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# Component Information Is Preserved in Glomerular Responses to Binary Odor Mixtures in the Moth *Spodoptera littoralis*

**Mikael A. Carlsson[1,2]*, Kwok Ying Chong[3]*, Wiltrud Daniels[2], Bill S. Hansson[2,4,†] and Tim C. Pearce[3,†]**

[1]Department of Safety Pharmacology, AstraZeneca R&D Södertälje, SE-151 85 Södertälje, Sweden, [2]Department of Crop Science, Division of Chemical Ecology, Swedish University of Agricultural Sciences, PO Box 44, SE-230 53 Alnarp, Sweden, [3]Centre for Bioengineering, University of Leicester, Leicester LE1 7RH, UK and [4]Department of Evolutionary Neuroethology, Max Planck Institute for Chemical Ecology, Hans-Knoell-Strasse 8, D-07745 Jena, Germany

*Correspondence to be sent to: Kwok Ying Chong, Centre for Bioengineering, University of Leicester, Leicester LE1 7RH, UK. e-mail: kyc11@leicester.ac.uk or Mikael A. Carlsson, AstraZeneca R&D Södertälje, SE-151 85 Södertälje, Sweden. e-mail: mikael.a.carlsson@astrazeneca.com*

*Authors contributed equally.
†Authors share joint seniority.

## Abstract

Natural odors are often complex mixtures of different compounds. These mixtures can be perceived to have qualities that are different from their components. Moreover, components can be difficult to distinguish within a blend, even if those components are identifiable when presented individually. Thus, odor components can interact along the olfactory pathway in a nonlinear fashion such that the mixture is not perceived simply as the sum of its components. Here we investigated odor-evoked changes in $Ca^{2+}$ concentration to binary blends of plant-related substances in individually identified glomeruli in the moth *Spodoptera littoralis*. We used a wide range of blend ratios and a range of concentrations below the level at which glomerular responses become saturated. We found no statistically significant cases where the mixture response was greater than both component responses at the same total concentration (synergistic interactions) and no statistically significant cases where the mixture response was less than either component presented individually (suppressive interactions). Therefore, we conclude that, for the plant mixtures studied, information of their components is preserved in the neural representations encoded at the first stage of olfactory processing in this moth species.

**Key words:** glomeruli, mixture interaction, moth, olfaction, optical imaging



## Introduction

Natural odor stimuli rarely occur as single compounds but are, in fact, very often complex mixtures of different molecular components. Chemically mediated behavior of both aquatic and terrestrial animals is often driven by such complex mixtures (Laing 1989), which comprise both compound and mixture information that is behaviorally relevant. In addition, these odors are often present in a background ''soup'' of irrelevant and interfering compounds. Despite the chemical complexity and low signal to noise ratio, the olfactory apparatus can filter out relevant information, for example, about food acceptability, presence of predators, and sexual status of the opposite sex. Yet, little is known about how mixtures are encoded within the olfactory pathway to support this type of processing.

Mixtures of odors are often perceived as having unique synthetic qualitative properties, and it is generally difficult to distinguish the individual components of the blend (Moskowitz and Barbe 1977; Laing and Francis 1989; Laing and Livermore 1992). Hence, the neural representation of individual components within the context of a mixture is believed to interact nontrivially along the olfactory pathway in a manner that may hinder the deconvolution of component information from mixture responses. When the neural response to a blend is not the simple sum of the responses

to its constituents, then a nonlinear mixture interaction has occurred. Such nonlinear interactions can contribute to the synthetic perception of a blend, which includes suppression, where the blend response is weaker than that of one or more of the components at the same concentration, and synergy, where the blend response is higher than that of the most strongly responding component at the same total concentration (Duchamp-Viret et al. 2003). Neural representations of odor components within a mixture may start to interact nontrivially even at the olfactory receptor neuron (ORN) level, with individual ORN responses to odor mixtures that exhibit suppression or synergy: in invertebrates, such as insects (Akers and Getz 1993; Carlsson and Hansson 2002; Ochieng et al. 2002) and crustacean (Steullet and Derby 1997), and in vertebrates, such as fish (Kang and Caprio 1991, 1997) and mammals (Duchamp-Viret et al. 2003; Oka et al. 2004). More commonly, mixture interactions have been observed in second-order neurons in vertebrates as well as invertebrates (De Jong and Visser 1988; Christensen et al. 1991; Tabor et al. 2004).

Nonlinear coding of mixtures at the periphery would seem to make identifying the individual components of a blend from the mixture representation a difficult task, and yet information relating to the components can persist in the perception of odors, as has been observed in rats (Linster and Smith 1999) and honeybees (Hosler and Smith 2000). This suggests a so-called nonconfigural, elemental aspect to odor perception in addition to the synthetic paradigm. This together implies that neural representations of complex odor mixtures comprise both configural and elemental properties. It is, however, unclear where and how mixture interactions take place in the olfactory pathway and its neural representation.

