© 2020. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2020) 223, jeb206748. doi:10.1242/jeb.206748

RESEARCH ARTICLE

Experience-dependent tuning of early olfactory processing in the adult honey bee, *Apis mellifera*

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ABSTRACT

Experience-dependent plasticity in the central nervous system allows an animal to adapt its responses to stimuli over different time scales. In this study, we explored the impacts of adult foraging experience on early olfactory processing by comparing naturally foraging honey bees, *Apis mellifera*, with those that experienced a chronic reduction in adult foraging experience. We placed age-matched sets of sister honey bees into two different olfactory conditions, in which animals were allowed to forage *ad libitum*. In one condition, we restricted foraging experience by placing honey bees in a tent in which both sucrose and pollen resources were associated with a single odor. In the second condition, honey bees were allowed to forage freely and therefore encounter a diversity of naturally occurring resource-associated olfactory experiences. We found that honey bees with restricted foraging experiences had altered antennal lobe development. We measured the glomerular responses to odors using calcium imaging in the antennal lobe, and found that natural olfactory experience also enhanced the inter-individual variation in glomerular response profiles to odors. Additionally, we found that honey bees with adult restricted foraging experience did not distinguish relevant components of an odor mixture in a behavioral assay as did their freely foraging siblings. This study highlights the impacts of individual experience on early olfactory processing at multiple levels.

KEY WORDS: Antennal lobe, Experience-dependent plasticity, Glomeruli, PER, Learning, Olfactory restriction

INTRODUCTION

Odors are homologously encoded in primary olfactory processing neuropils – the olfactory bulb (OB) or antennal lobes (AL) – in the brains of animals as diverse as insects and mammals, including humans (Hildebrand and Shepherd, 1997; Sinakevitch et al., 2017). Canonically, in these brain regions, the combinatorial nature of activity patterns that encode different odors is species specific and conserved across animals (Galizia et al., 1999b). These olfactory codes result from binding of volatile chemicals to peripheral olfactory receptor neurons (ORNs) that then project, via axon terminals, to glomeruli in the OB in mammals or the AL in insects (Buck and Axel, 1991; Hildebrand and Shepherd, 1997; Sinakevitch et al., 2017). Glomeruli are spheroidal, synapse-rich areas in the OB and AL. Odor processing in glomeruli is influenced by several different types of excitatory and inhibitory local interneurons (LNs) as well as by modulatory neurons that encode, among other things, reward (Sinakevitch et al., 2017; Galizia and Sachse, 2010; Hammer and Menzel, 1998). Projection neurons (PNs) or mitral cells from each glomerulus then transmit processed sensory patterns to higher order brain centers, such as mushroom bodies and the lateral horn in insect brains (Abel et al., 2001; Kirschner et al., 2006; Sinakevitch et al., 2018) or the piriform cortex in mammals (Sinakevitch et al., 2018; Bekkers and Suzuki, 2013).

It has been well established in both insects (Fernandez et al., 2009; Locatelli et al., 2013; Chen et al., 2015) and mammals (Doucette et al., 2007; Doucette and Restrepo, 2008; Jones et al., 2008; Litaudon et al., 1997; Mandairon and Linster, 2009; Sullivan and Leon, 1986) that non-associative and associative plasticity in olfactory circuits shape the output of activity patterns to odors. Presumably, this plasticity improves the contrast in neural representations required to help animals detect and discriminate important odors from background odors (Fernandez et al., 2009; Locatelli et al., 2016, 2013; Rath et al., 2011). This experience-dependent plasticity also implies that the neural representation of an odor could manifest differently in animals with different experiences. The glomerular activity patterns elicited by odors are canonically conserved across individuals of the same species and are established at the level of ORNs early in adult life prior to adult foraging in honey bees (Wang et al., 2005). However, adult honey bees show slight deviations in the patterns of PN activation in glomeruli activated by the same odors (Arenas et al., 2012; Carcaud et al., 2012; Galizia and Kimmerle, 2004; Sachse and Galizia, 2002; Fernandez et al., 2009). These individual differences could arise as a result of different foraging experiences with floral resources (Chen et al., 2015; Fernandez et al., 2009). Honey bees freely fly in all directions within a several-mile radius of the colony to collect pollen and nectar resources from a diversity of floral sources that produce different perfumes to signal these rewards (Wright and Schiestl, 2009; Menzel, 1985). Alternatively, these mature adult differences in the AL's PN responses to odors could represent random variability across individuals owing to genetics or stochastic developmental programming.

Chronological age is correlated with a shift in behavioral task and experience in honey bees, and these factors affect the morphological development of the AL and mushroom body neuropils in the honey bee brain (Fahrbach et al., 2003, 1995, 1998; Farris et al., 2001; Brown et al., 2004, 2002; Coss et al., 1980). The mushroom body contains approximately 340,000 intrinsic Kenyon cells, which integrate inputs from visual, mechanosensory and taste modalities in addition to the olfactory inputs from the AL PNs (Rybak and

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Received 8 May 2019; Accepted 19 November 2019

Journal of Experimental Biology (2020) 223, jeb206748. doi:10.1242/jeb.206748

Menzel, 1993; Strausfeld, 2002). Age and foraging experience have been correlated with region-specific volume changes and more complex dendritic arbors in the mushroom body calyx collar region, which receives inputs from visual neuropils (Durst et al., 1994; Fahrbach et al., 2003, 1998; Farris et al., 2001). Additionally, foraging experience correlates with changes in the volume and synaptic density of glomeruli within the AL (Brown et al., 2004, 2002; Winnington et al., 1996).

LNs play a modulatory role in the antennal lobe and synapse onto both other LNs and the AL output PNs. These neurons may be involved in the plasticity observed in the AL (Sinakevitch et al., 2011, 2013; Fernandez et al., 2009; Locatelli et al., 2013; Sachse and Galizia, 2002). It is speculated that some of this plasticity is modulated via octopamine, which is involved in olfactory associative conditioning in both fruit flies, *Drosophila melanogaster* (Gerber et al., 2009; Burke et al., 2012; Schwaerzel et al., 2003), and honey bees (Farooqui et al., 2003; Hammer and Menzel, 1998; Rein et al., 2013). In the honey bee AL, the octopamine 1 receptor (AmOA1) is expressed in GABAergic, inhibitory LNs, and the AmOA1 labeling patterns also exhibit inter-individual variability between experienced forager ALs (Sinakevitch et al., 2011). Thus it is possible that AmOA1 may be involved in differences between individual honey bee foragers with different olfactory experience.

Previous studies have described inter-individual differences in the glomerular representation of odors in the AL (Brown et al., 2004, 2002; Galizia et al., 1999a; Sinakevitch et al., 2011; Winnington et al., 1996). In addition, acute odor exposures in the laboratory have shown that olfactory experiences modify the way in which odors are encoded in the AL (Chen et al., 2015; Fernandez et al., 2009; Locatelli et al., 2013; Rein et al., 2013; Sachse et al., 2007). However, we do not yet know the extent to which the experiences while performing natural behaviors affect the way odors are encoded and perceived. Toward those ends, we experimentally controlled the environmental complexity experienced by age-matched and genetically similar groups of forager honey bees and then measured inter-individual differences in morphology, physiology and behavior. We show that foraging environment experienced as an adult significantly affects the tuning of olfactory responses in the honey bee AL, adaptive AL odor processing, and odor-guided behavior in honey bees.

MATERIALS AND METHODS

Beekeeping and rearing

A single open-mated Carniolan honey bee (*Apis mellifera carnica* Pollman 1879) queen was caged on an empty frame for 2 days and allowed to lay eggs on a single frame. All progeny from an open-mated queen will be full- or half-sisters depending on whether they share a paternal genotype (Page, 2013). After egg laying, the queen was released to move freely in the hive. Just prior to adult emergence, the frame was placed in an incubator. Newly emerged (eclosed) adult honey bees were collected from the frame within 24 h of eclosion and were paint marked with a non-toxic pen (Sharpie, oil based). A total of 1000 newly emerged honey bees were marked and split into two groups of approximately 500 honey bees every 2–4 weeks. Each of these groups of ∼500–700 honey bees was introduced into one of two 10-frame queenright host hives, designated as restricted (R) and free (F). We used one pair of host hives for both the calcium imaging and immunohistochemical experiments, and two pairs of host hives for the behavioral experiments.

The hives were placed within 30 m of one another in a shaded courtyard. The first set of restricted hives (R) were enclosed by individual 15.9 m^3 mosquito net tents in which all of the sucrose and pollen resources were artificially provided. The honey bees inside the tent were not olfactorally isolated from the ouside world; however, they did have only one odorant associated with provided resources (1-hexanol). The second set of free hives (F) was left outside of the tents and foraged freely at the Tempe, AZ campus of Arizona State University (Fig. 1).

The food resources in the tents were marked with a single-odor stimulus 1-hexanol as an artificial CS. This was done as follows: The 50% sucrose (w/w) solutions provided to the restricted honey bees contained 0.01% (w/w) odor. Around both the sucrose and pollen resources, a 10% odor solution diluted in mineral oil was applied to an absorbent material placed around both resources. Sucrose solutions were replaced every 24 h and a new 10% odor mixture was placed around the resources once in the morning and once in the afternoon. To the human nose, all materials retained the smell of the odor, even after 24 h.

Honey bee collection

Paint-marked honey bee foragers were all collected when returning to the hive from a foraging trip approximately 7 days after the first paint-marked honey bee was observed foraging. Honey bees were identified as foragers by placing a mesh cover over the entrance, and those that returned more than 5 min after the mesh was placed were assumed to be foragers.

Immunostaining collection

Paint-marked bees from all hives were collected simultaneously over 3 days and immediately processed for fixation with the following immunocytochemistry procedures. These bees were between 34 and 40 days old from adult emergence.

Calcium imaging collection

Paint-marked honey bees were collected from all hives simultaneously over a 3-week time frame and were immediately harnessed and prepared for calcium imaging. The honey bee cohort used in experiments was switched from the original 1000 newly emerged bees to a cohort 2–4 weeks younger halfway through the experiment, in order to keep the length of foraging experience more consistent across bee subjects. All bees were between 31 and 40 days old from adult emergence.

Variance learning proboscis extension response (PER) assay collection

Approximately 7500 paint-marked bees were collected between December 2016 and June 2017. Paint-marked newly emerged bees were placed into each hive at regular 3–4 week intervals. Age groups were switched approximately every 3–4 weeks, depending upon the dominant foraging paint-marked cohort at the time of collection. Eight bees were collected from each hive experience treatment (restricted and free) on each training day and they were divided equally into each training protocol group (see PER variance learning assay, below).

