

# The glomerular code for odor representation is species specific in the honeybee *Apis mellifera*

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Odors are coded by glomerular activity patterns in the insect antennal lobe (AL) and in the mammalian olfactory bulb. We measured glomerular responses to 30 different odors in the AL of honeybees using calcium-sensitive dyes. By subsequently staining glomeruli and identifying individual glomerular outlines, we were able to compare the patterns between animals. Regardless of whether the odors were mixtures or pure substances, environmental odors or pheromones, their representations were highly conserved among individuals. Therefore, it may be possible to create a functional atlas of the AL in which particular molecular receptive ranges are attributed to each glomerulus.

Physiological experiments have shown that odors evoke spatially organized activity patterns in both the vertebrate olfactory bulb<sup>1–6</sup> and the insect antennal lobe<sup>7–12</sup>. Evidence is mounting that this spatial organization is conserved within a species: in rodents, axons of receptor neurons expressing the same receptor gene converge to equivalent positions in different individuals<sup>13</sup>. Physiological responses to particular odors also show similar species-typical activity patterns<sup>14</sup>; this was demonstrated using electrophysiological recordings in rabbits<sup>15</sup>, radioactively labelled 2-deoxyglucose in rats<sup>3,4</sup> and optical recording in zebra fish<sup>5</sup>. However, up to now, mapping activity patterns to morphologically identified glomeruli was possible only in pheromone processing systems<sup>6,16</sup>.

We show that activity patterns are conserved between individuals at the level of identified, 'ordinary' glomeruli in the AL of the honeybee. Such a dedicated role of glomeruli previously has been shown only for the macroglomerular complex in insects<sup>17</sup> and the modified glomerular complex in mammals<sup>16</sup>. The AL of the worker honeybee consists of 156–166 glomeruli arranged peripherally in a single layer. The glomeruli possess stereotypical, species-specific morphology<sup>18</sup>. They range in diameter between 30 and 50  $\mu\text{m}$  and differ in shape and relative arrangement. This has made it possible to create a three-dimensional atlas of the honeybee AL<sup>19,20</sup>, in which each glomerulus is given a name composed of its afferent receptor axon tract (T1 to T4) and a number (for example, T1-45 or T3-45).

## RESULTS

To physiologically characterize identified glomeruli in an *in-vivo* preparation of the honeybee AL, we monitored intracellular calcium with Calcium Green<sup>9,10</sup>. Previous measurements showed that elicited activity patterns are odor-specific, stable for repeated stimuli<sup>9</sup> and bilaterally symmetrical<sup>11</sup>. When the AL is stained with Calcium Green, glomerular borders cannot be anatomically identified (Fig. 1a). Therefore, to visualize the glomerular layout, we stained the AL with the membrane-soluble dye RH795 fol-