The insect antennal lobe (AL) and the mammalian olfactory bulb consist of a species-specific number of glomeruli, each of which represents the input from all ORNs housing a specific receptor (Mombaerts et al. 1996; Vosshall et al. 2000). A combinatorial across-neuron pattern among the ORNs (Malnic et al. 1999) is thus represented as glomerular activity patterns. This has been confirmed in a number of optical imaging studies in vertebrates (Friedrich and Korsching 1997; Rubin and Katz 1999; Uchida et al. 2000; Meister and Bonhoeffer 2001) as well as insects (Joerges et al. 1997; Galizia et al. 1999; Carlsson et al. 2002). It has been suggested that glomerular activity patterns constitute a spatial olfactory code (Galizia et al. 1999). Such a code or representation is dependent not only on the chemical structure of the odor molecule but also on the concentration (Carlsson and Hansson 2003; Sachse and Galizia 2003). A handful of imaging studies have tested odor blends and the results diverge. Joerges et al. (1997) reported strong suppressive interactions between components using optical imaging in the honeybee. Other studies have reported responses to mixtures that represent the linear sum of responses to the components (Belluscio and Katz 2001). Recently, Tabor et al. (2004) demonstrated that mixture interactions were weak or negligible in the ORN presynapses in the zebrafish glomeruli, whereas both suppressive and synergistic interactions were observed in olfactory bulb output neurons.

Our model animal, the noctuid moth *Spodoptera littoralis*, is a broad generalist species found on at least 84 different host-plant species (Brown and Dewhurst 1975). Anderson et al. (1993) demonstrated that *S. littoralis* is strongly deterred by a complex mixture (but not to submixtures) of odorants induced by larval feeding. This indicates that synergistic blend interactions occur along the olfactory pathway in this species. In the present study, we exposed the animal to 3 plant-related compounds (and their binary mixtures) common to many of the moth's host plants. Odorants were chosen as they have previously been shown to activate either overlapping or nonoverlapping subsets of ORNs and glomeruli depending on the binary combination (Anderson et al. 1995; Jönsson and Anderson 1999; Carlsson et al. 2002; Carlsson and Hansson 2003). Thus, mixture phenomena due to either agonistic or antagonistic interactions could potentially occur. We studied responses in individually identified glomeruli by means of $Ca^{2+}$ imaging. This method basically reports input activity in the glomeruli (Galizia et al. 1998). In an attempt to mimic natural conditions in which an animal may realistically find itself, we used plant odors in a narrow range of biologically relevant concentrations (Carlsson and Hansson 2003) and mixtures at several different ratios. We investigated if the responses to the blends showed suppressive or synergistic effects. Responses to the mixtures were, however, predictable from the responses to the individual constituents. We did not observe any statistically significant cases of suppression or synergy, leading us to conclude that component information for some common plant–plant odors is preserved in the neural representation at the first stage of olfactory processing for this moth species.

## Materials and methods

### Animals and staining

We recorded neural activity optically in the AL of male *S. littoralis* by imaging $Ca^{2+}$ dynamics. A detailed description of the preparation of the animals and the experimental setup can be found elsewhere (Carlsson et al. 2002; Carlsson and Hansson 2003), but the following gives a brief summary of the experimental procedures. Experiments were performed on 1–5 days post emergent male moths. The head capsules were cut open between the compound eyes, and extraneous material removed to expose the ALs. A calcium-sensitive dye (CaGR-2-AM, Molecular Probes, Eugene, OR) was bath applied to the uncovered brain. The dye was dissolved in 20% Pluronic F-127 in dimethyl sulfoxide (Molecular Probes) and diluted in moth saline (Christensen and Hildebrand 1987) to a final concentration of ~30 μM. After

incubation (~60 min in 10–12°C) and rinsing in moth saline, recordings were done in vivo.

We used TILL Photonics air-cooled imaging system (Gräfelfing, Germany) with a 12-bit slow-scan CCD camera. Filter settings were dichroic: 500 nm, emission low pass 515 nm, and the preparation was excited at 475 nm. Sequences of 40 frames and a sampling rate of 4 Hz (200 ms exposure time) were recorded through an upright microscope (Olympus, Hamburg, Germany) with a 20× (NA 0.50; Olympus) water immersion objective. On-chip binning (2 × 2) was performed, which resulted in a pixel size corresponding to ~1 × 1 μm. Execution of imaging protocols and initial analyses of data were made using the software Till-vision (TILL Photonics).

