

Olfactory Coding in the Honeybee Lateral Horn

Edith Roussel,^{1,2,4} Julie Carcaud,^{1,2,3,4} Maud Combe,^{1,2} Martin Giurfa,^{1,2} and Jean-Christophe Sandoz^{3,*}

¹Research Center on Animal Cognition, Université de Toulouse (UPS), 118 Route de Narbonne, 31062 Toulouse Cedex 9, France

²Research Center on Animal Cognition, Centre National de la Recherche Scientifique (CNRS), 118 Route de Narbonne, 31062 Toulouse Cedex 9, France

³Evolution, Genomes, and Speciation Lab, CNRS (UPR 9034), 1 Avenue de la Terrasse, 91198 Gif-sur-Yvette Cedex, France

Summary

Olfactory systems dynamically encode odor information in the nervous system. Insects constitute a well-established model for the study of the neural processes underlying olfactory perception. In insects, odors are detected by sensory neurons located in the antennae, whose axons project to a primary processing center, the antennal lobe [1, 2]. There, the olfactory message is reshaped and further conveyed to higher-order centers, the mushroom bodies and the lateral horn [3–5]. Previous work has intensively analyzed the principles of olfactory processing in the antennal lobe and in the mushroom bodies [6–9]. However, how the lateral horn participates in olfactory coding remains comparatively more enigmatic. We studied odor representation at the input to the lateral horn of the honeybee, a social insect that relies on both floral odors for foraging and pheromones for social communication [10, 11]. Using *in vivo* calcium imaging, we show consistent neural activity in the honeybee lateral horn upon stimulation with both floral volatiles and social pheromones. Recordings reveal odor-specific maps in this brain region as stimulations with the same odorant elicit more similar spatial activity patterns than stimulations with different odorants. Odor-similarity relationships are mostly conserved between antennal lobe and lateral horn, so that odor maps recorded in the lateral horn allow predicting bees' behavioral responses to floral odorants. In addition, a clear segregation of odorants based on pheromone type is found in both structures. The lateral horn thus contains an odor-specific map with distinct representations for the different bee pheromones, a prerequisite for eliciting specific behaviors.

Results

Olfactory Coding in the Lateral Horn

Using *in vivo* calcium imaging, we specifically recorded the responses of axon terminals of a well-defined population of projection neurons (PNs; belonging to the lateral antenno-protocerebral tract, I-APT), which conveys olfactory information from the antennal lobe (AL) to both the lateral horn (LH) and mushroom body (MB) (Figure 1A). Retrograde staining of these neurons with Fura 2-dextran produced, along with the

well-described staining of their dendrites within AL glomeruli (Figure 1A1), a homogeneous staining composed of numerous fine axon terminals throughout the LH (Figure 1A2). We found that clear odor-evoked activity could be measured at this location (Figure 1B). Bees were presented with ten different stimuli: general odorants of various chemical structures (e.g., 1-hexanol, 1-nonanol, hexanal, octanal, 2-octanone, and 2-nonanone) and constituents of social pheromones (e.g., geraniol, citral, isopentyl acetate, and 2-heptanone). All compounds elicited clear calcium signals in the LH characterized by phasic-tonic time courses ($n = 4–9$; Figure 1C). For all odors tested, the amplitude of the calcium responses was significantly higher than for the air control (Figure 1D; Wilcoxon matched pairs test; in all cases, $z > 2.20$, $p < 0.03$). Typically, the shape of the activated LH region (see Figure 1B) corresponded to the pattern of projections of I-APT neurons, which innervate the most rostral region of the LH [12].

We then analyzed the spatial organization of odor signals in the LH. Figure 2A shows activity patterns recorded in one animal upon three stimulations with 1-hexanol and octanal, two odorants that evoked equally strong calcium signals in the LH (see Figure 1D). Although some variability could be observed from one presentation to the next, topical regions were systematically activated for each odorant. These activity regions were different for the two recorded odorants, as shown by the comparison between the two average patterns. To quantify the specificity of odor coding in the LH, we computed pixelwise Euclidian distances between any two presentations of the same odorant (1-hexanol versus 1-hexanol or octanal versus octanal) or of different odorants (1-hexanol versus octanal). We found that the average distance for two 1-hexanol patterns or two octanal patterns was significantly shorter than the average distance between 1-hexanol and octanal patterns (Figure 2B; Wilcoxon signed-rank test, $z = 2.66$, $p < 0.01$). The same result was found when extending the analysis to the ten odorants in the odor panel (Figures 2C and 2D; Wilcoxon signed-rank test, $z = 2.66$, $p < 0.01$). We thus conclude that the LH contains specific activation maps at its input, which can be measured using *in vivo* calcium imaging.

