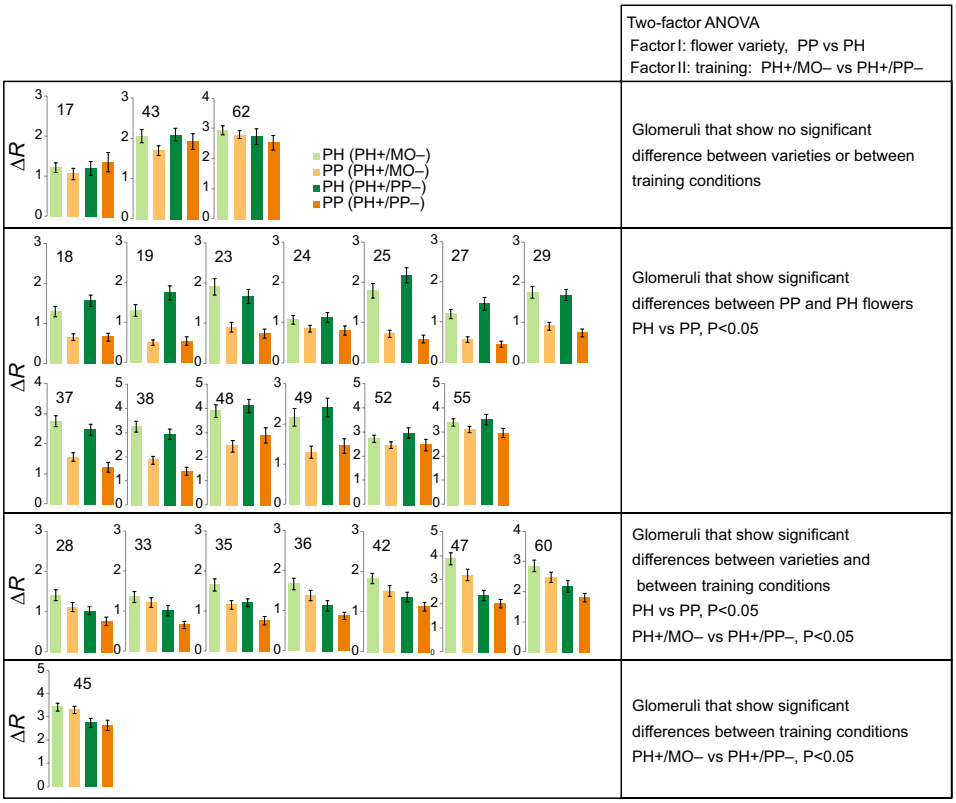

Fig. 7. Contribution of individual glomeruli to the different activity patterns elicited by both odor varieties and differentially affected by the training condition. Two-factor ANOVA was performed for each individual glomerulus to identify the glomeruli that are responsible for the different patterns among varieties and to identify glomeruli that are differentially affected by training. Each group of four bars shows the mean±s.e.m. of PH+/MO− (n=10) and PH+/PP− (n=7) honey bees for PH and PP varieties for each specific glomerulus. Glomeruli are grouped according to significant differences in the ANOVA factors. No glomerulus showed a significant interaction between factors.

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sequence. This test design allowed us to make three types of comparisons between the patterns measured in each individual animal. First, we calculated the correlation between patterns elicited by the same blend from one test to the next (e.g. PH1 to PH1, PP1 to PP1 etc.). Second, we calculated the correlations from all blends to all others of the same variety (PH1 to PH2–PH6 and PP1 to PP2–PP6 etc.). Third, we calculated the correlations from each of the blends of one variety to all other blends from the other variety (PH1 to PP1–PP6 etc.; Table S2).

Fig. 4A shows images representative of activity patterns in the AL induced by each of the 12 synthetic floral blends. Qualitatively, even slight differences in odor ratios evoke different activation patterns (Fig. 4B). As might be expected, differences within blend type (PH or PP) were subtle relative to differences between them. These differences were reflected in lower correlation coefficients in pairwise correlations between PH and PP blends than within PP or within PH blends (Table S2).