### Odor stimuli

Each animal was tested with a set of binary blend stimuli consisting of mixtures of 2 particular compounds. Three host-plant compounds—geraniol, linalool, and phenylacetaldehyde (PAA) were used. Previous electrophysiological and optophysiological studies have shown that these odorants evoke strong responses in ORNs and glomeruli (Anderson et al. 1995; Carlsson et al. 2002; Carlsson and Hansson 2003). In addition, both larvae and adults could be trained to respond behaviorally to these compounds (Carlsson et al. 1999; Fan and Hansson 2001). Odorants were dissolved in paraffin oil, which does not evoke a detectable response in the AL (Carlsson and Hansson 2003). Ten microliters of the solvent containing the respective odorant were applied on a filter paper (5 × 15 mm). For the control stimulus, paraffin oil solvent was applied alone onto filter paper. Two filter papers were inserted in a Pasteur pipette attached to a plastic pipette tip at the proximal end (total volume ~4.5 ml), each containing an odorant or pure paraffin oil. This means that blends were constructed by mixture of the headspace. The pipettes were sealed with Parafilm (American National Can Co., Chicago, IL) and stored in a freezer until the start of an experiment. Odorants were delivered in a randomized order. We allowed at least 60 s between stimulations to reduce potential adaptation effects. Altogether, 10 different stimulus loads were used of each compound (2.5–80 μg) and 12 different binary mixtures. In addition, the third compound that was not included in the binary mixtures was tested at 4 doses in order to physiologically identify the corresponding glomerulus. Thus, including a control stimulus, in each experiment, we used 37 different stimuli. We have in a previous study shown that the concentrations lie between threshold of optophysiological detection and saturation (Carlsson and Hansson 2003).

A moistened and charcoal-filtered continuous air stream (30 ml $s^{-1}$) was ventilating the antenna ipsilateral to the recorded AL through a glass tube (7 mm inside diameter). The glass tube ended ~10 mm from the antenna. An empty Pasteur pipette was inserted through a small hole in the glass tube, blowing an air stream of ~5 ml $s^{-1}$. Another air stream (ca. 5 ml $s^{-1}$) was blown through the odor-laden pipette by a computer-triggered puffer device (Syntech, Hilversum, The Netherlands) during 1 s (starting at frame 12) into the continuous stream of air. During stimulation, the air stream was switched from the empty pipette to the odor-laden one in order to minimize the influence of added air volume.

### Data processing

To correct for bleaching, a bleaching baseline function of time, $F_b(t)$, was taken from a nonresponding region of the AL. The bleach-corrected response, $r(t_i)$, was taken to be the relative change in fluorescence $\Delta F(t_i)/F_b(t_i)$, where $\Delta F(t_i)=F(t_i) - F_b(t_i)$ for each time step $t_i$ at each frame $i = 1, \cdots, 40$. In addition, over stimulus trials, the response magnitude often decreased. This caused a strong time dependency that added extra variability in the data. The stimuli were presented in a random order, and so the weakening was not stimulus dependent. Thus, this weakening over stimulations could be fitted (in a least-squares sense) with an exponential decay of the form $ae^{-n/\lambda}$, where the amplitude $a$ and the decay constant $\lambda$ are free parameters, and $n$ is the chronological index of the stimulation. This artifact was then normalized from the data series by dividing the magnitude of each response by this exponential fit evaluated at $n$. This normalization also had the effect of adjusting to 1 the mean of maximum response magnitudes over all the trials.

Finally, to attain an overall response to a stimulus, the response was integrated over time. In practice, this meant the sum $R= \sum_{i=12}^{40} r(t_i)\delta t$, where $\delta t = 0.25$ s. Because stimulus was applied at the 12th frame, this summation starts from the frame 12.

### Automated location of glomeruli

Glomeruli were located by an automation of the techniques that have been employed previously (Carlsson et al. 2002; Carlsson and Hansson 2003). First, a threshold of 50% of maximum response magnitude was used to determine which pixels were of active glomeruli. Glomeruli are known to be convergent sites for ORNs expressing the same type of receptor (Gao et al. 2000; Vosshall et al. 2000; Couto et al. 2005; Fishilevich and Vosshall 2005). Because the activity we observe is dominated by presynaptic activity of these receptor neurons, each glomerulus can be considered as a single functional unit with highly correlated activity within each glomerulus. First, we define a response profile space as follows. To each pixel, denoted by the location, $(i, j)$, of that pixel, we assign the response profile vector $\vec{P}_{ij} =(R_{ij}(S_1) \cdots R_{ij}(S_k) \cdots R_{ij}(S_{37})$, where $R_{ij}(S_k)$ is the mean response of the 5 × 5 pixel region around pixel $(i, j)$ to the stimulus $k = 1, \cdots, 37$. Hereafter, this will be called the pixel response. Pixels that have correlated response profiles are located in neighboring regions of this profile space.