Comparison of Odor Coding in the Lateral Horn and in the Antennal Lobe

Previous work showed that odor coding rules may be transformed between the dendrites of PNs in the AL and their axon terminals in the lips of the MB calyces [13]. We reasoned that through possible rewiring and local processing, the quantitative and qualitative principles of olfactory coding may change from the AL to the LH. We thus compared odor-evoked signals at the two ends of PNs. Using the same staining technique in a new set of animals, we recorded odor-induced calcium signals in the AL for the same panel of ten odorants ($n = 6$ bees). As found in previous studies [3, 14, 15], specific sets of AL glomeruli were activated by each odor, but not by the air control (Figure S1A available online). We found a highly significant correlation between the intensity of calcium signals in the LH and in the AL (Figure 3A; $R^2 = 0.92$, $p < 0.001$, 8 degrees of freedom [df]). Thus, the more an odor activates the AL, the more it activates the LH. Similarly, we found a clear correlation between similarity measures among odorants in the AL and in

⁴These authors contributed equally to this work

*Correspondence: sandoz@legs.cnrs-gif.fr



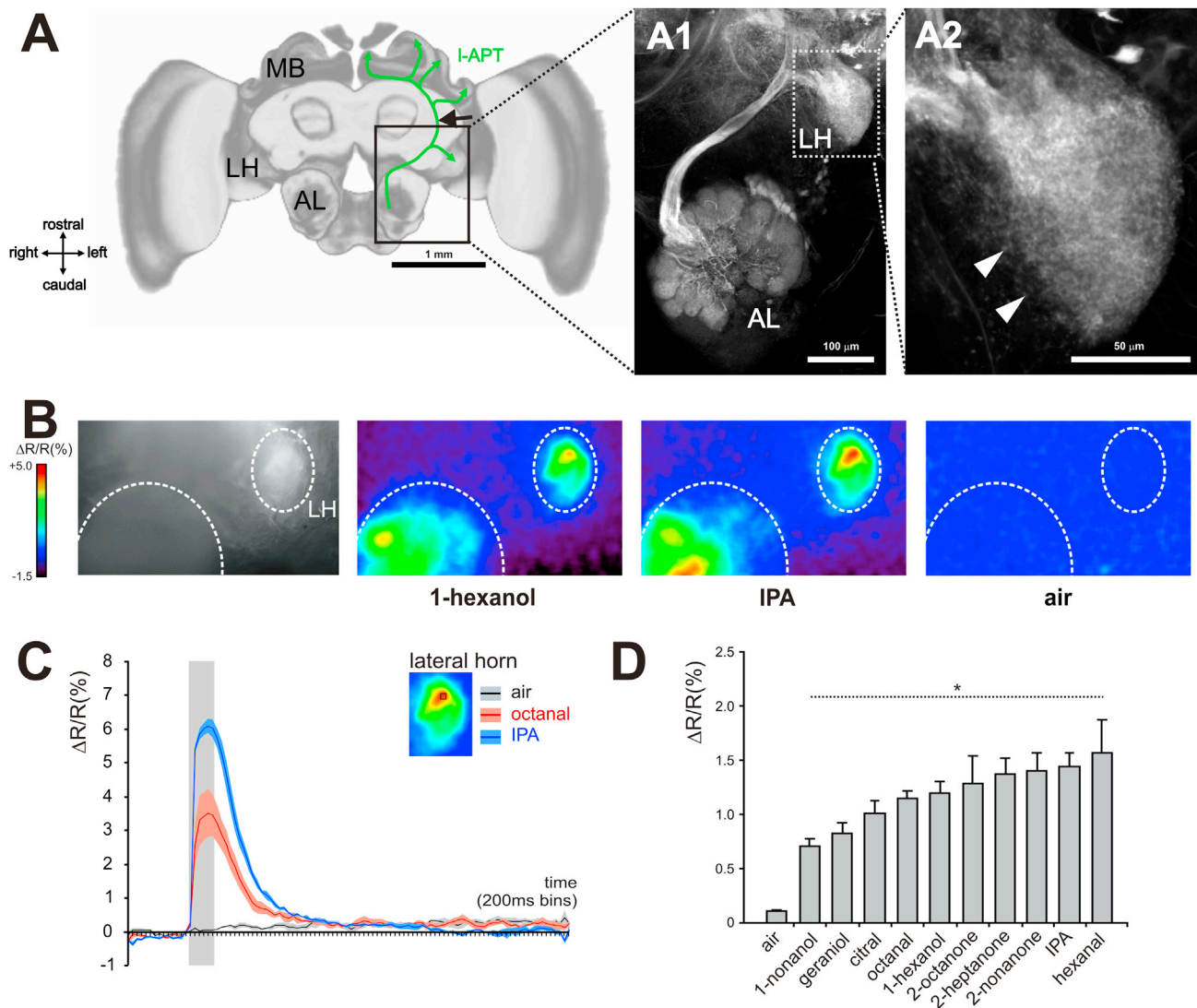


Figure 1. Calcium Imaging of Projection Neurons in the Lateral Horn

(A) Left: honeybee brain showing, in green, the innervation of PNs of the lateral tract (I-APT) from the AL to the LH and the MB calyces. Dye injection location is shown with a black arrow. Right: retrograde staining (revealed by tetramethylrhodamine dextran injected together with Fura-2 dextran) of I-APT PNs showing their innervation in AL glomeruli (A1) and their terminals in the LH (A2). Note the homogeneous mesh of fine PN processes in the LH. (B) False-color-coded activity maps of the recorded region containing the AL and the LH. Relative fluorescence changes ($\Delta R/R(\%)$) are presented in a false-color code, from dark blue to red. PNs show calcium signals to