Calcium imaging

Bee preparation and *in vivo* PN staining

Paint-marked honey bees were captured, briefly cooled on ice and restrained in custom-made individual holders suited for calcium imaging (Galizia and Vetter, 2004). After recovery from cooling, the bees were fed with 1.0 mol l^{-1} sucrose solution and left undisturbed until staining shortly after. A window was cut in the top of the head capsule, dorsal to the joints of the antennae and rostral to the medial ocellus. The hypopharyngeal glands and trachea near the

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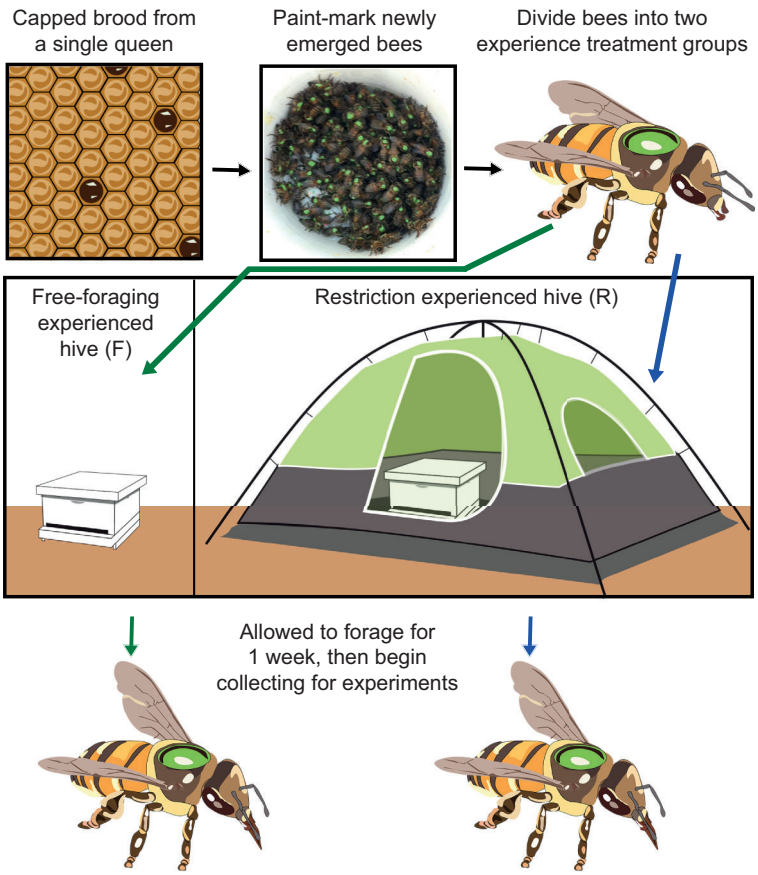

Fig. 1. Experimental treatments for each experiment. Newly emerged honey bee workers were collected from brood frames of a single colony and then paint-marked. Paint-marked workers were split into two treatment hives. Foragers from one hive were allowed to freely fly in the environment (F). Foragers from the second hive were restricted to forage inside of a tent for pollen and sucrose resources, associated with a single pure odor, 1-hexanol, diluted in mineral oil (R). After foragers had been observed foraging for 1 week, painted foragers were collected for calcium imaging at 31–40 days post-eclosion, behavioral experiments (3–11 weeks post-eclosion) and immunolabeling (34–40 days post-eclosion).

vertical lobes (Rybak and Menzel, 1993) were moved and served as visual reference for the staining (Sachse and Galizia, 2002). The tip of a glass electrode coated with fura2-dextran (potassium salt, 10,000 MW, Thermo Fisher Scientific) was inserted into both sides of the protocerebrum, dorsolateral to the vertical lobes, aiming for the antennal lobe tract (ALT) or lateral antenno-protocerebral tract (l-APT) that contains the axons of uniglomerular PNs (Galizia and Rössler, 2010). A few seconds later, after the dye had dissolved, we closed the window in the head capsule using the piece of cuticle that had been previously removed. The dye was left to travel along the l-APT tracts until the next day, roughly 10–18 h later. Before imaging, the antennae were fixed pointing toward the front, where odor will be delivered, using a low-temperature melting wax (Eicosane). Body movements were prevented by gently compressing the abdomen and thorax with a piece of foam held in place by a piece of tape. The brain was then rinsed with Ringer's 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 chemicals from Sigma-Aldrich), and glands and tracheae covering the ALs were removed. When necessary, we also cut a small hole between the antennae and mandibles, and then pulled out a small section of a compact structure of muscles, esophagus and supporting chitin. We did this to put this structure under slight tension and pull it away from the brain to prevent accessory movements in the AL (Mauelshagen, 1993). Only ALs that presented homogenous staining of all visible glomeruli were used for imaging. Stained honey bees were then mounted on the microscope and were allowed to recover for 15 min before imaging. We collected 18 full recordings, 10 recordings from honey bees that received the free-flying treatment and 8 from honey bees that received the restricted treatment.

Odor stimulation and imaging session

The focus of this analysis was to determine whether a given glomerulus was recruited by a given odor, and whether this glomerular response profile varied across animals within a given treatment group. For that aim, the glomerular responses were measured for pure odors, mixtures and different concentrations of both. Odors were diluted in mineral oil (all mol l^{-1}): 1-hexanol 2×10^{-2}, 1×10^{-2} and 1×10^{-3}; acetophenone 1×10^{-2} and 1×10^{-3}; mixture 1 (1-hexanol 2×10^{-2}+acetophenone 1×10^{-2}); mixture 2 (1-hexanol 1×10^{-2}+acetophenone 1×10^{-2}); mixture 3 (1-hexanol 1×10^{-3}+acetophenone 1×10^{-3}); 2-octanone 1×10^{-2} and 1×10^{-3}; geraniol 1×10^{-2}; lemon oil 1×10^{-2}; and linalool 1×10^{-2}. Ten microliters of odor solution were loaded onto a filter paper strip (0.5×4 cm) that was put into a 1 ml glass syringe, which served as an odor cartridge. The odor-delivery device had 14 identical channels, each composed of a three-way solenoid valve (LFAA1200118H; The LEE Company) and an odor cartridge. Valve opening was synchronized with the optical recordings using the acquisition software TILLVisION (Till-Photonics). When the valve opened, the air volume inside the cartridge was delivered (~50 ml min^{-1}) into a continuous charcoal filtered air stream (~500 ml min^{-1}), which in turn directed the air toward the honey bee head. Thus, the final concentration of odors reaching the honey bee was actually approximately one-tenth of the concentration in the headspace of the cartridge. Imaging acquisition trials lasted 10 s and were separated from each other by 1 min. Odor stimulation lasted 1 s and started 3 s after onset of acquisition. Each odor was tested two times

in each animal, making a total of 28 stimulations, including blank trials with mineral oil. Odor order was randomized, with the only restriction being to not use the same odor in two consecutive trials. Behind the honey bee, an exhaust continuously removed air, keeping the arena clean of olfactory stimuli.

Data acquisition and analysis

A Polychrome V (Till-Photonics, Gräfelfing, Germany) was used to emit excitation at two wavelengths alternating between 340 and 380 nm. Imaging data were then collected at 5 Hz using a CCD camera (SensiCamQE, Till-Photonics) mounted on an upright fluorescence microscope (Olympus BX-50WI, Japan) with a 20× objective NA 0.95 (Olympus) using a 505 DRLPXR dichroic mirror and a 515 nm LP emission filter (Till-Photonics). The final spatial resolution of each image was 1376×1040 pixels with a pixel side length equaling 2.6 µm. The exposure times during excitation were 8 ms at 340 nm and 2 ms at 380 nm. The image analysis was performed using custom software written in the Interactive Data Language (IDL; Research Systems) using routines created by Giovanni Galizia (University of Konstanz, Germany). Measurements from each animal consisted of a sequence of 50 fluorescence images, obtained at each excitation wavelength (F_{i340}, F_{i380}, where subscript i is the number of images 1 to 50, and subscript denotes measurements at the excitation wavelengths 340 or 380 nm). Calcium responses were calculated as the ratio $R_i=(F_{i340}/F_{i380})\times100$. We subtracted the background responses (R_b) from these ratios. We calculated R_b by averaging the R_i values 1 s immediately before the odor onset, where $R_b=1/5(R_{11}+\ldots+R_{15})$. The resulting relative calcium response measure (ΔR) represents a percentage change from the odor-free reference window (R_{11}–R_{15}). This measure has previously been shown to be directly proportional to changes in intracellular calcium concentration (Galizia and Kimmerle, 2004). Next, we identified glomeruli based upon their morphology and relative position using our own AL reconstructions and the digital atlases of the honey bee AL (Flanagan and Mercer, 1989a; Galizia et al., 1999a). We also visualized glomeruli using the raw fluorescence images obtained at the 380 nm excitation wavelength. For an additional confirmation of glomeruli locations, we created images that represent the degree of correlation between neighboring pixels with a tool provided by Mathias Ditzen (Freie Universitaet Berlin, Germany). Pixels stemming from the same glomerulus are highly correlated over time and pixels from different glomeruli are not. We finally ended up with a common set of 23 glomeruli that could be identified across all animals. All glomeruli corresponded to the dorso-rostral side of the AL innervated by the antennal nerve T1 tract (glomeruli 17, 23, 24, 25, 28, 29, 33, 35, 36, 37, 38, 42, 43, 47, 48, 49, 52, 54, 56, 60 and 62) and the T3 tract (glomeruli T3-54 and T3-52) (Flanagan and Mercer, 1989a; Galizia et al., 1999a). The activity for each glomerulus was calculated by averaging mean ΔR activity over a 9×9 pixel square area that corresponds to approximately a 23.4×23.4 µm square that fits within the center of each glomerulus. We then averaged this activity for each glomerulus in each animal over the 1-s odor stimulation period. These values were then used for the final comparisons of odor-elicited activity across animals and experience treatments.

In order to measure the degree of inter-individual variation within each treatment, we created correlation matrices to compare bees within each treatment group (restricted N=8, free N=10). We considered two scenarios: (A) the odor-response variation observed across individuals in a given glomerulus, and (B) the same variation but pooled across all glomeruli. For scenario A, each of the cells in the correlation matrices were the Pearson correlation value of the measured mean odor-evoked glomerular responses to all tested odors, as described above, between two individual bees within the same treatment group, considering each glomerulus in a different correlation matrix (23 restricted matrices of size 8×8 and 23 free matrices of size 10×10). We used a bootstrapping test of means to compare the correlation matrices between the two treatment groups. All P-values were adjusted for multiple comparisons according to the false discovery rate (Benjamini and Hochberg, 1995). For scenario B, each of the cells in the matrix was a pooled correlation across all glomeruli between two individual bees within the same treatment group (one 8×8 restricted matrix and one 10×10 free matrix). We used a paired t-test to compare the responses of pooled glomeruli.

Immunocytochemistry

After collection, honey bees were immobilized by cooling on ice for a maximum of 3 min, the heads were cut from the abdomen and placed into the 4% paraformaldehyde in phosphate buffered saline (PBS) pH 7.4, and the brains were removed and placed in 1 ml of fixative overnight at 4°C.