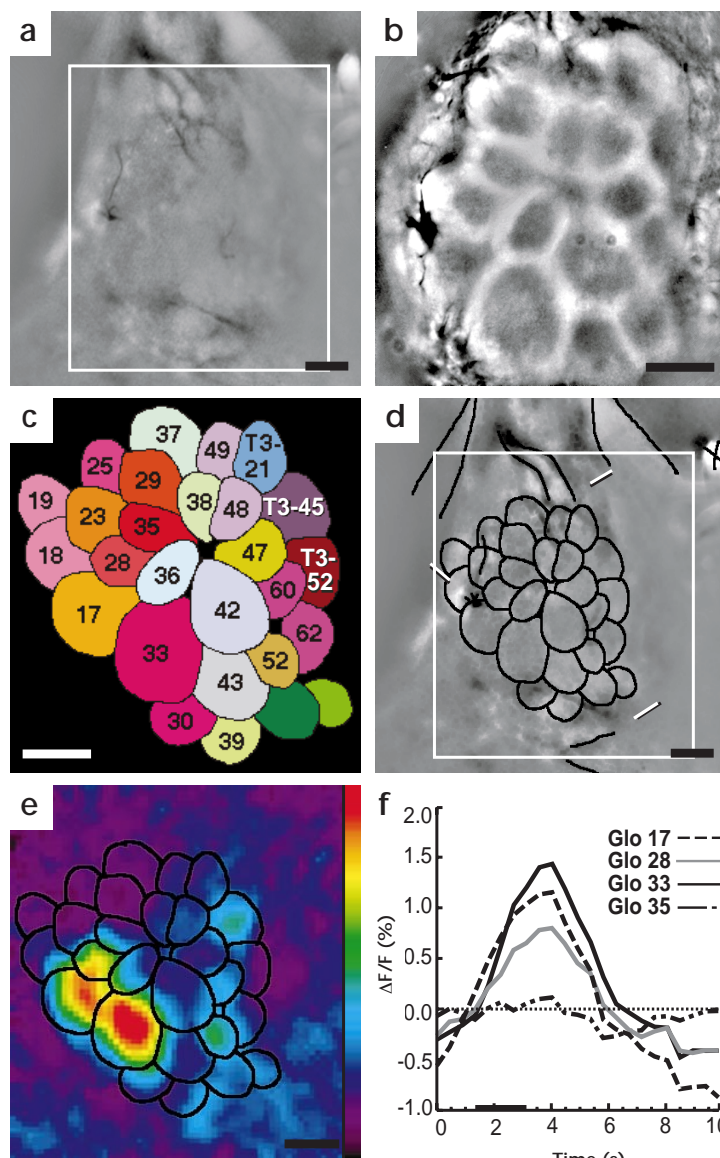
lowing physiological measurements (Fig. 1b). We then identified individual glomeruli using the AL atlas ([www.neurobiologie.fu-berlin.de/honeybeeALatlas](http://www.neurobiologie.fu-berlin.de/honeybeeALatlas)<sup>20</sup>). This allowed us to create a map of identified glomeruli for each bee tested (Fig. 1c). Activity patterns imaged with the calcium dye were then superimposed onto the map of identified glomeruli of the AL (Fig. 1d). Because the AL was removed for RH795 staining, accessory structures (tracheae, for example, marked with arrows in Fig. 1d) were used as landmarks for correct positioning of the mask. With this procedure, measured responses to each odor tested were attributed to each morphologically identified glomerulus. For example, octanol elicited the strongest response in glomeruli 33 and 17 and weaker responses in 28, 52 and 60 of T1, as well as in T3-45 (Fig. 1e). Note that the optical projection of AL combining several focal levels differs slightly from the projection obtained by the 20 $\times$  objective used in the physiological measurements at only one focal plane. This mismatch can be seen in glomerulus T1-17, for instance; because part of the glomerulus is out of focus, activity seems relegated to only one part of the glomerulus (Fig. 1e).

The activity elicited by odors was thus compared among individuals at the level of homologous glomeruli. For example, patterns of response to 1-hexanol appear to be conserved among the 21 individuals measured (Fig. 2): 1-hexanol elicits the strongest response in glomerulus T1-28 in 17 of 21 individuals and weaker, less consistent activity in glomeruli T1-38 and T1-52.

How similar are these patterns between individuals? To statistically address this question, we applied a multivariate discriminant analysis to the normalized data (Table 1). Discriminant analysis allowed us to judge whether odors clustered in multidimensional space (where each dimension represents a glomerulus). If individual observations for two odors had representations that overlapped in multidimensional space, then the discriminant analysis would be unable to discriminate between the two odors and, hence, unable to determine the correct odor from the measured response. We found that most measurements (86%) could be attributed unam-

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**Fig. 1.** Mapping identified glomeruli of the honeybee AL by calcium imaging. **(a)** View of the AL stained with Calcium Green AM, as seen in the experiment. Note poor contrast. Objective, 20 $\times$ ; scale bar, 50  $\mu$ m. **(b)** After calcium measurements, the tissue was stained with RH795, revealing glomerular boundaries. Objective, 40 $\times$ ; scale bar, 50  $\mu$ m. **(c)** Reconstruction of the glomerular map from views as in **(b)** at different focal planes and using a 20 $\times$  objective. Each glomerulus is represented by a unique, arbitrary color. The two green glomeruli at the bottom right could not be reliably identified. All glomeruli without tract labels are innervated by T1. **(d)** View of the AL as in **(a)** with a superimposed, reconstructed glomerular map. Objective, 20 $\times$ ; scale bar, 50  $\mu$ m. **(e)** False-color-coded spatial maps for responses of an individual to 1-octanol. Red indicates strongest response, blue, weakest (false-color bar is to the right). The reconstructed glomerular map of this individual is superimposed onto the response map. Compare with Fig. 1c for glomerular identity and with Fig. 3a. Objective, 20 $\times$ ; scale bar, 50  $\mu$ m. **(f)** Time traces of the response of individual glomeruli (T1-17, T1-28, T1-33, T1-35) to 1-octanol, plotted in  $\Delta F/F$  over time after correction for bleaching. Apparent increase toward zero before stimulus onset is a correction artifact (see Methods). White bar indicates the stimulus.



bigously to the correct odor. The mistakes are also telling: although most (90%) patterns evoked by 1-octanol (OC1) were correctly identified, one measurement was attributed to 2-octanol (OC2) and one to *n*-octane (OCT), chemically similar substances. The odor that evoked the worst performance was linalool (LIO), which was mistaken once for citral (CIT), once for lime blossom (LND) and once for orange (ORG). Indeed, linalool evoked a very diffuse pattern of activity (Fig. 4) with only limited enhancement in glomeruli T1-17 (shared with lime blossom), T1-30 (shared with orange) and T1-48 (shared with citral and orange).