Thus, because a glomerulus is a single functional unit, so pixels from a mutual glomerulus can be expected to cluster together. Moreover, pixels from different glomeruli, which respond differently to this stimulus set, will have differing pixel responses and so will be separated in response space. Thus, the glomeruli form distinct clusters. We applied cluster analysis to these pixel responses in order to identify functionally equivalent pixel regions. We used Ward's linkage method for calculating the separation between clusters in response space. This approach is akin to that of analysis of variance (ANOVA). First, for a given cluster $G$ of pixel responses, we define the centroid to be $\bar{\vec{G}} = \frac{1}{N_G}\sum_{\vec{P}_{ij}\in G}\vec{P}_{ij}$, where $N_G$ is the number of points in $G$. We define the sum of squares of the deviation from the centroid to be $\sigma^2(G) = \sum_{\vec{P}_{ij}\in G}\left|\vec{P}_{ij} - \bar{\vec{G}}\right|^2$. The distance $D_W$ between 2 clusters, $G$ and $H$, is calculated as the increase in the sum of squares if these 2 clusters were combined into one cluster: $D_W(G,H) = \sigma^2(G\cup H) - [\sigma^2(G)+\sigma^2(H)]$. The algorithm is started with all the pixel responses as separate points. At each iterative step, the Ward method combines those 2 points or clusters into a single cluster that causes the smallest increase in the sum of squares of deviances. The iterations stop when a predetermined number of clusters has been reached. For agglomerative clustering methods, the number of clusters must first be specified a priori. This number has been obtained iteratively to minimize the number of clusters, yet still portrays the main features.

### Criteria for mixture interactions

We used a definition for suppression, where the blend response is weaker than that of either of the responding components (same concentration singly or in mixture), and synergy, where the blend response is stronger than that of the most strongly responding component at the same total loading as the blend. The following criteria were used:

$$Synergy: R(A_x + B_y) > \max[R(A_{x+y}), R(B_{x+y})]$$

$$Suppression: either\ R(A_x + B_y) < R(A_x)\ or\ R(A_x + B_y) < R(B_y)$$

where $R$ is the normalized response, $A$ and $B$ are the 2 components used, $x$ and $y$ the doses, and max[·, ·] gives the largest of the 2 values.

For statistical comparisons, we used a 1-way ANOVA or a Student's $t$-test.

## Results

In each animal, glomeruli were located from localized and coherent regions of activation using an adaptation of an existing method (Carlsson et al. 2002; Carlsson and Hansson 2003). This new method allowed the automatic detection of glomeruli without the need to manually decide the location of glomeruli by viewing activity maps to all stimuli. From these glomeruli, 3 were identified in each animal based on the respective physiological response. The 3 compounds we used (geraniol, linalool, and PAA) each activated a single glomerulus most strongly. In addition, several other glomeruli displayed weaker activity. We refer to the glomerulus that gave the greatest response magnitude for a compound as the "best" glomerulus for that component. The "PAA-best" glomerulus, with strongest activity for PAA, was located in the medial part of the AL, whereas the other 2 glomeruli were located more laterally (see also Carlsson et al. 2002; Carlsson and Hansson 2003). The linalool-best glomerulus was located ventral of the "geraniol-best" glomerulus. The latter glomerulus actually responded similarly to geraniol and linalool, but during geraniol stimulation this glomerulus was the one with the greatest response magnitude. During linalool stimulation, this glomerulus gave only the second strongest response of all the glomeruli. These 3 glomeruli could be unambiguously identified in all animals and allowed us to pool data across individuals. Figure 1 shows a typical example of false color–coded responses to geraniol, linalool, and the binary mixtures of these in a single animal. In all images, identical intensity scales were used for ease of comparison.

Only animals where a complete or near-complete stimulus panel (see Odor stimuli) that could be tested were further analyzed. Out of 28 animals with recorded responses, 13 animals were used in the analysis.

Dose–response curves for individual components and their binary mixtures were constructed based on the total stimulus load (Figure 2). The geraniol-best glomerulus was strongly activated by both geraniol and linalool and only weakly by PAA at the highest concentrations. The "linalool-best" glomerulus, on the other hand, was only strongly activated by linalool, whereas geraniol and PAA evoke detectable responses only at much higher concentrations. Finally, the PAA-best glomerulus was only activated by the PAA within the concentration range used. Therefore, we show only dose–response curves for blends with at least one potent compound. The dose–response curves for the blends all lay between the curves for the individual components, except for blends of geraniol and linalool in the geraniol-best glomerulus, which elicit equal responses to either of the components or to the blend. For a clearer view, the responses are shown in a bar chart (Figure 3). In each section, the bars show responses to stimuli with the same total load. The differences between the mean responses within each section were tested with an ANOVA. In the geraniol-best glomerulus, responses to stimuli containing the same total load of geraniol, linalool, or a blend of these did not differ ($P$ = 0.226–0.997). All other combinations were, however, highly significantly different ($P$ = 0.014–0.000).