Primary antibodies

Affinity-purified goat polyclonal anti-AmOA1 antibodies (21st Century Biochemicals, Inc., Marlborough, MA, USA) were raised against a synthetic peptide acetyl-AMRNDRSPSYSMQVPQQGC-amide, which corresponds to amino acids 547–564 of the honey bee AmOA1 receptor. These antibodies were previously used to study the distribution of the AmOA1 receptor in the honey bee brain (Sinakevitch et al., 2011, 2013). Mouse monoclonal anti-synapsin antibodies (SYNORF1, 3C11) were purchased from Data Bank Hybridoma. Anti-synapsin binds to protein associated with presynaptic sites of neurons and is largely used for labeling the synaptic neuropil. Phalloidin conjugated with TRITC (tetramethylrhodamine isothiocyanate; Invitrogen) binds to polymerized actin (F-actin).

Secondary antibodies

We visualized primary anti-AmOA1 using F(ab′)2 fragments of donkey anti-goat antibodies conjugated to Cy5 (Jackson ImmunoResearch Laboratories). F(ab′)2 fragments of donkey anti-mouse antibodies conjugated to Alexa 488 were used to visualize anti-synapsin (Jackson ImmunoResearch Laboratories).

Anti-AmOA1 staining procedures on brain sections

Fixed brains were washed in phosphate buffered solution and embedded in 8% (w/v) agarose (low melting point, Sigma-Aldrich) in water. The 80 µm sections of brains were made using a vibrating blade microtome (Leica VT1000S, Leica Biosystems, Germany) in PBS. Sections were washed (6×20 min) in 0.5% Triton X-100 in PBS (PBSTX) and then were pre-incubated with normal donkey serum (Jackson ImmunoResearch Laboratories) for 15 min. Next, the primary antibodies goat anti-AmOA1 in the PBSTX were added to brain sections at a 1:16 dilution for overnight incubation at room temperature. Next, sections were incubated overnight at room temperature with secondary antibodies (donkey anti-goat antibodies conjugated with Cy5 at 1:200) and Phalloidin-TRITC (1:160 in PBSTX). Sections were then washed in PBS (6×10 min) and embedded on slides in mounting medium (Fluoro-Gel with PIPE Buffer, EMS). The specificity and control tests for the goat anti-AmOA1 stains are described in detail in prior work (Sinakevitch et al., 2011, 2013). In the present study, we performed additional control tests to demonstrate that neither the primary goat anti-AmOA1 antibodies nor the secondary anti-goat Cy5 antibodies

interacted with the Phalloidin staining. In the first control, the primary AmOA1 antibodies were omitted from the protocol and we added the secondary antibodies as described above. In the absence of the primary AmOA1 antibody, we observed no differences in the Phalloidin staining patterns compared with Phalloidin stained alone preparations. Additionally, in the reverse control, omitting the anti-goat Cy5 secondary antibodies there was no difference compared with the Phalloidin staining observed (data not shown). This indicated that neither the primary goat anti-AmOA1 antibody nor the secondary anti-goat Cy5 interacted with the conjugated Phalloidin staining.

Anti-synapsin staining procedures on whole-mount brains

After fixation procedures described above, brains of free-flying honey bees (*A. m. carnica*) were washed in PBSTX (6×20 min). Following washes, brains were pre-incubated with normal donkey serum for 15 min and incubated for three nights at room temperature with anti-synapsin at 1:800 in PBSTX solution. The following day, brains were washed again in PBSTX (6×20 min) and incubated for three nights at room temperature with donkey anti-mouse antibodies conjugated with Alexa 488 at 1:270 and then with Phalloidin-TRITC at 1:160 in PBSTX overnight. After staining with secondary antibodies, brains were washed with 4% paraformaldehyde fixative for 10 min and then put through a dehydration protocol using increasing steps of ethyl alcohol. Following full dehydration and three washes in 100% ethanol, brains were cleared in methyl salicylate. They were then mounted on slides in methyl salicylate.

Confocal image collection and processing

Images were collected using a Leica TCS SP5 confocal laser scanning microscope (Leica, Bensheim, Germany) with a Leica HCX PLAPO CS 40× oil-immersion objective (1.25 NA) using the appropriate filter and laser setting for each fluorescent molecule used (see sections above). Image stacks were collected using 1 µm optical sections through a depth of approximately 20 µm. Image stacks were then flattened using maximum intensity functions in the Leica software. Image size, intensity and resolution were adjusted using Adobe Photoshop CC.

Three-dimensional reconstructions of the antennal lobe and whole brain

To identify the glomeruli number in sectioned preparations, we used AVIZO software (FEI) to make a 3D reconstruction of the AL from a whole-mount preparation. We then identified glomeruli by comparison with the honey bee brain atlas (Rybak, 2012; Flanagan and Mercer, 1989a; Galizia et al., 1999a) and made virtual (sagittal) sections of this reconstruction. We also reconstructed the whole brain of one honey bee forager for general reference.

Tiff files of whole-mount brain confocal scans were taken every 10 µm throughout the right and left honey bee AL reconstructions and scans were taken every 2 µm for the whole brain reconstruction. These files were imported into AVIZO software, and the voxel dimension outputs of each image stack were imported to provide correct dimensions (Thermo Scientific, FEI). Using the image segmentation function, we identified brain structures based upon the honey bee brain atlas and identified individual glomeruli on the dorsal surface of the left and right ALs (Flanagan and Mercer, 1989a; Galizia et al., 1999a; Rybak, 2012). Brain neuropils and glomeruli were individually labeled by hand throughout the image stacks. A volume rendering of these labels was created, and exported into Photoshop CC. In Photoshop, we created a manipulatable 3D image using the 3D volume function.

PER variance learning

Eight harnessed honey bees from each odor experience treatment group (free and restricted) were collected and presented with an odor mixture paired with 50% sucrose (w/w) at a 10-min inter-trial interval using an odor delivery system each training day (Smith and Burden, 2014; Wright and Smith, 2004). Stimuli were delivered by passing air through a glass cartridge containing 20 µl of an odor mixture on a small strip of filter paper. Each odor cartridge was used for no more than six presentations.

Odor blends of three monomolecular odorants were used and mixed with hexane as a solvent. Two classifications of odor mixtures were produced. The first blend comprised acetophenone, geraniol and 2-octanone. The second blend consisted of phenylacetaldehyde, nonanal and α-farnesene.

As in Wright and Smith (2004), in the variable mixtures, one of the three odors was presented consistently over all trials at a constant concentration. Other odors were varied from trial to trial, using either a high (H: 2.0 mol l^{-1}) or low (L: 0.0002 mol l^{-1}) concentration (Wright and Smith, 2004). Four mixtures were prepared for each odor set as follows – LLH, LHL, LLL and LHH – with the target odor, phenylacetaldehyde or acetophenone, presented consistently at the low concentration for each blend. Ratios of each odor mixture were then confirmed using GC/MS. It is important to note that the total mean odor concentrations across all presentations were equal for both protocols. Each subject was presented the above odor mixtures over 16 trials in a pseudorandomized order, with each odor being presented four times over the process of training.

An additional group of honey bees was trained in the same way as above; however, the odor mixture ratios were held constant across the entire training process. Honey bees from both the natural free-flying experienced group and the restriction experienced group were trained under either the varied or constant mixture conditions. At the end of conditioning, the honey bees were placed in a humidified container. After 2 h, the subjects were tested for a PER to the individual component odors that comprised the training odor mixtures, separately, at the low concentration (0.0002 mol l^{-1}). Each odor component was presented without sucrose reinforcement, and PERs were recorded (Wright and Smith, 2004). Test odors were presented in a randomized order.

We analyzed responses for acquisition using a generalized linear model (GLM), and for recall using a generalized linear mixed model (GLMM) to allow honey bee identity to be included as a random factor. Finally, we compared differences in recall responses between the variable and constant acquisition protocols between treatment groups using the non-parametric Mann–Whitney *U*-test.

RESULTS

Experience-dependent maturation of the antennal lobe circuits in adult honey bees

The honey bee olfactory system comprises the antennae, the antennal nerve (AN), the AL and higher-order olfactory centers such as the mushroom body, lateral horn and lateral protocerebral lobe (Fig. 2A). The AL consists of ∼170 glomeruli and an aglomerular region (Fig. 2B) (Robertson and Wanner, 2006; Sinakevitch et al., 2017). The glomeruli are divided into a cortex (or the outer region of the glomeruli) and a core region (Galizia and Sachse, 2010; Hildebrand and Shepherd, 1997; Nishino et al., 2009; Sinakevitch et al., 2017). The cortex contains arborizations of ORNs, PNs and LNs and is located on the peripheral surface of each glomerulus (Nishino et al., 2009; Flanagan and Mercer, 1989b; Zwaka et al., 2016). The core region contains arborizations composed of LNs and PNs and is

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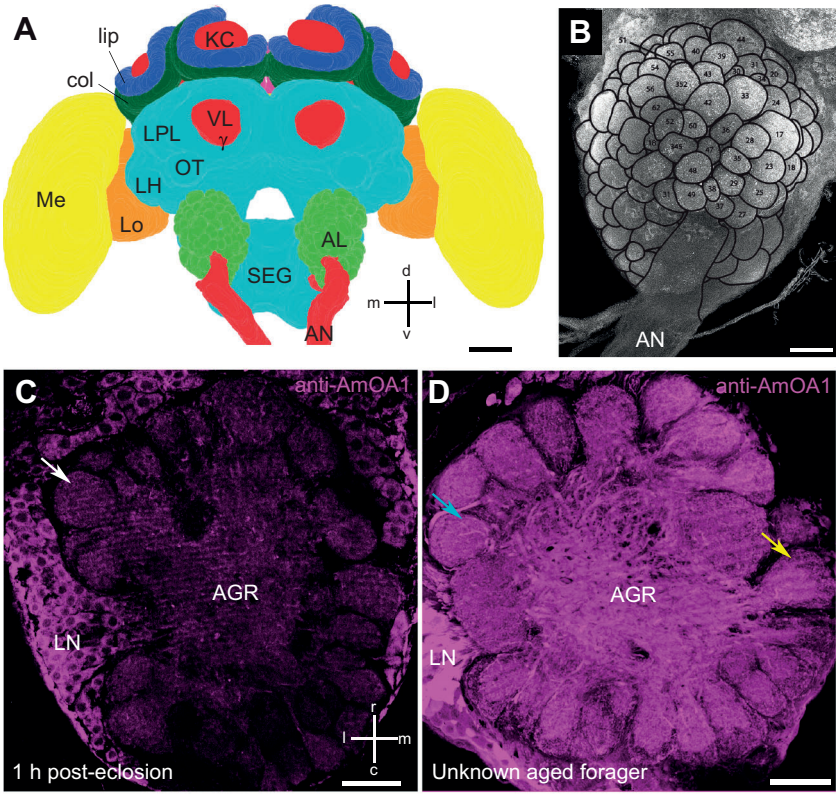

Fig. 2. Honey bee brain and antennal lobe (AL) developmental plasticity with anti-AmOA1 staining. (A) The schematic of the honey bee brain with major neuropilar areas. (B) Honey bee AL immunostained with anti-synapsin with an overlaid digital reconstruction of the AL identifying dorsal glomeruli that receive ORNs from T1–T3 tracts. Glomeruli are identified and labeled according to Galizia et al. (1999a). (C,D) Natural variation in anti-AmOA1 staining in (C) a newly emerged adult honey bee (D) and an unknown aged forager. Arrows denote example glomeruli. AGR, aglomerular region of the AL; AN, antennal nerve; col, mushroom body collar region; KC, mushroom body Kenyon cells; LH, lateral horn; lip, mushroom body lip region; Lo, lobula; LPL, lateral protocerebral lobe; Me, medulla; OT, optic tubercle; SEG, subesophageal ganglion; VL and γ, vertical lobe and gamma lobes of the mushroom body. d, dorsal; l, lateral; v, ventral; m, medial. Scale bars: (A) 250 μm, (B) 50 μm, (C,D) 20 μm.

located in the center of the glomerulus (Nishino et al., 2009; Zwaka et al., 2016; Sinakevitch et al., 2017).