odors but not to the air control in the AL and the LH. (C) Typical time course of calcium signals in the LH (average of three presentations) measured in the rostral part of the LH, shown by a black square in the inset. (D) Mean amplitude of activation for the rostral part of the LH (as in C) to ten odorants and the air control ($n = 4-9$ bees). Odor-induced signal amplitude was always higher than that of the air control. * $p < 0.05$.

the LH (Figure 3B; $R^2 = 0.64$, Mantel test, $p < 0.001$, 44 df). Thus, similarity relationships are mostly conserved between the two levels. As AL signals predict the bees' behavioral responses in a generalization experiment after appetitive conditioning [16, 17], we asked whether odor-response maps recorded in the LH also have this property. Seven of the odorants tested here were also included in a prior appetitive conditioning study which established a generalization matrix among aliphatic odorants [16]. Figures S1B and S1C present odor-similarity matrices for all pairs of these seven odorants for the AL and for the LH, as well as for the behavioral study. We found many similarities among the three matrices, interodor distances being mostly determined by chemical similarity (chain length and carbon chain length; Figures S1B and S1C).

Accordingly, distances computed in the AL and the LH correlated significantly with behavioral distances (Figure 3C; $R^2 = 0.20$, Mantel test, $p < 0.05$, 19 df and $R^2 = 0.21$, $p < 0.05$, 19 df, respectively). Thus, odor-response maps in the LH predict whether honeybees treat odorants as similar or not in an appetitive conditioning experiment.

Pheromone Coding in the Lateral Horn

We then asked how pheromonal odorants are encoded in the LH of bees and how this coding relates to that in the AL. Bees were presented with a large odor panel containing 21 components of the bees' most important pheromones: queen mandibular pheromone (QMP) (five compounds and the QMP mixture), brood pheromone (two compounds), alarm