We tested for synergistic interactions according to the criterion as stated in Materials and methods. We compared the blend response with single component responses where the single component is at the same concentration load as the total concentration load of the blend. This ensured that

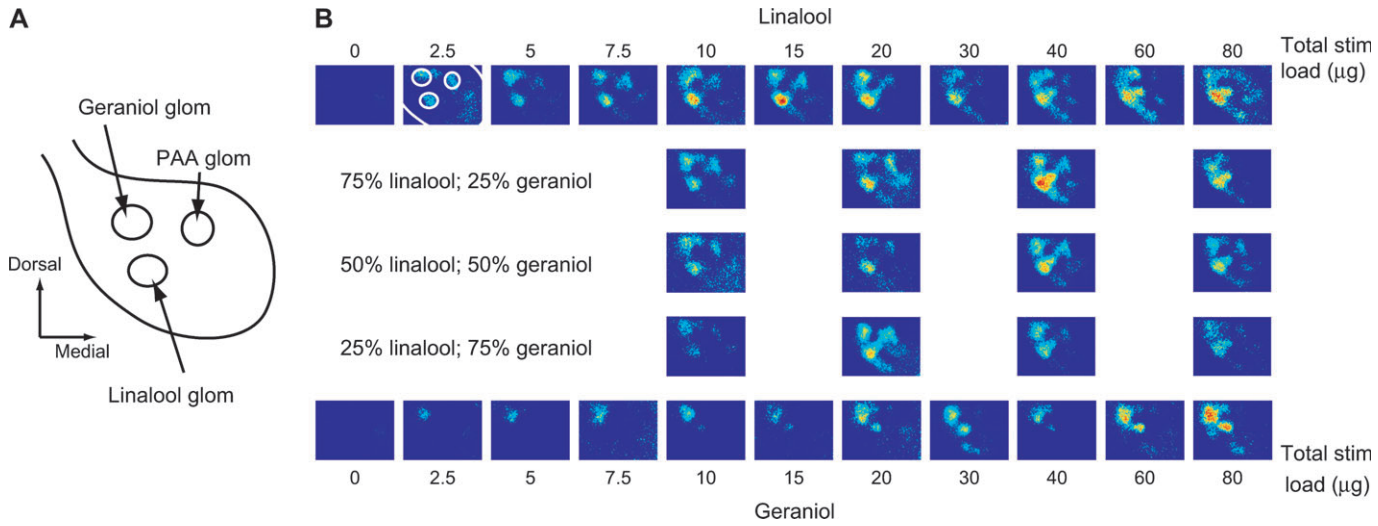


**Figure 1** Example of responses recorded in a single animal. **(A)** A map of the AL indicating regions that were used to attain glomerular activity, with an estimated outline for the AL. To indicate the relative positioning of these glomeruli against the activity images, the map has been superimposed onto the response to linalool at 2.5 μg stimulus load. **(B)** After initial image processing, we attain spatial activity maps for each of the odors tested in one binary combination of odorants. In this case, the binary combination was of linalool and geraniol. Generally in this diagram, columns represent the total stimulus load (micrograms), increasing from left to right, and rows represent different ratios from pure linalool at the top to pure geraniol at the bottom. In this diagram, we can see the regions of activity develop in the series as loading increases, particularly for the pure odorant stimuli. The increase in activity and the spatial spreading of activity in the recruitment of more glomeruli can be observed. Also evident is the progression of the spatial activation as the odor ratios move between one pure odorant to the other. There appears to be a smooth transition in general.

we did not identify a synergistic effect when an increase in response was merely due to an increase in concentration load as 2 components were added together. We statistically tested all pairs (strongest responding compound vs. mixture) containing identical total loads (Figure 4). No responses to the blends were significantly stronger than the responses to the corresponding single components (84 comparisons, all $P > 0.05$, Student's $t$-test). That is, no synergistic effects were observed. Finally, we tested the occurrence of suppressive interactions (Figure 5). In this test, we compared the blend responses with the component responses such that the components were at the same concentration load as that from which the blend was comprised. In this way, any decrease in the response to the individual components compared with the response to the blend cannot be the result of lowering the concentration load of either component. Every combination where the component had the same concentration singly or in a mixture was compared. Out of 168 comparisons, we find 62 meet the suppression criterion, and only in 4 cases did we find a mixture that evoked a significantly weaker ($P < 0.05$, $t$-test) response than the component alone. The observed suppressions were not dependent on mixture, concentration, or ratio (see Figure 5). Furthermore, when a Bonferroni correction for multiple tests is applied, a $P < 0.05$ threshold level for the whole suppression experiment requires individual tests to give a $P$ value less than $0.05/62 \approx 8 \times 10^{-4}$. This only takes into account the cases where the criterion was met and a Student's $t$-test was performed, yet none of the individual tests were significant to this level.