Averaged response patterns of 24 bees to 30 odors were used to estimate the glomerular representation of each odor in *Apis mellifera*. (Most bees were tested with a subset of odors; see Methods.) We show the spatial position of individual activated glomeruli for the odors 1-octanol (Fig. 3b) and clove oil (CLV; Fig. 3c), as well as the pattern of glomeruli activated by each odor tested (Fig. 4). Of 34 entries with strong responses, 17 had a s.d. <15%, 13 between 15% and 25% and 4 between 25% and 35%. No entries with variability greater than 55% were found. An illustrative example of this predictability is given: 2-octanone (OCN) consistently elicited strong activation in glomerulus T1-33 (black color, average maximum response of 10.03 from 11 individuals), with responses in T1-28 that varied from equal to weaker than those of T1-33 (same circle size, but blue color). Less-active components of the pattern were T1-17, T1-29, T1-36, T1-38, T1-39, T1-48, T1-52 and T3-45. There were weak responses in all glomeruli. In contrast, pentanoic acid (PES) gave much weaker responses (average maximum response 1.51) in glomerulus T1-21 of all of six individuals tested (black) and in T1-8 in most individuals (blue); a weak response was measured from T1-12. However, this glomerulus could only be identified in one animal (white circle). No responses above noise were measured in the other glomeruli (no symbol).

## DISCUSSION

This study showed that associations of individual glomeruli with characteristic molecular receptive ranges are conserved among conspecific individuals. Thus, odors are represented by characteristic, species-specific mosaics of activated glomeruli.

We have measured a substantial part of the honeybee olfactory code (Fig. 4). The complete code is certainly more complex: first, of a total of 160 glomeruli, only 38 could be imaged so far (24%). Interestingly, T1 innervates 34 of these, almost 50% of the 70 glomeruli of this part of the AL. Second, dynamic aspects such as the sequence of glomerular activation<sup>9,10</sup> or fast temporal components<sup>21</sup> are likely to be part of the complete olfactory code as well. In this study, glomeruli were each assigned an overall activity measure based on slow calcium measurements, thus ignoring any temporal features of the code. Third, each odor was consistently delivered at the same, non-saturating stimulus concentration routinely used in behavioral assays. Previously, we showed that elicited activity patterns are stable over a concentration range of two orders of magnitude<sup>9</sup>. In preliminary studies, however, we have found





**Table 1. Intraspecific variability of odor representations.**

Odor tested	<i>n</i>	Number correct	Percent correct	Misplaced odor	Mistaken
ISO	4	4	100%	—	—
CIT	4	3	75%	OCT	LIO
GER	9	7	77.8%	OCT, LIO	—
CIN	4	3	75%	PFM	—
EUG	4	4	100%	—	—
CIO	3	3	100%	—	GER, OCT
MNT	3	3	100%	—	—
LIM	4	4	100%	—	—
LIO	7	4	57.1%	CIT, LND, ORG	—
HX1	21	18	85.7%	OC2(2), OCN	OC2
OC1	21	19	90.5%	OC2, OCT	OC2(3)
OC2	18	14	77.8%	HX1, OC1(3)	HX1(2), OC1
OCA	10	10	100%	—	OCN
OCT	9	7	77.8%	CIO, CLV	CIT, GER, OC1
CLV	5	5	100%	—	OCT
LND	3	3	100%	—	LIO
PFM	4	4	100%	—	CIN
ORG	4	3	75%	LIM	LIO

Discriminant analysis of the glomerular activity patterns elicited from all measured individuals. The first column (odor) gives the odor tested (see Methods for abbreviations); *n*, number of animals tested; number correct, measurements correctly attributed to test odor; percent correct, percentage of measurements correctly attributed; misplaced odor, odors to which measured activity patterns were wrongly attributed—if more than one, the number of instances is indicated in brackets; mistaken odor, instances in which patterns were wrongly attributed to this odor. (For example, the row 'CIO' shows that all three measurements of CIO have been attributed to CIO by the analysis; however, one GER and one OCT measurement have also been mistaken for CIO.)