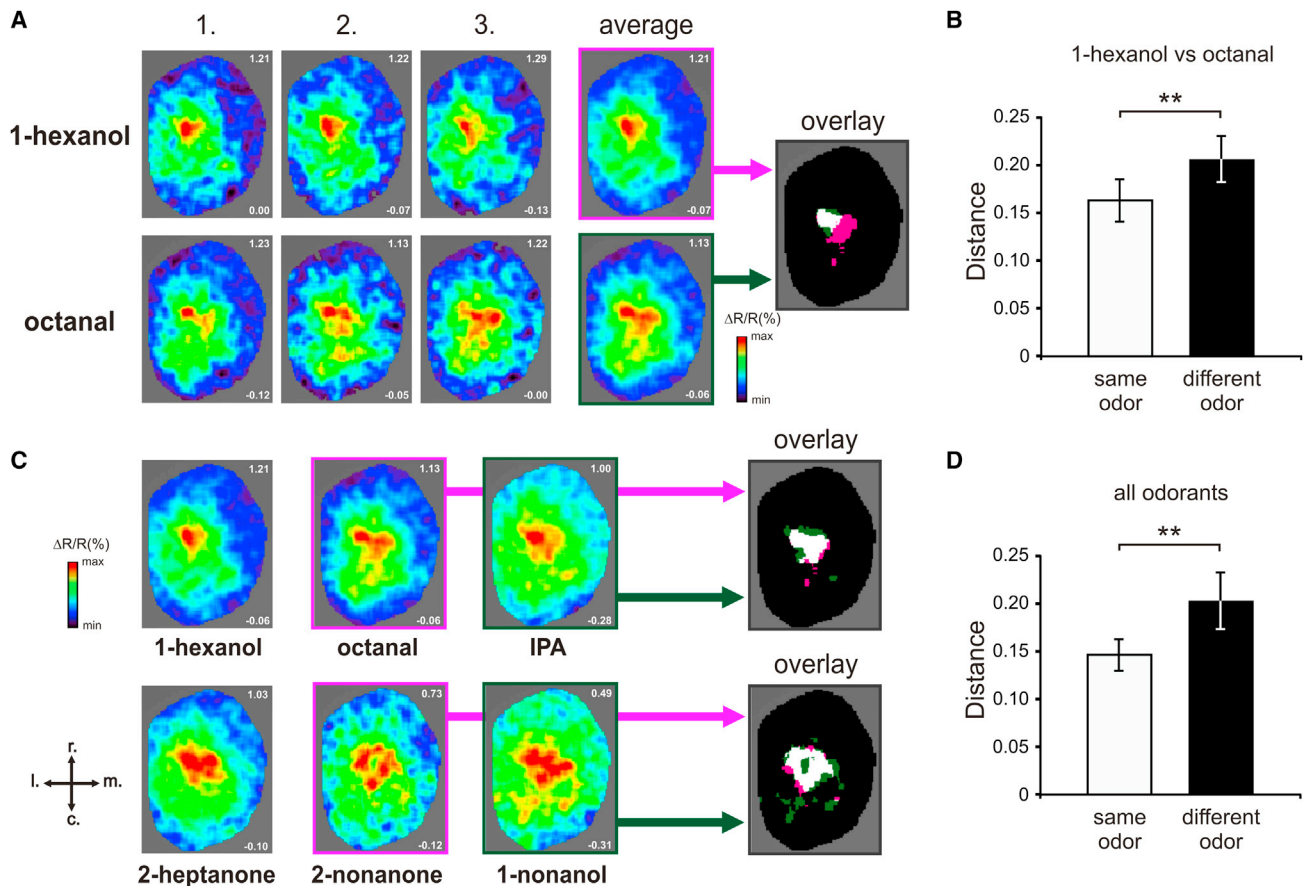


Figure 2. Olfactory Coding in the Lateral Horn

(A) Three presentations of two odorants (1-hexanol and octanal) in the LH. Each odorant induces reproducible activity patterns in the LH. Each map is scaled to its own minimum and maximum ($\Delta R/R\%$ values are indicated in white). The average maps of the three presentations of each odorant are represented on the right, along with an overlay of most strongly activated pixels by both odorants.
 (B) Similarity between presentations of the same odor or of different odors with these two odorants (1-hexanol and octanal) in nine bees. Activity maps are more similar (i.e., distances are shorter) when the same odor is presented than when a different odor is presented.
 (C) Calcium signals in the LH to six odorants (1-hexanol, 1-nonanol, octanal, 2-nonanone, 2-heptanone, and IPA). Different odors induce different activity patterns in the LH. Overlays for example pairs of odorants are shown on the right.
 (D) Same as (B), but including all ten odorants. Again, distances are shorter when the same odor is presented than when a different odor is presented, showing specific odor coding in the LH.
 Error bars in (B) and (D) represent the SEM. See also Figure S3.