## Discussion

Mixture interactions in moths have been frequently observed, both in behavioral studies (Arn et al. 1980; Löfstedt et al. 1982; Wu et al. 1995) and in electrophysiological recording from second-order neurons of the AL (Christensen and Hildebrand 1987; Christensen et al. 1989, 1991, 1995; Anton and Hansson 1995; Wu et al. 1996; Anton et al. 1997; Hartlieb et al. 1997). It is, however, unclear whether these interactions originate in peripheral receptor cells, by computational processes in the AL or by feedback from higher brain regions. Here we studied responses in single glomeruli representing the convergent input from hundreds of ORNs likely housing identical receptor proteins (Gao et al. 2000; Vosshall et al. 2000). Potential blend interactions detected by $Ca^{2+}$ imaging of the glomeruli could result from nonlinear activation at the receptor site, by feedback activity in the ORN terminals, or a combination of both. Two conservative approaches were used to identify possible mixture interactions. Suppression was defined as a response to a blend that was significantly weaker than the strongest responding component alone applied at the same concentration as in the mixture. Synergy, on the other hand, was defined as a blend response that is stronger than that of the most strongly responding component at the same total loading as the blend. Using this approach, we could not identify any synergistic or suppressive interactions, irrespective of concentration or ratio. That is, responses to binary blends of plant-related compounds in single glomeruli could be predicted based on the