Newly eclosed adult workers show very little labeling of the honey bee octopamine 1 receptor (AmOA1) within the glomerular (Fig. 2C, white arrow) or the aglomerular regions (Fig. 2B). As a honey bee ages and begins performing foraging behaviors, there is a change in anti-AmOA1 receptor labeling, with a higher level of heterogeneous immunolabeling within and across glomeruli in the glomerular and aglomerular regions of the AL (Fig. 2D). For example, the two glomeruli marked by the teal and yellow arrows (Fig. 2D) highlight this variation across glomeruli in one AL. In this forager, the rostro-lateral glomerulus (teal arrow) is labeled by anti-AmOA1 in both the core and cortex. However, in the medio-caudal glomeruli (yellow arrow), only the core is labeled by anti-AmOA1 (Fig. 2D).

In order to explore the AL network changes that occur as a result of foraging experience, we placed bees in one of two experiential treatment groups (Fig. 1). We then immunolabeled the brains of foragers in our two treatment groups as well as newly emerged adult bees from the same cohort used to establish the treatment colonies. The AmOA1 receptor is known to be important for associative plasticity in the AL (Farooqui et al., 2004), thus we used anti-AmOA1 antibodies to study its distribution in the AL in relation to our treatments and age. Simultaneously, we used Phalloidin-TRITC to visualize polymerized actin (F-actin) (Farris and Sinakevitch, 2003). The presence of F-actin has been correlated with neurite growth and development and with the outgrowth of dendritic spines in honey bees and other animals (Groh et al., 2012; Kaech et al., 1997).

To identify glomeruli in sectioned brains labeled with anti-AmOA1 and Phalloidin, we first established a protocol using 3D reconstructions of the AL from whole-mount preparations in which we identified glomeruli and made virtual sagittal sections (Fig. 3A). To ensure we were comparing similar synaptic regions across individuals, we only used sagittal sections cut in a plane in which we could visualize both the T1 tract of the antennal nerve and distinct glomeruli, such as glomerulus 44 and its neighbors (Fig. 3B). Using this method to identify glomeruli, we moved forward and immunolabeled sectioned ALs against both anti-AmOA1 and F-actin (Fig. 4). As expected, we found a shift in the patterns of anti-AmOA1 labeling across AL development from newly emerged adult (Fig. 4Ai,Bi) to aged forager (Fig. 4Ci,Di). In newly emerged adult honey bees, we primarily observed anti-AmOA1 labeling in the cell bodies around the AL (Fig. 4Ai,Bi). In contrast to anti-AmOA1 labeling, Phalloidin broadly labeled F-actin in olfactory receptor neuron tracts and glomeruli at this stage of development (Fig. 4Aii,Bii).

Also expected, we observed a characteristically heterogeneous anti-AmOA1 labeling across both the aglomerular and glomerular regions in aged adults (Sinakevitch et al., 2011) (Fig. 4Ci,Di). Freely foraging honey bees appeared to have a more porous and sparse F-actin labeling within glomeruli (Fig. 4Cii,Dii). Overall, the merged images show very few fibers with close co-localization of AmOA1 and F-actin. However, technical limitations do not allow identification of AL cell types that express F-actin in glomeruli of these preparations (Fig. 4Cii,Dii).

In the restricted treatment (R), we observed very similar anti-AmOA1 labeling patterns to those found in bees with free-flying experience (Fig. 4Ei,Fi). However, we observed differences in the F-actin labeling. We observed a higher level of F-actin labeling in both the core and cortex of all glomeruli, very unlike their naturally foraging, age-matched sisters (Fig. 4Cii,Dii). Restricted honey bees instead had labeling patterns that were more consistent with those observed in newly emerged adult bees (Fig. 4Eii,Fii).

Experience-dependent effects on antennal lobe PN tuning

To assess experience-dependent effects on AL odor responses, we visualized PN responses within glomeruli of the rostro-dorsal

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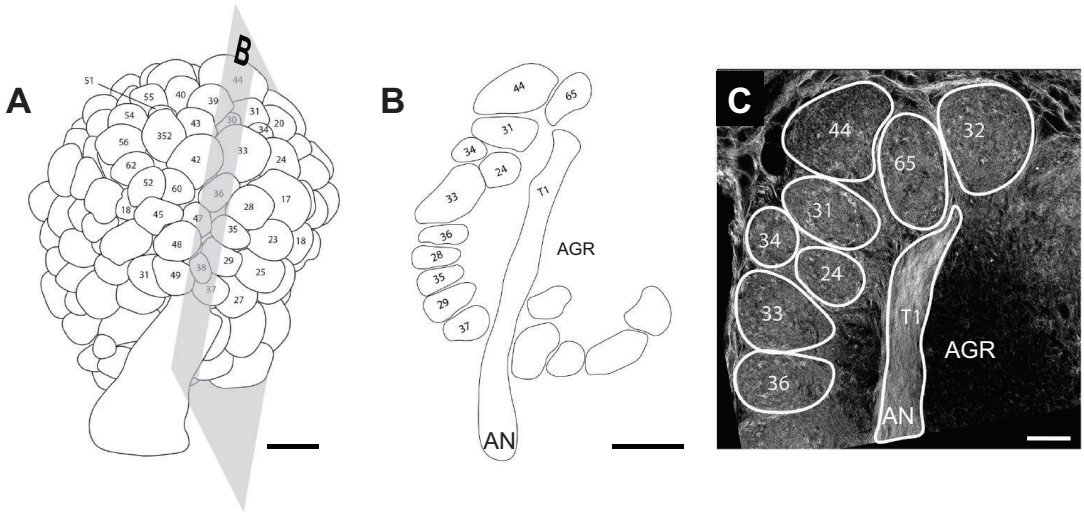

Fig. 3. Antennal lobe glomeruli identification. (A) A digital reconstruction of the AL in Fig. 2B. Glomeruli are identified and labeled according to Galizia et al. (1999a). Shaded plane illustrates sagittal section displayed in B. (B) A digital, sagittal section taken from the reconstruction in A. Section clearly identified by the T1 ORN tract from the antennal nerve. (C) A digital overlay with labeled glomeruli and T1 ORN tract on sagittal sectioned AL. Tissue has F-actin labeled with Phalloidin-TRITC. AGR, aglomerular region of the AL; AN, antennal nerve. Scale bars: (A,B) 50 μm, (C) 25 μm.

portion of the honey bee AL using a fura-2 dextran dye injection for age-matched honey bees that had one of our two experiential treatments (Fig. 1). We then calculated the mean calcium responses in the PNs of each glomerulus in each bee during the odor stimulation period (Fig. S1). A set of 14 different stimulations including different odorants, concentrations and mixtures were used to define the tuning profile of each glomerulus. In addition, every stimulation with a given odor was repeated two times in a randomized order in each bee, thus we could also measure how consistent the tuning profile for a given glomerulus was within and across bees. The glomerular responses were highly consistent between the two presentations within bees in both treatments (response profile correlations: F=0.879±0.028, T=0.896±0.0119). We next averaged these two presentations to obtain a mean glomerular tuning response for each bee for each of the 23 identified glomeruli during the odor stimulation period (Fig. S1). We then compared this mean odor response across bees. Fig. 5 shows the variability observed in the tuning, or response profile across presented odorants, of glomeruli 36 and 37 across free-flying bees at the level of output from the AL. The precise glomerular PN response profiles to the panel of odors were variable across bees (Fig. 5). For example, glomerulus 36 responded consistently to 2-octanone (Fig. 5A) and glomerulus 37 responded to acetophenone (Fig. 5B) in all bees. However, the other odorants to which glomerulus 36 and 37 also responded were variable between bees. Glomerulus 36 in bee 1 responded to both 1-hexanol and acetophenone in addition to 2-octanone. In bee 2, glomerulus 36 also responded to linalool and lemon oil (Fig. 5A). Additionally, glomerulus 37 in bee 2 also responded to 1-hexanol, 2-octanone and linalool (Fig. 5B). These inter-individual response deviations at the level of output from the AL (PNs) demonstrate differences in odor processing within the AL network between bees.

We hypothesized that inter-individual variation in the glomerular response tuning was a consequence of dissimilar foraging experiences across bees. Thus, we predicted that bees with the more homogeneous experience in the restricted treatment would show less variation in glomerular response tuning across animals. To assess this, we computed the correlation of odor-evoked glomerular responses between bees. We found that there was a significantly higher correlation in the odor-evoked glomerular response profiles between the restricted (R) bees than between the freely flying (F) bees (all glomeruli: paired t-test, P<0.001, mean±s.e.m. correlation value: restricted=0.619±0.027, free=0.545 ±0.021). This increased correlation between bees indicates that neural responses exhibited less inter-animal diversity across bees raised in the restricted condition, in support of our hypothesis.

We next wanted to determine whether the reduced inter-individual variation was driven by all of the 23 measured glomeruli or, alternatively, by only a few glomeruli that show exceptionally homogeneous or heterogeneous response profiles across subjects. We computed the same correlation measure as before, but restricted to odor-evoked responses in single glomeruli (Fig. 6). Again, glomeruli for restricted foragers tend to have lower response diversity (higher correlation) than the corresponding glomeruli in free-flying foragers (Fig. 6A). This effect was significant in 12 of the measured 23 glomeruli (bootstrap test of means, four cases P<0.05, eight cases P<0.01; Fig. 6, $R_{\text{Restricted}}$<R_{Free} highlighted blue). We found one glomerulus with the opposite pattern, a higher response diversity (lower correlation) in the tent compared with the free-flying foragers (bootstrap test of means, P<0.05; Fig. 6, $R_{\text{Restricted}}$>R_{Free} highlighted red). Ten of the 23 glomeruli showed no significant difference in the inter-individual variation between the two experiential treatments, versus 22 expected under the null hypothesis (bootstrap test of means, P>0.05; Fig. 6, $R_{\text{Restricted}}$=R_{Free} highlighted gray).