pheromone (nine compounds), and aggregation pheromone (four compounds). In each bee, either the LH ($n = 10$ bees) or the AL ($n = 10$ bees) was imaged. Recordings were carried out at higher magnification to obtain more highly resolved maps of LH activity (Figure 4A). All pheromonal odorants induced a significant calcium response in the bee LH ($n = 10$ bees; Figure S2A; Wilcoxon signed-rank test, all $p < 0.05$). Pheromonal odorants followed the same rules described above for floral odorants: odor response maps in the LH were more similar for the same odorant than for different odorants (Figure S2B; Euclidian distances; Wilcoxon signed-rank test, $z = 2.70$, $p < 0.01$), and signal intensity and similarity relationships were correlated between LH and AL (Figures S2C and S2D; $R^2 = 0.79$, $p < 0.001$, 19 df and $R^2 = 0.29$, Mantel test, $p < 0.001$, 208 df, respectively). However, for odor similarity, the correlation coefficient was moderate ($R^2 = 0.29$), and the dispersion of the distances around the regression line suggested rearrangements of odor representations in the bees' olfactory space. To study this possibility, we next observed how the different types of odorants are arranged in the neural spaces

defined by the pixelwise responses within each structure. To this aim, we performed proximity analyses (Figure 4B), which extract from a distance matrix principal dimensions explaining most of the variance in the data. For the LH, the first two dimensions (71% of overall variance) mostly segregated the different types of pheromones, with all queen pheromonal components being grouped together and separated from brood components and worker-produced pheromones. These alarm and aggregation pheromones were only partly resolved, with substantial overlap between them. Such segregation could either be acquired through rewiring and presynaptic processing or be inherited from the AL. In the AL (Figure 4B, bottom), the first two dimensions (55% of overall variance) showed a rather similar arrangement of odor representations as in the LH. Queen, brood, and aggregation pheromones were clearly segregated, while alarm components were widely spread and overlapped with the three other pheromone types. According to these representations, rearrangements between the two processing levels are rather subtle. We thus compared the relative similarity among pheromone types in these two structures

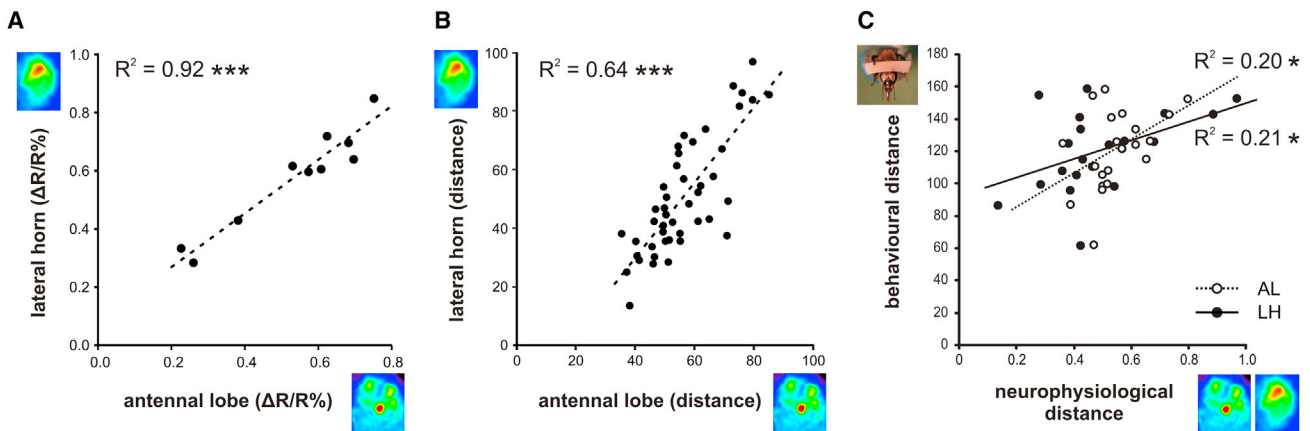


Figure 3. Comparison of Odor Coding in the Antennal Lobe and the Lateral Horn

(A) Correlation between the amplitude of activation for each odor in the AL and in the LH. The correlation is positive and highly significant ($R^2 = 0.92$, $p < 0.001$), suggesting a conservation of response intensity at the two processing levels.
 (B) Correlation of Euclidian distances between odor pairs calculated in the AL and in the LH. The correlation is positive and highly significant ($R^2 = 0.64$, $p < 0.001$), suggesting a conservation of similarity relationships at the two processing levels.
 (C) Correlation of distances between odors from optophysiological measurements in the AL (white circles) or in the LH (black circles) with distances obtained from behavioral experiments measuring perceptual similarity among odorants using a generalization procedure [16]. Both correlations are significant (with AL measures: $R^2 = 0.20$, $p < 0.05$; with LH measures: $R^2 = 0.21$, $p < 0.05$), indicating that neural distances measured in the AL and in the LH can predict bees' olfactory behavior.