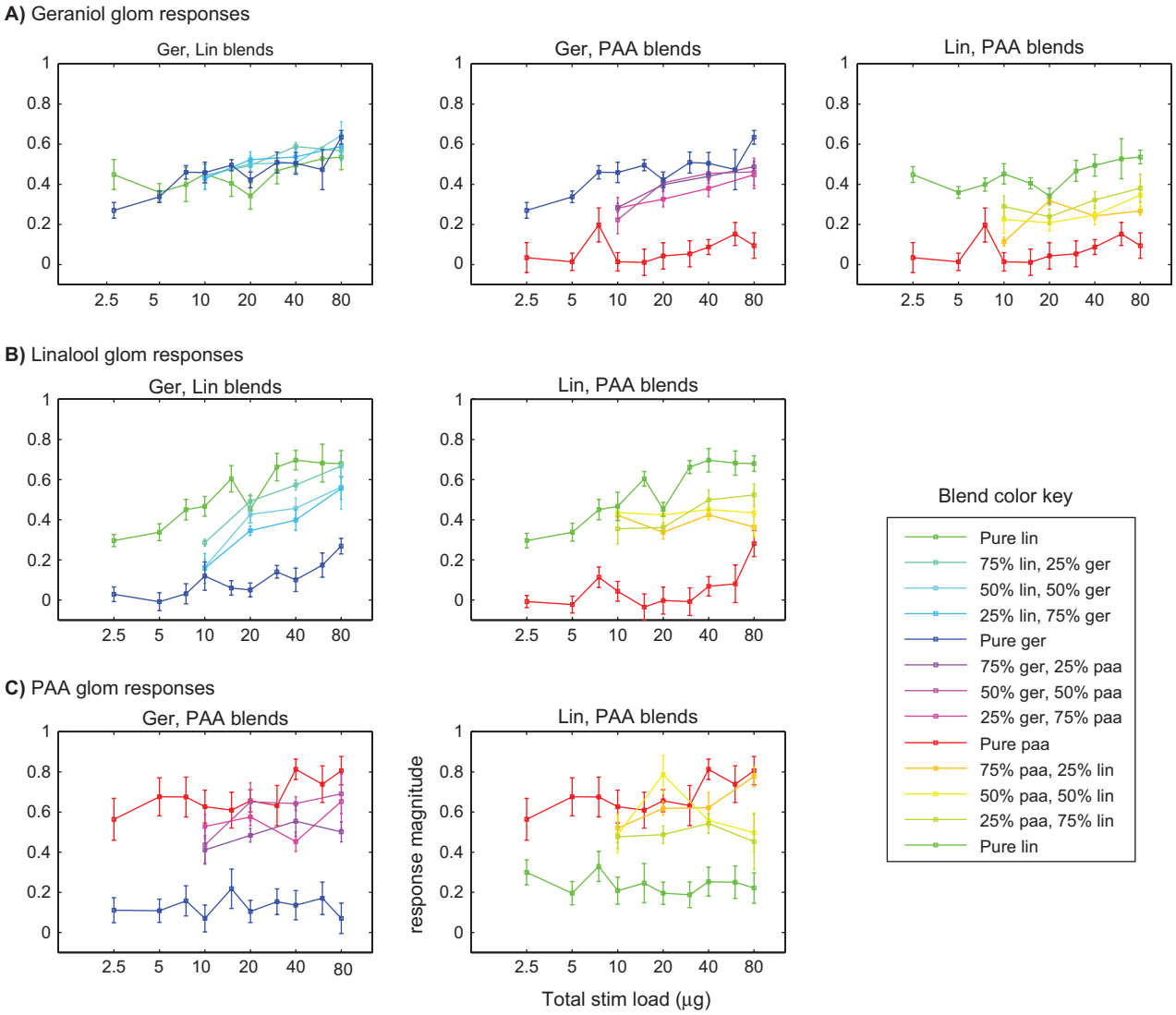


**Figure 2** The responses to all odors pooled across animals. The graphs show the concentration curves for each ratio mean averaged over identified glomeruli across animals, with error bars showing the standard error. The colors are chosen to represent the different pure odorants and their mixtures. Linalool is assigned green, geraniol assigned blue, PAA assigned red, and the colors for the binary blends are the colors of the respective constituent components mixed in the corresponding ratios (see legend). Each panel shows the response of an identified glomerulus to binary blends of one pair of odorants for **(A)** the geraniol-best glomerulus, **(B)** the linalool-best glomerulus, and **(C)** the PAA-best glomerulus. The glomeruli, identified in each animal as the one responding strongest to a pure odorant, show different response strategies. Responses were normalized between zero and one before pooling (see Materials and methods). In almost all cases, there is an upward trend by way of response magnitude increasing with stimulus load. However, we can see that the geraniol glomerulus will respond to geraniol and linalool indifferently and discriminating only on the total stimulus load, whereas the linalool glomerulus responds mainly to linalool alone until geraniol or PAA reaches higher loads, and the PAA glomeruli will respond only to PAA. It is also apparent that the responses to the varying ratios are roughly in the order you would expect as they graduate between one pure stimulus to another. The responses for the linalool glomerulus to geraniol–PAA blends and for the PAA glomerulus to linalool–geraniol blends have been omitted because these glomeruli did not respond to these blends.

responses to the individual components. It cannot, however, be excluded that less pronounced interactions may have been overlooked with this approach.

The nearly complete lack of suppressive interactions (and complete lack, after the Bonferroni correction) in our study as opposed to the study by Joerges et al. (1997), using the same imaging technique, can be explained by the differences in stimulus loads used. The doses in our study were chosen to be well below saturation level (Carlsson and Hansson 2003) to mimic naturally occurring concentrations. Joerges et al. (1997), on the other hand, used nondiluted substances, which in our model would elicit responses above saturation level (Carlsson and Hansson 2003). In a previous experiment, we showed that suppression in pheromone-sensitive ORNs in the moth *Agrotis segetum* only occurs at very high concentrations of mixtures (Carlsson and Hansson 2002). It is not unlikely that competition for a receptor site is significant when a large abundance of molecules is present. Even