Experience-dependent effects on odor mixture learning

We next used a behavioral assay to compare the restricted and free-flying odor experience groups to evaluate the effects of experience on odor learning. We chose a variant of an odor categorization problem reported in Wright and Smith (2004) in order to make the task more complex and thus more difficult to solve. This task required the animals to analyze a set of odor mixtures paired with reward, and then in a memory test determine whether the animals were capable of generalizing from the mixture to the components. Animals experienced mixtures that were either ‘variable’ from trial-to-trial in their component concentrations or mixtures that remained ‘constant’ in their component concentrations

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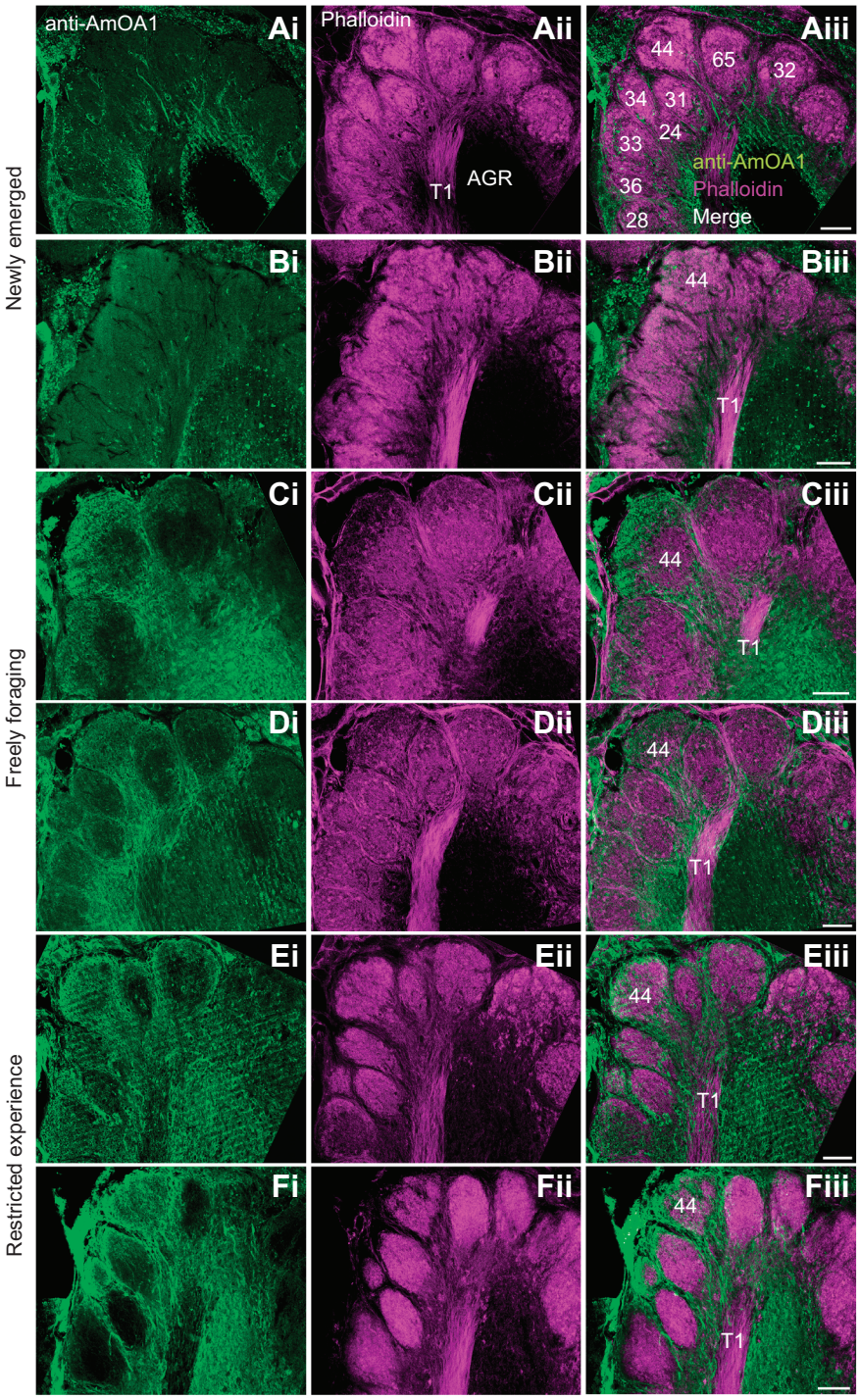

Fig. 4. Sagittal sections of left AL from honey bees with different degrees of olfactory experience. The left column shows anti-AmOA1 labeling in green (i), the center column shows F-actin bound by Phalloidin-TRITC in magenta (ii) and the right column shows merged images anti-AmOA1 and Phalloidin-TRITC labeling (iii). (A,B) Newly emerged adult honey bee workers. (C,D) Adult forager honey bee workers aged 4–5 weeks post adult emergence. (E,F) Adult forager honey bees age-matched and sisters to C and D that were tent restricted only experienced 1-hexanol associated with all resources. Glomeruli are numbered in Aiii and glomerulus 44 and T1 antennal nerve tract are labeled in all overlays for reference. Scale bars: 25 μm.

across trials. Under natural foraging circumstances, honey bees that experience a variable mixture are more capable of recognizing the mixture components.

To control for odor-specific effects, we trained bees on one of two different mixture blends made up of three odorants [mixture blend 1: acetophenone (target), geraniol, 2-octanone; or mixture blend 2: phenylacetaldehyde (target), nonanal, α-farnesene]. Each subgroup was further divided into bees receiving either a variable mixture training protocol (Fig. S2A) or a constant mixture training protocol (Fig. S2B). All groups received 16 presentations of the odor mixture paired with sucrose. Within the variable protocol, one odorant was held constant from trial-to-trial and was therefore the most reliable odorant signaling reward. This odorant is referred to as the 'target' odor. Bees were then tested for a proboscis extension response without reinforcement to each of the individual components that made up the associated mixture (Fig. S2A–C).

Bees with a restricted experience were able to associate mixture stimuli with sucrose just as well as free-flying adults over the 16 acquisition trials (GLM, family=binomial, experience×blend: Z=1.389, P=0.165, experience×protocol: Z=−1.339, P=0.181, experience×blend×protocol: Z=−1.762, P=0.078; Fig. S3). As was expected from the nature of the protocol, bees that experienced the variable odor mixture showed slower acquisition to the odor

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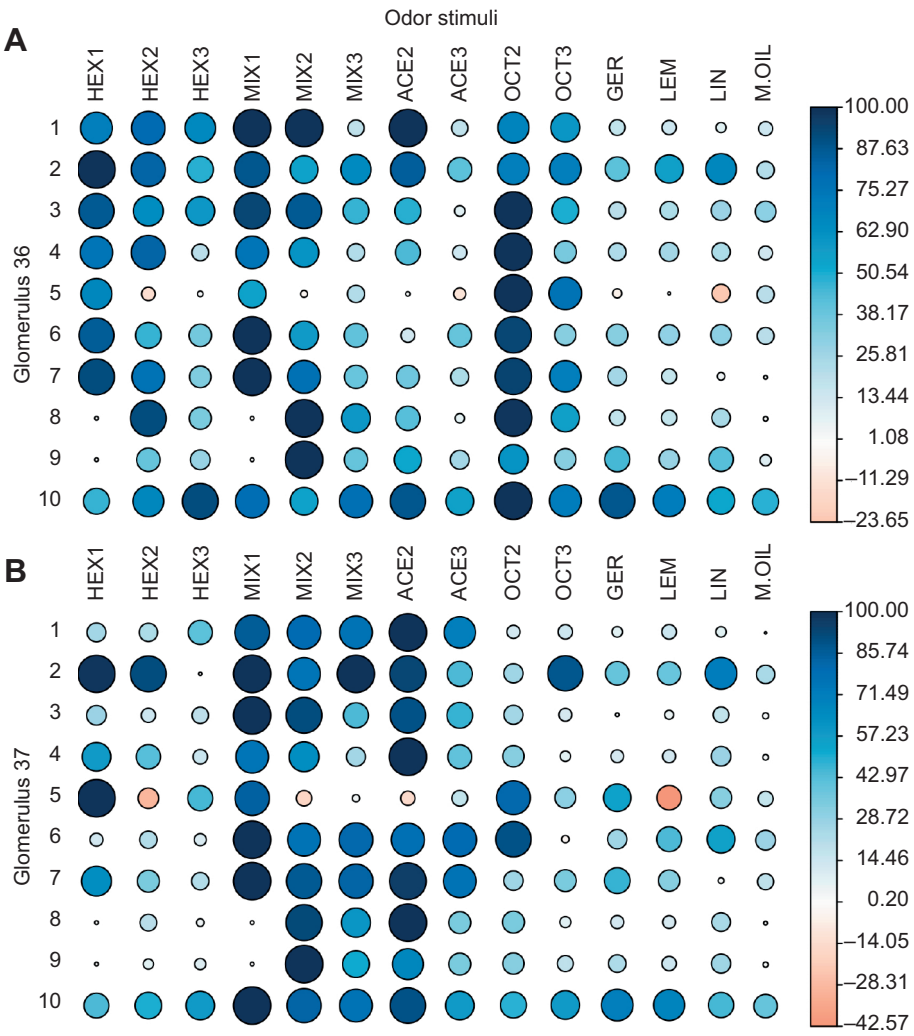

Fig. 5. Natural inter-individual variation in glomerular response profiles to odors in free-flying honey bees. Response profiles for glomerulus 36 (A) and glomerulus 37 (B) for 10 age-matched foragers with natural olfactory experience. Size of circle denotes the magnitude of the calcium response and color denotes the direction (excitation versus inhibition) of change. Odor labels: HEX1=2×10^{-2} mol l^{-1} 1-hexanol, HEX2=1×10^{-2} mol l^{-1} 1-hexanol, HEX3=1×10^{-3} mol l^{-1} 1-hexanol, MIX1=2×10^{-2} mol l^{-1} 1-hexanol and 1×10^{-2} mol l^{-1} acetophenone, MIX2=1×10^{-2} mol l^{-1} 1-hexanol and 1×10^{-2} mol l^{-1} acetophenone, MIX3=1×10^{-3} mol l^{-1} 1-hexanol and 1×10^{-3} mol l^{-1} acetophenone, ACE2=1×10^{-2} mol l^{-1} acetophenone, ACE3=1×10^{-3} mol l^{-1} acetophenone, OCT2=1×10^{-2} mol l^{-1} 2-octanone, Oct3=1×10^{-3} mol l^{-1} 2-octanone, GER=1×10^{-2} mol l^{-1} geraniol, LEM=1×10^{-2} mol l^{-1} lemon oil, LIN=1×10^{-2} mol l^{-1} linalool. All odors dissolved in mineral oil (M.OIL).

mixture than bees that received the constant protocol (GLM, family=binomial, variable protocol, $Z=-4.702$, $P<0.001$; Fig. S3).