* $p < 0.05$, *** $p < 0.001$. See also Figure S1.

independently of the distribution of distances within each data set, using a nonparametric ranking analysis. At each processing step, all possible odorant pairs were ranked from the shortest distance (rank 1, highest similarity) to the longest distance (rank 171, lowest similarity). Figure 4C presents average ranks in the AL and in the LH for odorant pairs belonging to the same pheromone or to different pheromones, for queen, alarm, and aggregation pheromones (brood pheromone was excluded because only two such components were tested, yielding only one distance measure). Figure 4D presents the same calculation for all pheromone types together. In the LH, distances between odorants belonging to the same pheromone always had lower ranks, i.e., response maps were more similar, than distances between odorants belonging to different pheromone types (Wilcoxon test; for each pheromone type, $z > 2.09$, $p < 0.05$; for all pheromones together, $z = 2.70$, $p < 0.01$). Thus, the LH clearly contains an olfactory representation that allows segregating honeybees' main pheromone types. However, this segregation was strongly inherited from the AL, as a similar effect was already found there (Wilcoxon test; for each pheromone type, $z > 2.80$, $p < 0.01$; for all pheromones together, $z = 2.80$, $p < 0.01$). Direct comparisons of the ranks obtained in the LH and the AL were almost all nonsignificant (Mann-Whitney test, $z < 1.59$, not significant). The only comparison that yielded a significant outcome was that comparing the distances between alarm pheromone and other pheromone types, which had lower ranks, i.e., lower similarity, in the LH compared to the AL (Mann-Whitney test, $z = -2.27$, $p < 0.05$). We conclude that the LH contains a segregated representation of the different pheromone types, which is inherited from the AL.

Discussion

We performed functional measurements of odor-evoked neural activity at the input of the LH to provide a first account of olfactory processing in this region of the honeybee brain. We show that this structure responds to a wide range of odorants,

including floral and pheromonal compounds, and that neural activity evoked by these odorants reflects an odor-specific code as they elicit different spatial activity maps. Comparison of the coding principles between AL and LH shows a strong conservation of response intensity and odor-similarity relationships. Consequently, similarity relationships recorded in the LH allow predicting honeybees' generalization behavior in an appetitive conditioning experiment. In addition, the LH contains a segregated representation for the bees' main pheromone types.

An Olfactory Code in the LH

The LH receives both direct olfactory information from the AL and processed information from the MBs and is thought to be a premotor center controlling fast odor-driven behaviors [18–20]. Previous neuroanatomical work in fruit flies described the wiring in the LH of PNs belonging to different AL glomeruli and found a stereotypical branching pattern of the PN terminals [21–23]. This organization could support an odor-specific map that could serve a basic odor recognition function. Our data demonstrate the existence of such odor-specific maps at the input to the LH. These maps are specific and allow predicting the bees' olfactory generalization.

The intensity of activation induced by different odors in the LH was highly correlated with intensities in the AL. We also found clear evidence for a general conservation of odor-similarity relationships among odorants in the two structures. These observations show that in the honeybee PN, signals from the AL are conserved upon entry in the LH [21, 22], which indicates that local LH neurons do not presynaptically reshape olfactory information. This is clearly different from the presynaptic boutons of the same neurons entering the MB calyx [13]. There, presynaptic inhibition from MB recurrent neurons (protocerebral-calycal tract neurons) reshapes olfactory input information, especially in I-APT neurons. These observations underline the clearly different organization of these PNs' inputs in the two higher-order centers of the bee brain.