Previously, we compared activity patterns without matching these patterns to the structures that generate them. In our first study<sup>9</sup>, we argued that the representation of pheromones (citral) was conserved between individuals whereas floral odors (for instance, hexanol) were more variable. We show here that the representations are equivalent for all odors, irrespective of their functional roles. Citral elicited a strong response in the medially positioned glomerulus T3-45 (Fig. 4), whereas the pattern for hexanol, involving a series of weaker glomerular responses, was more complex, changing in appearance with the orientation of the antennal lobe. Such differences between individuals may only be resolved by mapping the responses to morphologically identified glomeruli. Still, some variability overlay stereotypical glomerular activity patterns (Fig. 2). Several experimental constraints may have reduced pattern similarity between individuals, including errors in the glomerular borders introduced by projection artifacts (Fig. 1d), misidentification of glomeruli, spoiled single measurements due to movement artifacts, differences in relative responses between animals due to variable focal plane placement, poor signal-to-noise ratio resulting from low signal amplitude or minute differences in stimulus timing. Nevertheless, we believe a part of the observed variability to be a genuine consequence of biologically relevant processes. Genetic variation could, for example, result in structural variability between animals. Furthermore, there are plastic connections in addition to hard-wired connectivity in the AL. Indeed, calcium-activity patterns in the AL have been shown to change after appetitive training in the honeybee<sup>12</sup>. Also, glomerular size has been shown to be plastic, and the relative sizes of individual glomeruli to be reversibly dependent on the behavioral role (forager or

nurse bee) of the individual<sup>26,27</sup>. Therefore, in addition to dedicated molecular receptive ranges for each glomerulus, it seems that development and experience may modulate responses. Because the measured responses comprise components from all cells present in the AL, signal variability may arise from interneurons rather than receptor neurons. We cannot exclude the possibility that the variable components may depend on season of observation, ethotype or genetic background of individual bees.

Identified neurons and known, hard-wired neuronal circuits have long been established among insects<sup>28</sup>. However, stereotypical spatial organization of the olfactory bulb and conserved spatial activity patterns may also be a property of the olfactory bulb in mammals<sup>3,4,13</sup>, suggesting that homologous glomeruli may have identical molecular receptive ranges among mammals, as well. It is possible that olfactory maps optimize connection patterns between individual, active glomeruli. Indeed, measurements from neighboring mitral cells have shown similar receptive range profiles and response sharpening due to lateral inhibition from neighboring glomeruli<sup>29</sup>. We are currently investigating interactions between active glomeruli in honeybees.

## METHODS

**Measurement of odor-evoked activity patterns.** *In-vivo* calcium recordings were done as described<sup>11</sup>. All measurements were done between January and March, 1998. Bees came from four different hives kept in a flight chamber on a constant night/day cycle. After capture, bees were quickly anesthetized by chilling and placed in a plexiglass chamber. The head capsule was then opened and the brain exposed and floated in a solution of Calcium Green 1 or 2 AM (Molecular Probes, Eugene; 50 µg dye was first dissolved in 50 µl Pluronic in DMSO and then diluted in 950 µl Ringer's saline: 130 mM NaCl, 6 mM KCl, 4 mM MgCl<sub>2</sub>, 5 mM CaCl<sub>2</sub>, 160 mM sucrose, 25 mM glucose, 10 mM HEPES, pH 6.7, 500 mOsmol). After staining for 1 h, the brain was rinsed in fresh Ringer's and the recording chamber placed under the microscope under constant perfusion with Ringer's at room temperature (1 ml per min; 22°C). At all stages, great care was taken not to wet the antennae. Images were taken at a rate of ~2 images per second with 240 ms exposure time per image, and measured with a 12-bit CCD camera (Photometrics CH250A, Tucson, Arizona). The data were analyzed on a Macintosh computer with custom software using IDL (Research Systems, Colorado). Signals were calculated as relative changes in fluorescence ( $\Delta F/F$ ). First, the resulting time-courses were linearly corrected against bleaching, and then the control air stimulus was subtracted. Because bleaching follows an exponential function, this procedure leads to the apparent artifactual increase of signal from negative toward zero before the stimulus onset.

For all odors, 4 µl of the pure liquid substance was placed on filter paper (1 cm<sup>2</sup>), and the filter placed in a 1-ml plastic syringe. Stimuli were delivered manually by puffing 0.8 ml of odor-saturated air into a constant air stream directed to the animals' antennae. Stimulus timing was



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