We next tested for responses to all three components 2 h later. According to prior work from Wright and Smith (2004), honey bees that were trained on the variable protocol should respond more often to the component odors than those trained to the constant protocol, and they should also distinguish the target odor from the other mixture components. The target odor [acetophenone (Ace) or phenylacetaldehyde (PAA)] was held constant in each type of odor mixture during the variable training protocol. We found no difference between odor mixture blends (GLMM, family=binomial, bee ID random factor, mixture blend×experience×protocol, $t=2.877$, d.f.=458, $P=0.3$). As expected, the free-flying bees that received the variable protocol responded more frequently to the target odor than bees trained using the constant protocol (Fig. 7A), and the target odor elicited a higher response than the other components (GLMM, family=binomial, bee ID random factor, Ace: $t=2.544$, d.f.=457, $P=0.01$, PAA: $t=2.256$, d.f.=457, $P=0.025$; non-target odors, $P>0.05$; Fig. 7A, Fig. S4A). In contrast, the restricted bees showed no difference in response to components between the constant and variable protocols (GLMM, family=binomial, bee ID random factor, experience×protocol, $t=-2.819$, d.f.=458, $P<0.01$; Fig. 7B, Fig. S4A). This difference in response between the variable and constant training protocols across all odors was also significantly larger in the free-flying bees than the restricted bees across both mixture blends (Wilcoxon rank sum test, $W=1$, $P<0.01$; Fig. 7C). Unlike their freely flying counterparts, the bees with reduced olfactory experience did not distinguish the odor mixture components when trained to the variable protocol and instead responded at a high level across all odors irrespective of training protocol or odor blend.

DISCUSSION

Honey bees with natural foraging experiences may have foraged at multiple floral species, each of which has complex olfactory stimuli composed of many different volatile chemicals present at floral nectaries (Levin et al., 2003; Raguso, 2008). The problem for any forager is to analyze this high-dimensional, multi-component space of floral odors and extract the lower number of dimensions that are indicative of nectar and pollen content of flowers (Zhou et al., 2018). Our work was aimed toward understanding how the circuitry in early processing in the AL could potentially be tuned to help extract these features (Locatelli et al., 2016) through natural versus restricted foraging experiences. We show that experience affects anatomical structure, neural encoding and behavioral performance of bees. This comprehensive set of results may have underlying causal features that can now be investigated through further experimentation.

We have shown that by reducing the complexity of the foraging environment that a honey bee experiences, there is an overall

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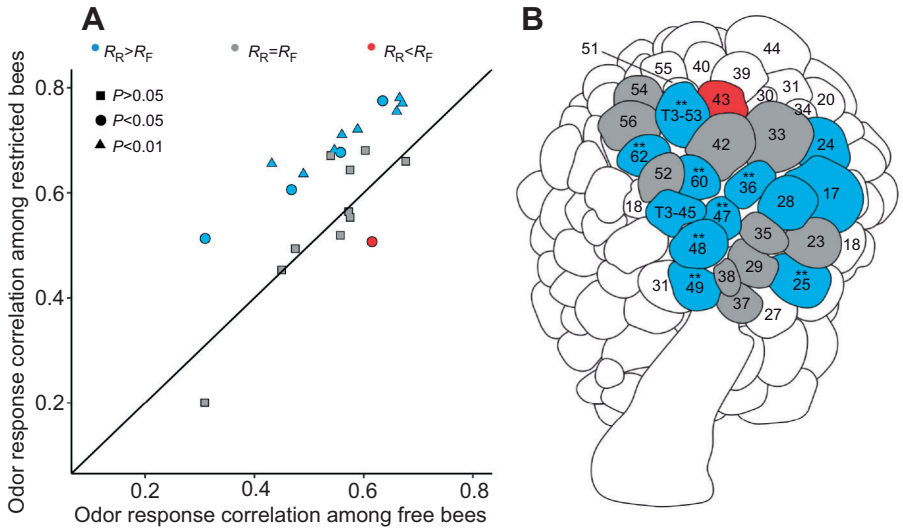

Fig. 6. Increased correlation among the glomerular response profiles to odors in restricted bees versus free-flying honey bees. (A) A comparison of the glomerular response similarity in bees with a free-flying experience plotted against similarity in bees with a restricted experience. Each point is the mean inter-animal correlation in odor-evoked responses for an identified glomerulus, for either the free-flying bees (horizontal axis) or for the same glomerulus in the restricted bees (vertical axis). The diagonal line represents the null hypothesis of no difference in response correlation across conditions. (B) Reconstruction of the honey bee AL and highlighted glomeruli denoting significant differences between experience treatments. Glomeruli with two asterisks above the number denote significance of $P<0.01$. A bootstrapping test of means was used to assess significance and P-values were adjusted for multiple comparisons. In both A and B, colors denote significant differences between experience treatments and in which direction, and the shape of the points in A denotes the level of significance.

reduction in the inter-individual variation in how AL projection neurons respond to odors. The chronic activation – or lack thereof – of specific glomeruli during sucrose reinforcement could have global effects on the synaptic weights of interneuron connections (LN–LN and LN–PN) in the AL, which could explain this result. This is supported by the fact that we observed different levels of F-actin staining observed in restricted bees compared with aged adults with natural foraging experience (Fig. 4, Phalloidin-TRITC). Specifically in mushroom bodies of insects, including honey bees, it has been shown that elevated levels of F-actin staining are indicative of active structural or synaptic plasticity (Frambach et al., 2004; Groh et al., 2006). Furthermore, bees with the restricted foraging experience were less apt than naturally foraging bees to pick one component out of a mixture (Fig. 7C), which indicates that experience

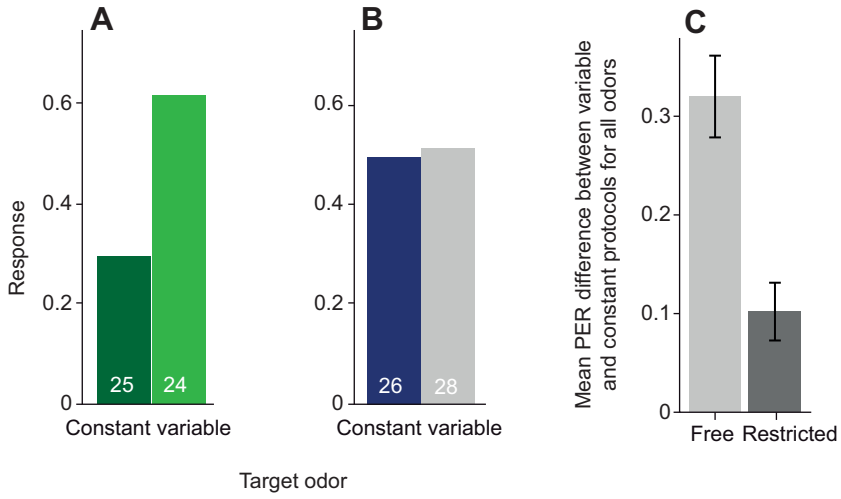

Fig. 7. High odor mixture component generalization with reduced foraging experience. Honey bees were trained using either a constant mixture (dark color) or a variable mixture (light color) protocol. The bars show the proportion of bees extending their proboscis 2 h after conditioning to the target odor (acetophenone). See Wright and Smith (2004) for more details. (A) Bees with a free-flying experience. (B) Bees with a restricted foraging experience. (C) Difference between the responses of bees trained using the variable mixture protocol and the constant mixture protocol for bees with a free-flying experience (light gray) and a restricted foraging experience (dark gray) for all odor components in both trained mixture blends. Error bars represent s.e.m. Numbers at the bottom of each bar represent the number of bees tested. Statistics: (A,B) GLMM with interacting factors of mixture (blend 1 or blend 2), odor components (phenylacetaldehyde, nonanal, α-farnesene, acetophenone, geraniol, 2-octanone), bee experience (free versus restricted) and training protocol (constant versus variable), and bee identity as a random factor. There was no statistical difference in the responses to either mixture blend, and we just show responses to mixture blend 1 target odor here (see Fig. S2 for full odor response details). (C) Mann–Whitney U-test, $W=1$, $P<0.01$, free constant $N=45$, free variable $N=48$, restricted constant $N=52$, restricted variable $N=53$.

affects the ability to analyze an odor mixture. This behavioral result is further supported by Cabirol et al. (2017), who recently found that deprivation of experience results in a reduced ability to perform more complex learning tasks such as reversal learning.

More recently, it has been shown via genetic knockout of the obligatory co-receptor to all olfactory receptors of two ant species – *Harpegnathos saltator* and *Ooceraea biroi* – that normal development of the AL requires not only olfactory receptor neuron axon presence, but also physiological responses to olfactory stimuli (Trible et al., 2017; Yan et al., 2017). Although we only explored the impacts of this olfactory restriction treatment on processing in the antennal lobe, there are also likely significant impacts of this treatment on higher-order processing centers downstream of the AL. For example, long-term, olfactory memory formation has transient impacts on synaptic bouton density in the mushroom bodies of the ant *Acromyrmex ambiggus* (Falibene et al., 2015). These short-term synaptic changes may result in broad network-level restructuring in these higher-order neuropils that can persist for months, as behaviorally, leaf cutter ants, *Atta colombica*, will avoid leaves in the field that were previously known to be harmful for upwards of 18 weeks (Saverschek et al., 2010). Therefore, we suggest that olfactory learning and experience have broad impacts on the development and functioning of the adult nervous system at all levels, not only in higher-order neuropils such as the mushroom bodies, but also in the earliest sensory processing neuropils such as the antennal lobe.

Post-eclosion input on antennal lobe maturation

Post-eclosion experience shapes the development of neural networks in honey bees in the visual processing centers of the mushroom bodies (Coss et al., 1980; Fahrbach et al., 1995; Farris, 2005; Farris et al., 2001) and to some degree the early network development of the AL (Brown et al., 2004, 2002; Arenas et al., 2012). Global AL responses to odors, primarily composed of ORN responses, continued to develop for up to 2 weeks of age but prior to adult foraging began, and that after foraging the volume of glomeruli can continue to change in a task-dependent manner (Wang et al., 2005; Winnington et al., 1996). In addition, recent studies have shown that there are physiological changes in the AL of adult foragers in response to odors associated with different kinds of olfactory experiences, in which PN responses to odors are modified to support discrimination of relevant odors in AL ensemble activity (Arenas et al., 2009, 2012; Chen et al., 2015; Fernandez et al., 2009; Hourcade et al., 2009; Locatelli et al., 2013).

Prior to the present work, in experiments with an experience-associated treatment, experimenters only isolated behavioral honey bee castes that did or did not have in-hive experience (Hourcade et al., 2010, 2009; Brown et al., 2004, 2002; Coss et al., 1980; Fahrbach et al., 1995; Farris, 2005; Farris et al., 2001), presented general odor experience in the hive for a fixed period of time (Arenas et al., 2009, 2012), measured age groups independent of caste or experience (Wang et al., 2005), or only focused on highly specialized odorant pathways (Sachse et al., 2007). They then measured physiological changes and/or region-specific morphological changes in volume and synaptic densities (Coss et al., 1980; Fahrbach et al., 1995; Farris, 2005; Farris et al., 2001; Brown et al., 2004, 2002; Arenas et al., 2009, 2012; Wang et al., 2005; Sachse et al., 2007). In the present study, we extend and complement prior studies in several ways. We allowed animals to develop within the hive normally, and we tested animals from the same genetic cohorts and from within the same caste that have different foraging experiences. We therefore add a comprehensive view of the effects of experience restriction on olfaction at the level of neuroanatomy, physiological responses to odors, and a complex odor recall assay.