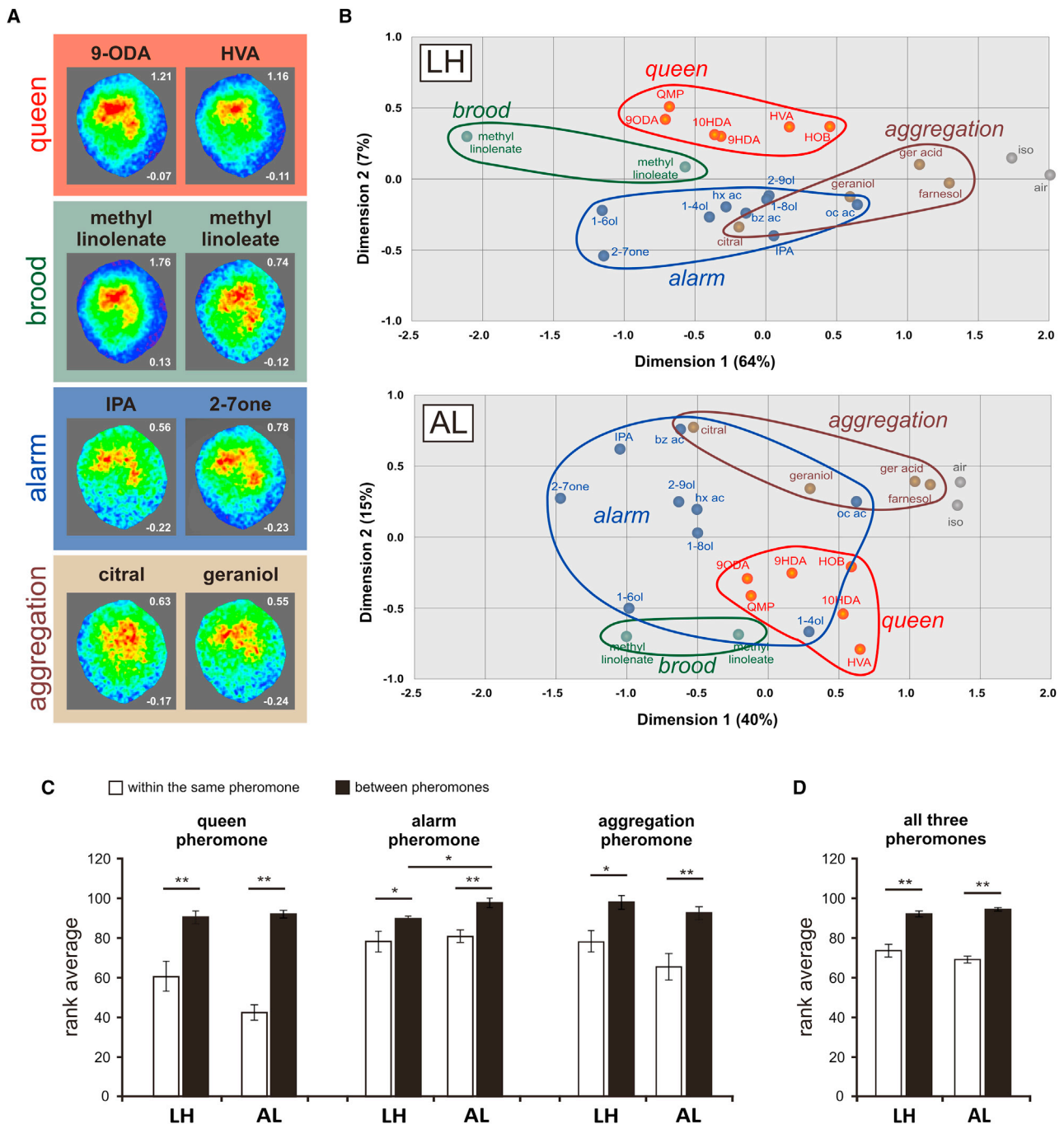


Figure 4. Representation of Pheromones in the Lateral Horn

(A) Example activity maps of components of the four main honeybee pheromone types (queen, brood, alarm, and aggregation pheromones) in the LH. Response amplitude ($\Delta R/R\%$) is presented in a false-color code, from dark blue to red. Each map is scaled to its own minimum and maximum ($\Delta R/R\%$ values are indicated in white).

(B) Proximity analysis showing similarity relationships among pheromonal components using Euclidean distances obtained for the 210 odor pairs, in the LH (top) and in the AL (bottom). In the LH, the different types of pheromones were mostly segregated, with a clear segregation of queen pheromonal components from brood components and worker-produced pheromones (alarm and aggregation). In the AL, a rather similar arrangement of odor representations as in the LH is found. Queen, brood, and aggregation pheromones were clearly segregated, while alarm components tended to be more widely spread and to overlap with the three other pheromone types.

(C) Ranking analysis of Euclidean distances among odor pairs within the same pheromone and between pheromones for three main pheromone types, as measured in the LH and in the AL ($n = 171$ odor pairs). All distances measured for odorants belonging to the same pheromone had a lower rank (i.e., a higher similarity) than distances for odorants belonging to different pheromones, both in the LH and the AL. Ranks obtained in the LH and in the AL were not different, except for distances between alarm pheromone and other pheromone types, which had lower ranks in the LH compared to the AL.