Differential conditioning de-correlates odor blends of different varieties

Correlations across the three types of comparisons depended on the training protocol (Fig. 5A,B). For both training protocols, the strongest correlations (R>0.9; similarity>1.5) were for retesting the same blends (Ph*i*-PH*i* and PP*i*-PP*i*; Table S2). Correlations between different blends from the same variety (PH*i*-PH*n* and PP*i*-PP*n*) in control honey bees were the same as retesting the same blend. Interestingly, differential conditioning reduced the Ph*i*-PH*n* (rewarded odor) correlation relative to the control bees. A similar, but not statistically significant, trend was observed for the non-rewarded cultivar (PP*i*-PP*n*). Finally, differential conditioning significantly reduced the correlations between varieties (PH-PP). In reference to the clusters in Fig. 2, differential conditioning using blends from the two varieties spread the points out slightly within each cluster (PH*i*-PH*n* and PP*i*-PP*n* correlations) and significantly spread the clusters apart (PH-PP correlations).

Decorrelation arises early during stimulation and is maintained for the stimulus duration

We analyzed the temporal evolution of the PN activity patterns across 24 glomeruli during the entire stimulus duration for all synthetic PH and PP blends in both training conditions (Fig. 6). The spatiotemporal patterns were analyzed as trajectories in a two-dimensional space defined by the first two principal components obtained after a Principal Component Analysis (Fig. 6A). The temporal evolution of the response to each odor is represented by a trajectory through this space (Fernandez et al., 2009; Galan et al., 2006). After stimulus onset, trajectories depart from rest near the origin and begin to separate. The trajectories slow down by 500 ms which is consistent with previous measurements. At that point, the trajectories remain at a fixed separation until odor offset, possibly having reached a fixed point (Mazor and Laurent, 2005). Qualitatively, the trajectories separate the PH and PP cultivars and suggest that activity patterns for the varieties are more clearly separated in the differentially conditioned group (PH+/PP−) (Fig. 6A). To quantify the relationships among PH and PP cultivars during the stimulus, we calculated the Euclidean distances (Fig. 6B) and the correlation coefficients (Fig. S1) among all possible PH*n*-PP*n* pairs at each 125 ms time point in the 24-dimensional space defined by the 24 glomeruli. We decided to use and show both measures because of disagreement in regard to which one of them provides a better indication of pattern similarity (Locatelli and Rela, 2014). Both measures show that the patterns evoked by PH and PP cultivars during the duration of the stimulus are more clearly separated in honey bees that had been differentially conditioned (PH+/PP− group).

Glomeruli show heterogeneous responses to blends and in plasticity

The mean changes reported above do not capture the diversity of patterns across all glomeruli. We therefore performed a more detailed analysis of the responses among the 24 glomeruli to the PH and PP blends in the two training protocols (Fig. 7). For this analysis, we averaged the responses from all PP*n* blends and from all PH*n* blends to get two unique PP and PH flower scents per bee. The glomeruli clustered into four categories according to their contribution to differentiation between cultivars and between training conditions. Three glomeruli (Fig. 7, top row) failed to show any difference in response to either cultivar or training conditions. Thirteen glomeruli (Fig. 7, second row) separated PP and PH varieties in both training conditions but failed to separate the two training conditions. Seven glomeruli (Fig. 7, third row) separated PP and PH as well as the training conditions. In all seven cases the responses were lower on average in the differentially trained group. Finally, in one glomerulus (Fig. 7, bottom) the responses were lower in the differentially trained group but the responses to PP and PH were not different.

DISCUSSION

Several studies have documented both non-associative and associative plasticity in early olfactory processing in mammalian and insect brains (Fernandez et al., 2009; Locatelli et al., 2013; Wilson and Linster, 2008). However, studies have not addressed why this plasticity exists in relation to the ecological problem it helps to solve. We have framed the ecological problem in terms of the variation from one odor object to the next using floral odors as a model. We define categorization as the separation of odor (floral) objects that have different meanings. The category is defined minimally by both the mean and variance of the blends encoded by activity patterns in AL. Any activity pattern that falls within the boundary defined by experience should be classified as having the same meaning – e.g. reward or no reward. These boundaries are set, in part, by generalization of excitation around the odor blends that have been directly associated with food. However, the boundaries are not simply the sums of all of the Gaussian distributions around these odor blends. Instead, we show here that the category boundaries can be changed by different types of experience and especially by the unrewarded trials.