In light of previous work, our data suggest that post-eclosion experience affects processing in the AL network in a way that influences performance of complex olfactory-based tasks. We do not yet know how foraging experience (e.g. flying, experience of more complex scenes) and specific olfactory experience (the hexanol exposure in our restricted treatment) interact to give rise to this effect, nor the identity of the specific neuronal sub-types and network connections affected during olfactory restriction. From work in *Drosophila melanogaster*, it is possible that inhibitory LNs may be responsible for these global changes in AL tuning as a result of experience (Sachse et al., 2007).

In our treatments, we manipulated the chronic foraging environment that adult honey bee foragers experienced. The restriction treated honey bees had access to all of the normal colony odors, but were lacking both natural resource-associated olfactory experiences and a natural foraging environment. In our treatments by restricting honey bees' activity to a tent, we were able to control the olfactory experiences but in the process we also manipulated the general foraging experience of honey bees by restricting flight distance and aerobic effort, visual diversity, nectar complexity and likely the colony forager dance communication profile. All of these factors could affect development of many areas of the brain. Nevertheless, the present study is the necessary first step describing the impacts of foraging experience on the tuning of the olfactory system at the morphological, physiological and behavioral levels.

Perception of odors with reduced experience

Honey bees with chronic exposure to an odor associated with a resource show a bias towards that odor in the form of an elevated initial proboscis response to that odor prior to any sucrose stimulation (Grosso et al., 2018). We wanted to understand the differences in the olfactory capabilities across our experiential treatments, and thus chose to test these animals on an olfactory learning task that is typically more difficult for bees than learning straightforward associations of an odor with reinforcement (Wright and Smith, 2004). The differences we report in recall of odor mixtures by honey bees with restricted versus free experience could have several possible explanations and require further study. First, bees with limited experience could have difficulty recognizing odors in general. These bees could also have less precise recall of odor mixtures compared with bees with free-flying experience, or they could have a higher degree of generalization to odor associations. These possibilities could be tested using a discrimination task across a number of odorants followed by a recall test to similar and dissimilar odorants, as done in Guerrieri et al. (2005). Coding of complex olfactory mixtures is not well studied in the AL, despite these stimuli being abundant in natural settings (Laloi et al., 2000; Strutz et al., 2014; Locatelli et al., 2016; Guerrieri et al., 2005; Zhou et al., 2018). More complex olfactory stimuli could be computationally more intensive to process. An AL network that has been globally altered via our restriction treatment might be less capable of processing and appropriately responding to multi-component odors compared with their natural foraging counterparts.

Conclusions

Our data show that adult foraging experience has significant impacts on the AL network in honey bees. We show that the development of the AL neuropil is delayed, inter-individual variation in the physiological responses to odors in this network is reduced, and

the capacity of animals to learn and behaviorally analyze odor mixtures is also reduced when foraging experience is reduced. Although the causal relationship among these effects needs to be established, these findings suggest that experience drives the normal inter-individual variation we observe in nature and that these experiences are necessary for the function of the AL in honey bees.

Acknowledgements
We thank Drs Ronald Rutowski, Jon Harrison, Stephen Pratt, Jason Newbern and Karla Moeller (Arizona State University) for their comments and suggestions.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: C.M.J., I.S., F.L., B.H.S.; Methodology: C.M.J., R.H., I.S., F.L., B.H.S.; Software: F.L.; Validation: C.M.J., I.S., F.L., B.H.S.; Formal analysis: C.M.J., R.C.G., F.L.; Investigation: C.M.J., R.H., I.S., F.L.; Resources: C.M.J., I.S., B.H.S.; Data curation: C.M.J., R.C.G., F.L.; Writing - original draft: C.M.J., R.C.G., I.S., B.H.S.; Writing - review & editing: C.M.J., R.C.G., I.S., F.L., B.H.S.; Visualization: C.M.J., I.S., F.L.; Supervision: C.M.J., I.S., F.L., B.H.S.; Project administration: C.M.J., B.H.S.; Funding acquisition: B.H.S.

Funding
This work was supported by awards from the National Institute of General Medical Sciences (GM113967) and the National Science Foundation (IOS 1556337) to B.H.S. R.C.G. was supported by the National Institute of Mental Health (1R01MH106674) and the National Institute of Biomedical Imaging and Bioengineering (1R01EB021711). F.F.L. was supported by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET; National Scientific and Technical Research Council, Argentina). Deposited in PMC for release after 12 months.

Supplementary information
Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.206748.supplemental

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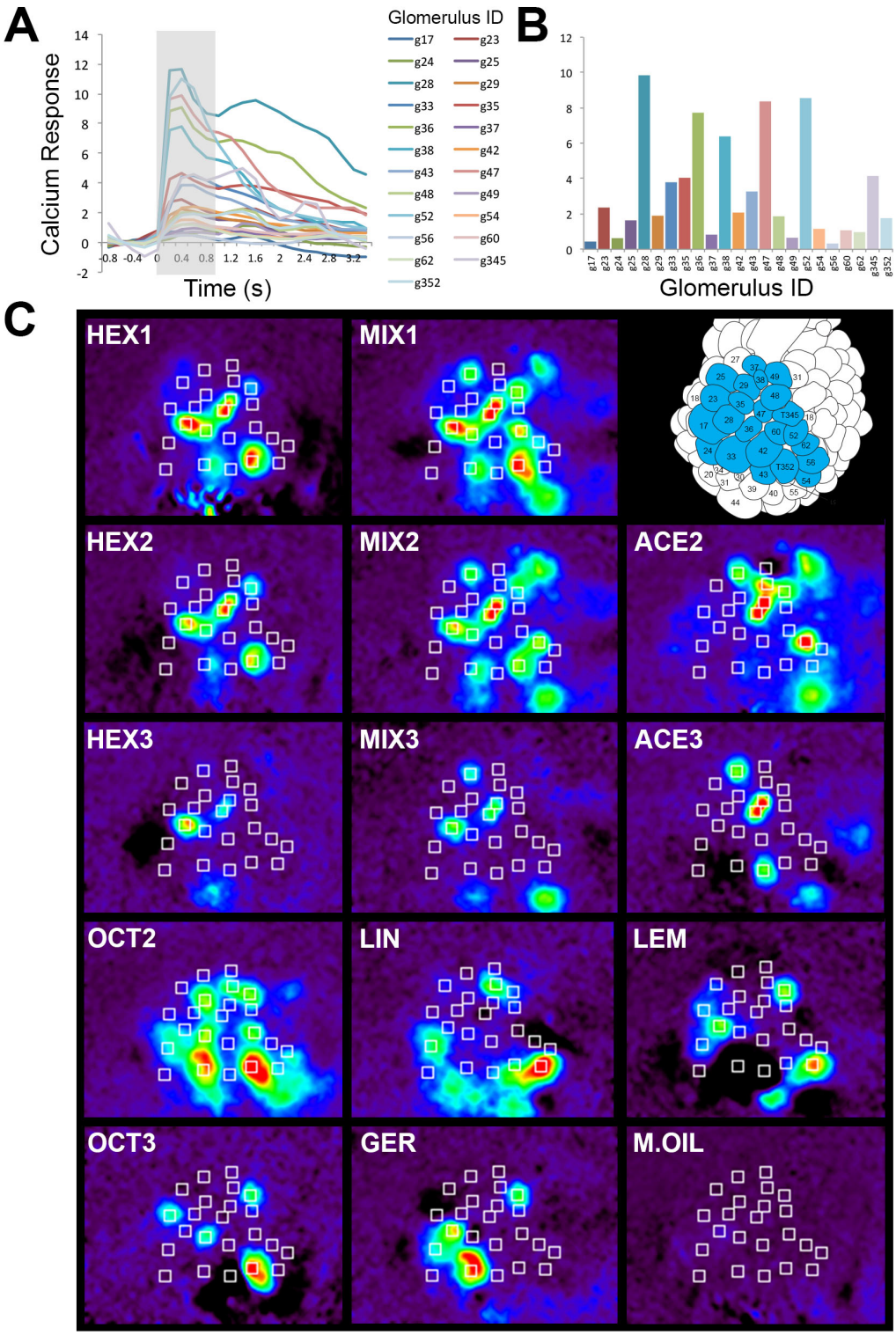

Figure S1. Calcium imaging data collection and processing protocol. Example data from a single free-flying worker bee. (**A**) Response of 23 identified glomeruli to Mix1 over time. Shaded region denotes odor stimulation. (**B**) Averaged response during odor stimulation (shaded region in a) for each glomerulus. (**C**) Example false colored average response, over the 1 second odor stimulation window, of the honey bee AL to each odor stimulus presented. Schematic reconstruction of the rostral portion of the AL and the 23 identified glomeruli analyzed in this experiment in top right panel. Odors labels: HEX1 = 2x10-2 M 1-Hexanol, HEX2= 1x10-2 M 1-Hexanol, HEX3= 1x10-3 M 1-Hexanol, MIX1= 2x10-2 M 1-Hexanol and 1x10-2 M Acetophenone, MIX2= 1x10-2 M 1-Hexanol and 1x10-2 M Acetophenone, MIX3= 1x10-3 M 1-Hexanol and 1x10-3 M Acetophenone, ACE2= 1x10-2 M Acetophenone, ACE3= 1x10-3 M Acetophenone, OCT2= 1x10-2 M 2-Octanone, Oct3= 1x10-3 M 2-Octanone, GER= 1x10-2 M Geraniol, LEM= 1x10-2 M Lemon oil, LIN = 1x10-2 M Linalool. All odors dissolved in mineral oil (M.OIL).