(D) Same calculations for all three pheromones together. No difference appeared between the AL and the LH. Thus, pheromone-specific maps in the LH are inherited from the AL.

* $p < 0.05$, ** $p < 0.01$. Error bars in (C) and (D) represent the SEM. See also Figures S2 and S3.

How fine is the LH odor representation compared to that at the level of the MB, in terms of the coding of odors' chemical structure? The MBs possess numerous and highly specific intrinsic neurons (~170,000 Kenyon cells per brain hemisphere in bees), which are only sparsely activated by particular combinations of inputs from many PNs [7, 8, 24]. Comparatively less is known about third-order LH neurons, but they are thought to be much less numerous than MB neurons (a few hundred in *Drosophila* [25]). Therefore, these neurons would probably only have the capacity to describe general differences among olfactory stimuli, i.e., in a less-specific fashion than Kenyon cells. One may thus imagine that while MB neurons would be critical for an *odor discrimination* function, differentiating between highly similar olfactory stimuli (odorants with highly similar chemical structures or odor blends with a slightly different compositions), LH neurons may be adapted for an *odor generalization* function, providing information about general similarity among odorants.

Pheromone Representation in the LH

The honeybee LH responded to all tested pheromonal components. In *Drosophila*, anatomical data suggested that PNs responding to fruit odors and sex pheromones are spatially segregated in the LH [18]. As a consequence, the LH would be organized according to the biological value of odorants rather than to their chemical nature. Our data in honeybees only partly substantiate this proposal. We indeed found that response maps elicited by honeybee pheromones in the LH are mostly segregated according to the type of pheromone considered. However, in our observations, most activity maps were widely spread over the whole LH, and we could not identify any specific region of the LH that would be specific for a single pheromone type. Moreover, the same activity regions were activated by odorants with a floral or with a pheromonal value for bees. Thus, we deem it unlikely that the honeybee LH would harbor clearly segregated compartments for the different pheromones or a clear separation between general odorants and pheromones. In contrast to fruit flies, honeybees are social insects, and their colony life is ruled by complex communication networks based on the use of many social pheromones conveying messages on alarm, aggregation and attraction, queen dominance, or brood presence [10, 11]. In addition, most pheromones in honeybees are complex chemical blends, in which the ratios of individual components may be critical for granting a specific biological value to a pheromone [10, 26, 27]. Coding such a plethora of pheromones following a labeled-line principle does not seem adaptive. Rather, our data suggest that the pheromonal message arriving in the LH contains information allowing segregation among pheromone types, but based on an across-fiber pattern strategy. In other words, third-order neurons need to read out information from several PNs simultaneously for classifying an odorant as a pheromone, especially for pheromonal mixtures.

From LH to Behavior

How is the olfactory message arriving in the LH translated into specific behaviors? In bees, neural networks within the LH are still unknown. Anatomical data in cockroaches suggest that descending neurons involved in behavior control do not take information directly from the LH, but that their dendritic arbors are broadly distributed in the lateral and medial protocerebrum [28]. Thus, several processing steps can take place before the olfactory message is transformed into motor output. A study in

locusts systematically stained and recorded the activity from many LH neurons and found a wide variety of different anatomical types of LH neurons, but all of these neurons tended to respond broadly to many odorants [29]. In *Drosophila*, different types of LH neurons have also been described, with some highly specific neurons but also some much more broadly tuned neurons [25, 30, 31]. The general idea stemming from these studies is that the LH cannot code the precise chemical quality of a wide range of odorants (this would be the MBs' task), but would rather provide for most odorants broad similarity information. As suggested above, this property could be instrumental for generalization among odors, for instance during feeding behavior. In addition, particular LH neurons would be highly specific to particular pheromonal compounds and would allow triggering highly stereotyped behaviors [25, 31]. In honeybees, whose rich social behavior strongly relies on the use of many complex pheromones (see above), we would expect the existence of many types of LH neurons, each tuned to particular PN patterns. However, only a careful study of LH neurons and their possible connections with descending neurons and/or MB output neurons may help understanding the exact role of the LH in shaping olfactory behavior. Our study indicates that the LH receives enough information both for showing generalization gradients to different odorants in appetitive learning experiments and for responding with specific behaviors (i.e., inducing specific motor output) for the different pheromone types.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at <http://dx.doi.org/10.1016/j.cub.2014.01.063>.

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