One of the fundamental questions about floral odors is whether several of the components in a blend are relevant for the code, or whether only a few more salient components dominate it (Riffell et al., 2013). We show that the honey bee AL encodes all of the odors we used in making synthetic blends and that most of the glomeruli activated by components are also activated in the blend. However, some glomeruli that were activated by a component failed to be activated, or were less activated, in the blend (e.g. glomerulus 17 in Fig. 1). These differences between components and the mixture, particularly in regard to suppression of glomeruli, most likely arose from local inhibitory interactions in the AL network (Linster et al., 2005; Sachse and Galizia, 2002; Shen et al., 2013).

Given the influence of inhibitory connections in shaping responses of the AL network, changing the weights of inhibitory connections could change activity after conditioning in ways that we and others have described (Chen et al., 2015; Linster and Smith, 1997). In the honey bee AL, an octopamine-based sucrose

reinforcement pathway (Hammer, 1993) specifically targets a group of local inhibitory (GABAergic) interneurons (Sinakevitch et al., 2011, 2013). Modeling studies have shown how modification of this type of inhibition in the network can produce associative changes in AL processing (Linster and Smith, 1997). It is of course still possible that octopamine drives plasticity through direct modulation of excitatory projection neurons rather than, or in addition to, modulating inhibition. Even though such direct action has not yet been described, experimental and modeling approaches cited above predict the modulation of inhibitory synapses that target inhibitory processes, which could result in more excitation of the PNs. The nature of the unreinforced experience also differed between treatment groups in our experiments (MO− versus PP−). Unreinforced exposure to odor reduces responsiveness to that odor (Chandra et al., 2010) and also alters processing in the AL, possibly via Hebbian (nonassociative) plasticity at specific synapses (Locatelli et al., 2013). Therefore it is likely that both nonassociative and associative mechanisms operate to produce the changes we report.

However, the different mechanisms of plasticity do not have a uniform impact on different glomeruli in our study or in previous ones (Fernandez et al., 2009; Locatelli et al., 2013). We observed many possible changes and combinations thereof, although the average change contributed to greater separation (categorization) of the odors we tested (Fig. 7). Some glomeruli show no changes as a result of odor exposure or reinforcement. Other glomeruli change in relation to the odors presented and/or in relation to the conditioning protocol. The changes we observed are similar to changes in AL activity patterns produced by application of octopamine (Rein et al., 2013). In their study Rein et al. (2013) applied octopamine, which produced changes (excitation and inhibition) that depended on the odor. These complex patterns are likely to be the result of network-level modulation of glomeruli that are differentially activated by different odors, rather than solely to a global effect such as excitation. But the nature of the specific mechanism is still undetermined.

We designed our experiments based on the assumption that both nonassociative and associative mechanisms of plasticity contribute to the changes we observed in behavior as well as in AL processing. When odors are associated with sucrose reinforcement, the PER response to the conditioned odors increases (Bitterman et al., 1983). However, with backward pairing (odor is delivered after sucrose) or after allowing too much of a gap between odor and sucrose when forward pairing, conditioned inhibition is produced (Hellstern et al., 1998; Rescorla, 1969). Furthermore unreinforced exposure to odor produces latent inhibition (Chandra et al., 2010; Lubow, 1973). Both types of inhibition can slow subsequent learning about an odor. All of the mechanisms can act in parallel to produce decisions about floral odors (Bazhenov et al., 2013), particularly as the frequency of flowers without nectar changes in a patch as a honey bee forages.

In the present work we trained the animals on one day and measured AL activity approximately 24 h later. In doing it in this way, we minimized learning and memory disruptions caused by stress associated with preparation for imaging. Interestingly, the fact that changes affecting mixture representation in the AL are evident 1 day after the experience (Chen et al., 2015; Fernandez et al., 2009; Sandoz et al., 2003) suggests that the changes may constitute a component of a consolidated, protein synthesis-dependent olfactory memory. It remains to be determined if changes we describe in the AL emerge immediately after conditioning or whether they only emerge in the interval from training to imaging that we used.

Our analyses highlight the need to understand more detail about the statistics of odor spaces, just as statistical analyses of visual scenes have contributed to an understanding of neural mechanisms of vision (Sharpee et al., 2004). We observed that plasticity in the AL enables a more efficient use of coding space by increasing the separation between rewarded and unrewarded odor blends along dimensions of variation that best separate the blends. Moreover, differential conditioning also increased separation among representations of blends from the same cultivar relative to non-differentially conditioned bees. We interpret these two effects as part of the same mechanism that expands the coding space of the AL in the region that represents the blends. Future studies should address how later stages of processing, for example, in the mushroom bodies, use the increased separation within and between clouds of floral odor signatures to categorize stimuli.