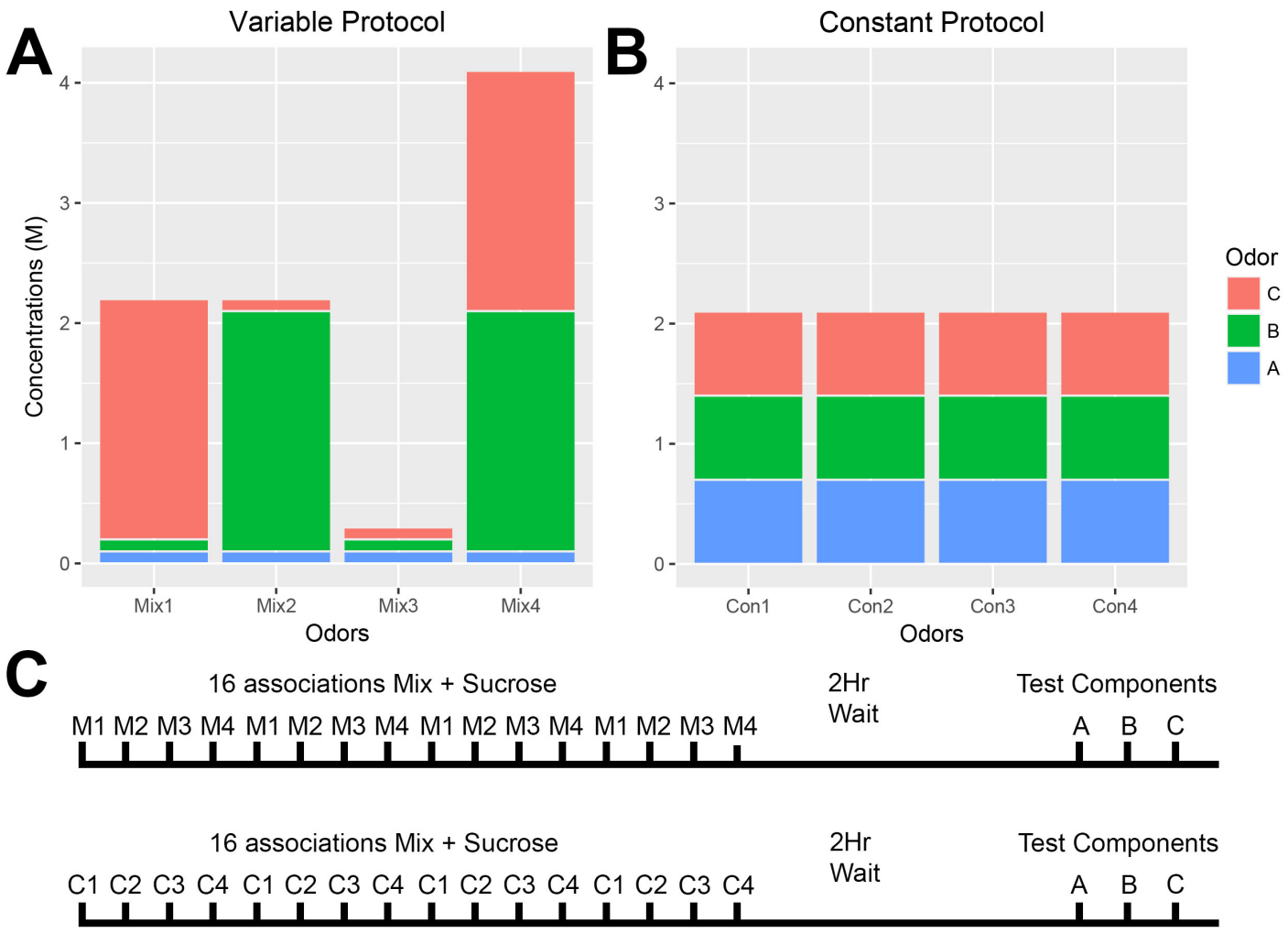

Figure S2. **Odor mixture component learning protocol**
The odor mixture component concentrations used during the (**A**) variable mixture protocol (**B**) the constant mixture protocol. (**C**) A schematic of the associative conditioning and component memory testing procedures. Bees that received the variable protocol received different mixtures (M1, M2, M3 or M4) in a pseudorandomized order over 16 associative acquisition trials. Bees that the constant protocol received identical mixtures (Co) over 16 associative acquisition trials. Mean total odorant exposure was equivalent across the two protocols. Memory test component order was randomized between bees for both protocols. We used a mixture blend made of three components: A=Acetophenone, B=Geraniol and C=2-Octanone. Acetophenone (A, the target odor) is held at a constant intensity even in the variable protocol. Across trials, the average mixture intensity is the same for both the constant and the variable protocols. We also tested a second mixture blend see methods and Wright and Smith (2004) for more details.

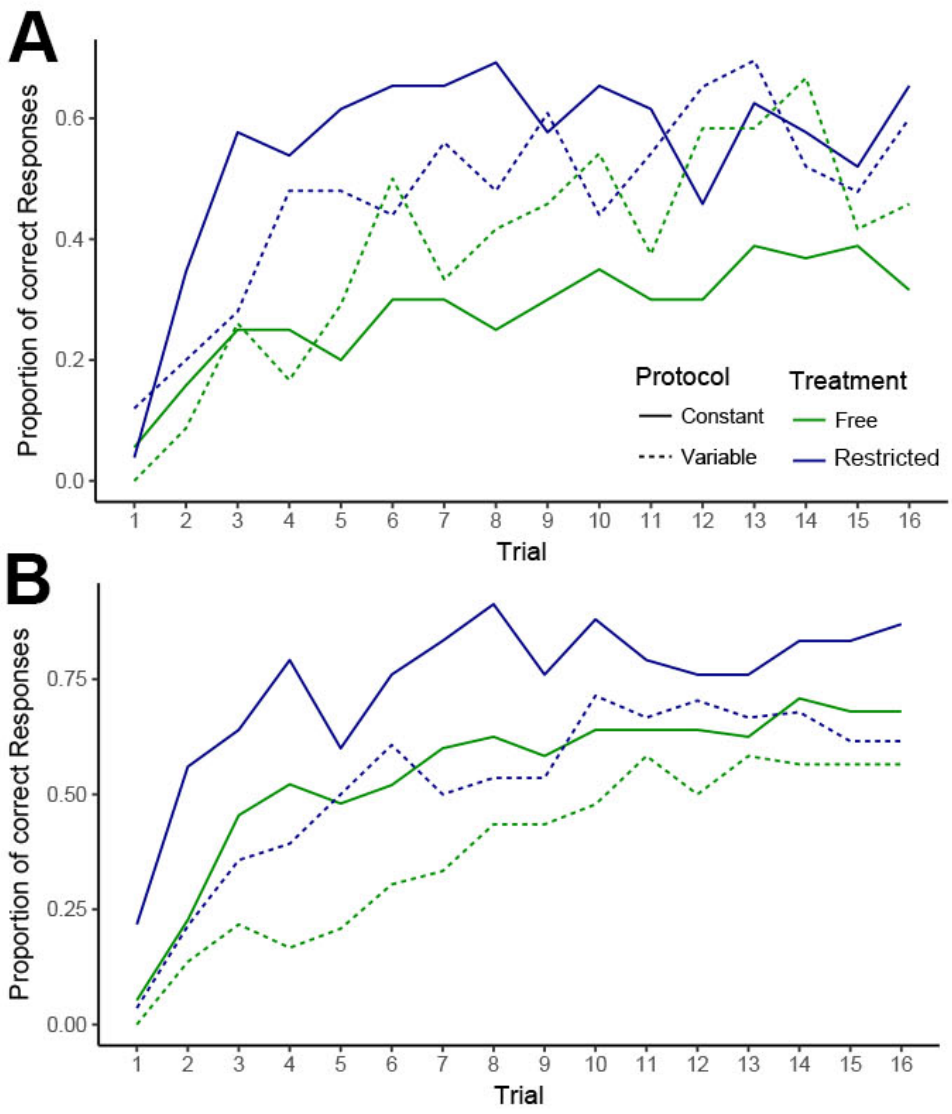

Figure S3. Acquisition of odor mixture blend 1 across freely foraging (green) and restricted bees (blue) which received either a constant (solid line) or a variable (dashed line) mixture protocol. (**A**) Mixture blend 2 odors (**B**) mixture blend 1 odors. Sample Sizes: free blend 2 constant N=20, free blend 2 variable N=24, free blend 1 constant N=25, free blend 1 variable N=24, restricted blend 2 constant N=26, restricted blend 2 variable N=25, restricted blend 1 constant N=26, restricted blend 1 variable N=28.

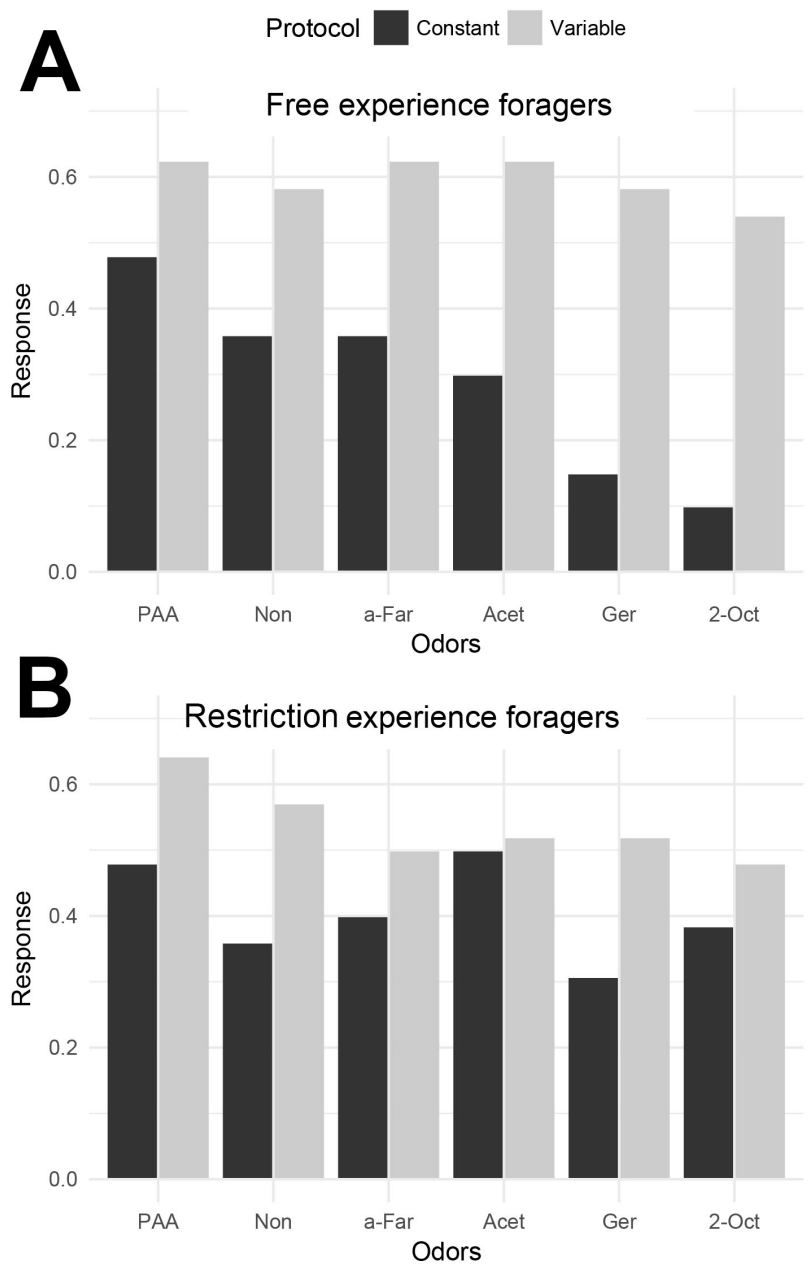

Figure S4. **The memory response rates for bees 2hrs after receiving the acquisition protocol.** Bees receieved either the constant protocol (black) or the variable protocol (gray) and had one of two prior experience treatments (**A**) a free-flying experience or (**B**) a restricted foraging experience. Sample Sizes: free blend 2 constant N=20, free blend 2 variable N=24, free blend 1 constant N=25, free blend 1 variable N=24, restricted blend 2 constant N=26, restricted blend 2 variable N=25, restricted blend 1 constant N=26, restricted blend 1 variable N=28. PAA= phenylacetaldehyce, Non= nonanal, a-Far= a-farnesene, Acet= acetophenone, Ger= geraniol, 2-Oct= 2-octanone.