In the honey bee there are approximately 170 odorant receptors expressed in the periphery (Robertson and Wanner, 2006). With combinatorial coding, this number allows the encoding of an enormous number of odors (Bushdid et al., 2014), although a precise estimate remains to be determined (Gerkin and Castro, 2015; Meister, 2015). However, only a few odors will be relevant to any animal at any point in its lifetime. Furthermore, those few odors may represent very similar clusters of blends – such as those we have used here – associated with different meanings, such as the presence or absence of nectar. Temporary expansion of the coding space used to represent similar odors, as we have shown here, would help to more efficiently use the vast coding space to represent and separate boundaries for odor categories that are very similar yet are important to differentiate. The space could then be reconfigured to solve a different problem when those odors lose relevance.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceived and designed the experiments: F.L., P.F., B.H.S. Performed the experiments: F.L., P.F. Analyzed the data: F.L., P.F. Wrote the paper: F.L., P.F., B.H.S.

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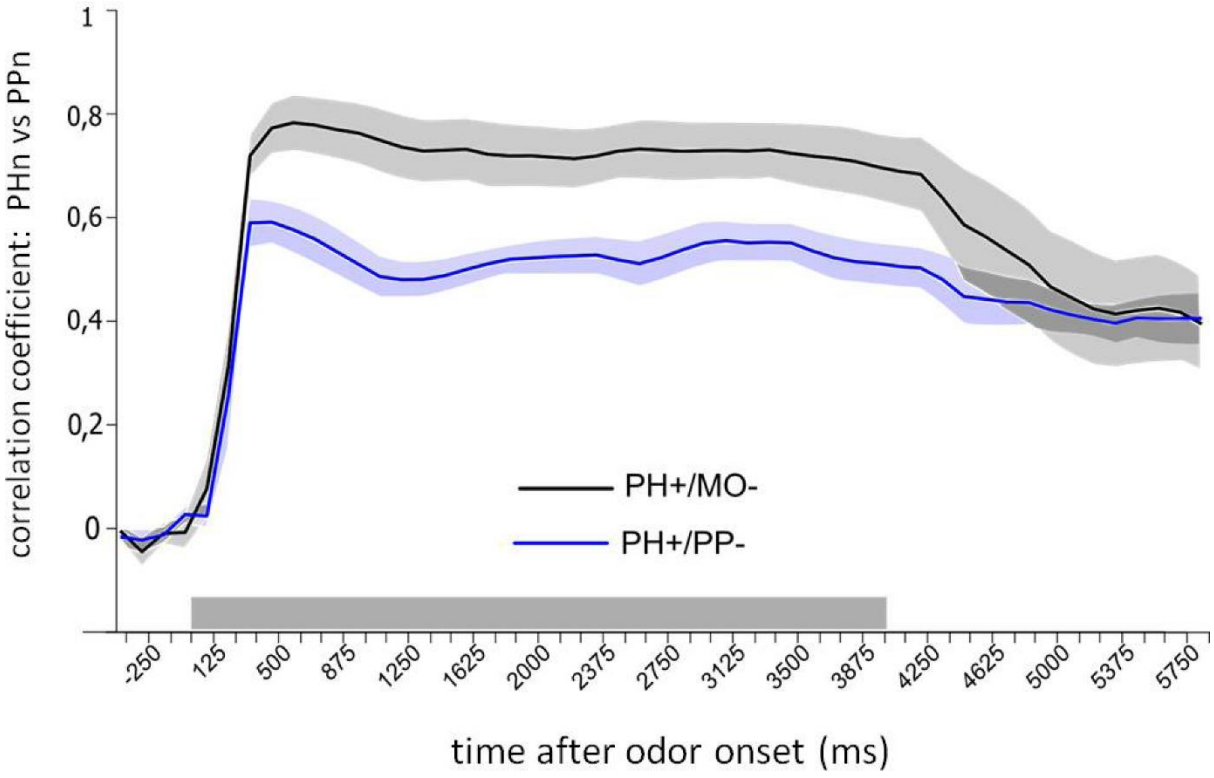

Fig. s1

Differential conditioning decorrelates the response patterns elicited by rewarded and non-rewarded varieties.

The Pearson's correlation coefficient between the response patterns elicited by flowers from the PH and the PP variety blends is lower for PH+/PP- trained bees along the whole duration of the olfactory stimulus. The Pearson's correlation coefficient was calculated, frame-by-frame for all possible pairs of PH and PP flowers (36 combinations per frame). The 36 correlation values obtained in each individual bee were averaged to get a unique value per bee that represents a general correlation between PH and PP blends. The traces represent the mean ± SEM of these values across bees. Black line: PH+/MO, n=10 and blue line: PH+/PP-, n=7.

Table s1

PCA of snapdragon odors.

A population of 99 flowers from the PH cultivar and 44 flowers from the PP cultivar were subjected to principal components analysis on the basis of their odor component content (acetophenone, trans-methyl cinnamate, ocimene, linalool, methyl benzoate and myrcene). The first two principal components explain 70% of the variance. The outcome of the analysis was further subjected to "Varimax" rotation. The table shows the loading of each of the original 6 factors onto the first two principal components.

	Factor 1	Factor 2
Acetophenone	0.846	-0.179
Trans Methyl Cinnamate	0.778	-0.121
Ocimene	0.835	0.330
Linalool	0.125	0.794
Methyl Benzoate	0.005	0.744
Myrcene	-0.421	0.781

Table s2

Matrix showing the similarity between activation patterns elicited by rewarded and non-rewarded blends and for both training conditions.

Correlation values were Fisher's Z transformed and used as measurement of similarity between patterns. 36 similarity values correspond to flowers from different cultivars (gray); 15 values for different flowers of the PH variety (light green) and 15 values for different blends of the PP cultivar (light orange). Since each blend was measured two times in each animal, we calculated the similarity between replicates of the same blend (dark green and dark orange). A matrix as the one the in figure was conformed for each animal. The values in the present table correspond to the average of all animals for training groups. The half matrix above the diagonal correspond to data from PH+/MO- trained bees (n=10) and the half matrix bellow the diagonal correspond to data from differentially trained bees PH+ /PP- (n=7). The bottom table is the same as the top but just color-coded to show degree of similarity.

	PH1	PH2	PH3	PH4	PH5	PH6	PP1	PP2	PP3	PP4	PP5	PP6	
		1,245	1,290	1,195	1,488	1,397	1,109	1,227	1,009	1,132	1,073	0,733	1,067	PH1
PH1	1,313		1,069	1,176	1,347	1,309	1,102	1,263	1,131	1,299	1,291	0,803	1,012	PH2
PH2	1,187	0,959		1,471	1,298	1,200	1,379	0,994	0,909	0,989	0,934	0,662	1,007	PH3
PH3	1,191	1,107	1,440		1,293	1,516	1,275	1,110	0,962	1,142	1,052	0,710	1,073	PH4
PH4	1,029	1,104	1,205	1,242		1,400	1,177	1,140	0,976	1,165	1,055	0,692	0,978	PH5
PH5	0,936	1,112	0,985	0,990	1,747		1,695	0,906	0,817	0,962	0,862	0,646	0,950	PH6
PH6	0,886	0,857	1,114	0,896	0,840	1,447		1,577	1,572	1,735	1,654	1,039	1,147	PP1
PP1	1,020	0,928	0,685	0,533	0,853	0,629	1,634		1,327	1,513	1,574	1,166	1,278	PP2
PP2	0,814	0,857	0,535	0,428	0,731	0,579	1,514	1,063		1,145	1,717	1,063	1,375	PP3
PP3	0,809	0,906	0,667	0,527	0,976	0,595	1,286	1,180	1,262		1,274	1,088	1,232	PP4
PP4	0,728	0,953	0,632	0,519	0,881	0,625	1,179	1,267	1,247	1,072		1,361	1,003	PP5
PP5	0,515	0,490	0,446	0,297	0,534	0,405	0,806	0,851	0,805	0,823	1,252		1,594	PP6
PP6	0,973	0,878	0,861	0,675	1,014	0,774	1,231	1,164	1,132	1,249	0,966	1,850		
	PH1	PH2	PH3	PH4	PH5	PH6	PP1	PP2	PP3	PP4	PP5	PP6		

PH retest
two different PH blends
any PH and PP blends
two different PP blends
PP retest