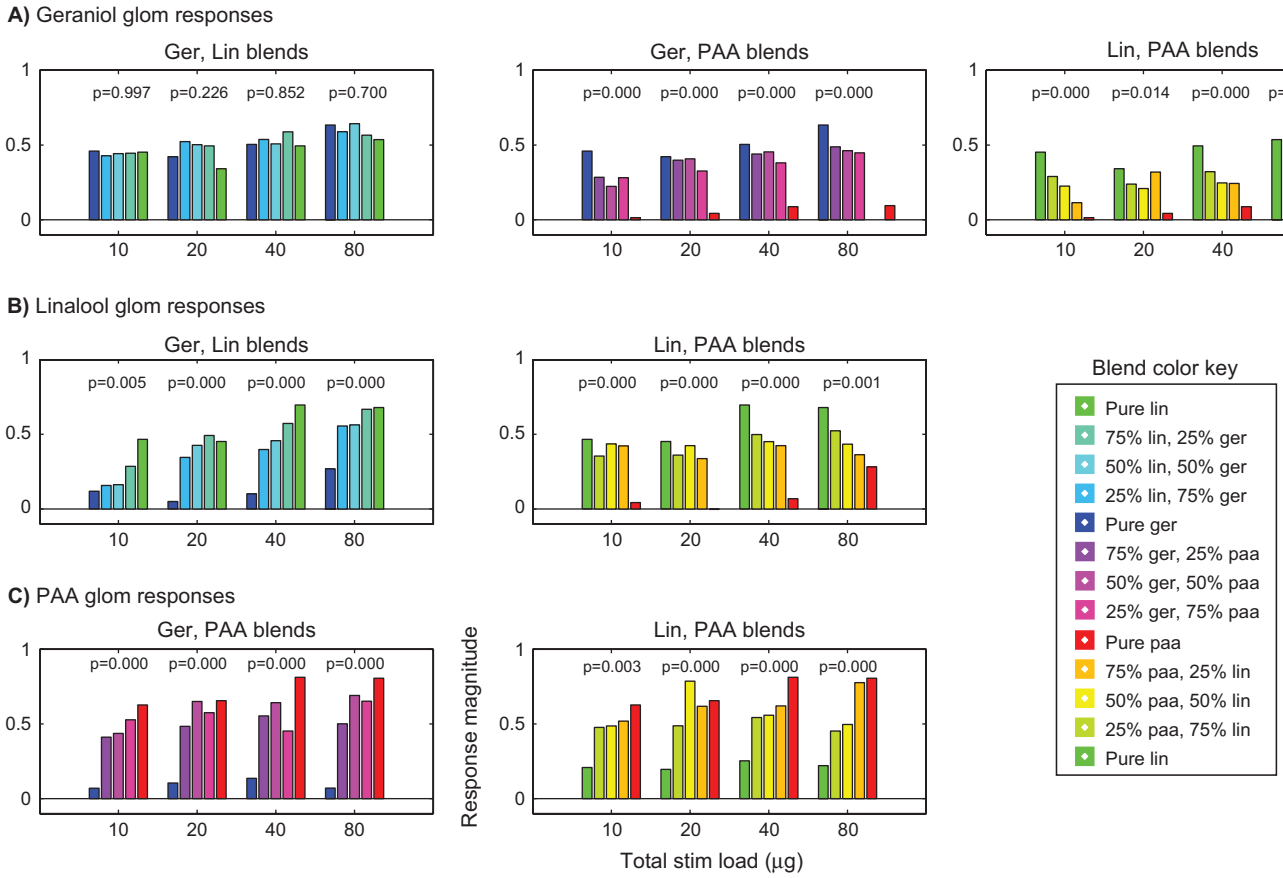


**Figure 3** Comparisons of the blend responses with the pure stimuli response. These bar graphs are color coded as in Figure 2, with green depicting pure linalool stimuli, blue geraniol, and red PAA. The panels are organized as in Figure 2, with the different identified glomeruli **(A)** the geraniol-best glomerulus, **(B)** the linalool-best glomerulus, and **(C)** the PAA-best glomerulus. Each small group of 5 bars are the responses to the odors of the same total stimulus load but at different ratios. These bars are organized such that left- and right-most bars of each 5-bar grouping are the pure odorants, and the 3 bars in between are the ratios graduating from one odorant to the other. The *P* values quoted for each group is of an ANOVA to test if the bars are statistically different. This arrangement allows one to observe 2 things: 1) whether the glomerulus responds differentially to the 2 odorants and 2) whether there are any cases of synergy. For all responses except for the geraniol glomerulus response to geraniol-linalool blends, it is clear that each glomerulus can distinguish between any 2 pure odorants at any load. The ratio responses generally fall on the slope connecting the responses of the pure odorants. ANOVA tests show that the difference between these responses overall are statistically significant. However, the geraniol glomerulus responses to geraniol–linalool blends (in a, left panel) are not statistically different for blends of the same load, and this is noticeable in the lack of slope within each 5-bar group because the responses are all of the same magnitude. Synergy can be seen here if the ratio responses exceed both the pure odorant responses at the same load. On the bar charts, this would mean any of the middle 3 bars being greater than the left- and right-most bars. One obvious example is the PAA glomerulus response to the blend 10 µg linalool plus 10 µg PAA (in c, right panel, 20 µg total stimulus, central bar), which exceeds both the pure linalool response at 20 µg and the pure PAA response at 20 µg. However, no responses to blends were significantly stronger than a response to the strongest component alone ($P > 0.05$).

though a compound does not activate the receptor itself, it may act as a competitive antagonist and block or inhibit the action of a responsive compound. Suppressive interactions in ORNs found in other experiments could be explained by the fact that one of the blend components excited the neuron, whereas the second component inhibited it. Such a dual function of ORNs has been observed in the pheromone-detecting subsystem in moths (Kaissling et al. 1989; Hansson et al. 1990).

Generally, ORNs are broadly tuned to a wide range of odor stimuli and have overlapping receptive fields. Thus, we might expect that chemically dissimilar odorants that stimulate non-overlapping subsets of receptors should display elemental properties, whereas similar odorants stimulating highly overlapping subsets of receptors would induce more interaction between the odorants, giving rise to configural properties in the representation. This is supported by behavioral studies that demonstrate the effects of odor component similarity on the ability of rats to distinguish blends from their components (Laing et al. 1989; Kay et al. 2003; Wiltrout et al. 2003). However, our results indicate that blends of either similar (geraniol and linalool) or dissimilar (geraniol and PAA; linalool and PAA) odorants are linearly represented at the first stage of olfactory processing.

Strong mixture interactions have been observed in the pheromone-processing subsystem in moths. Some output

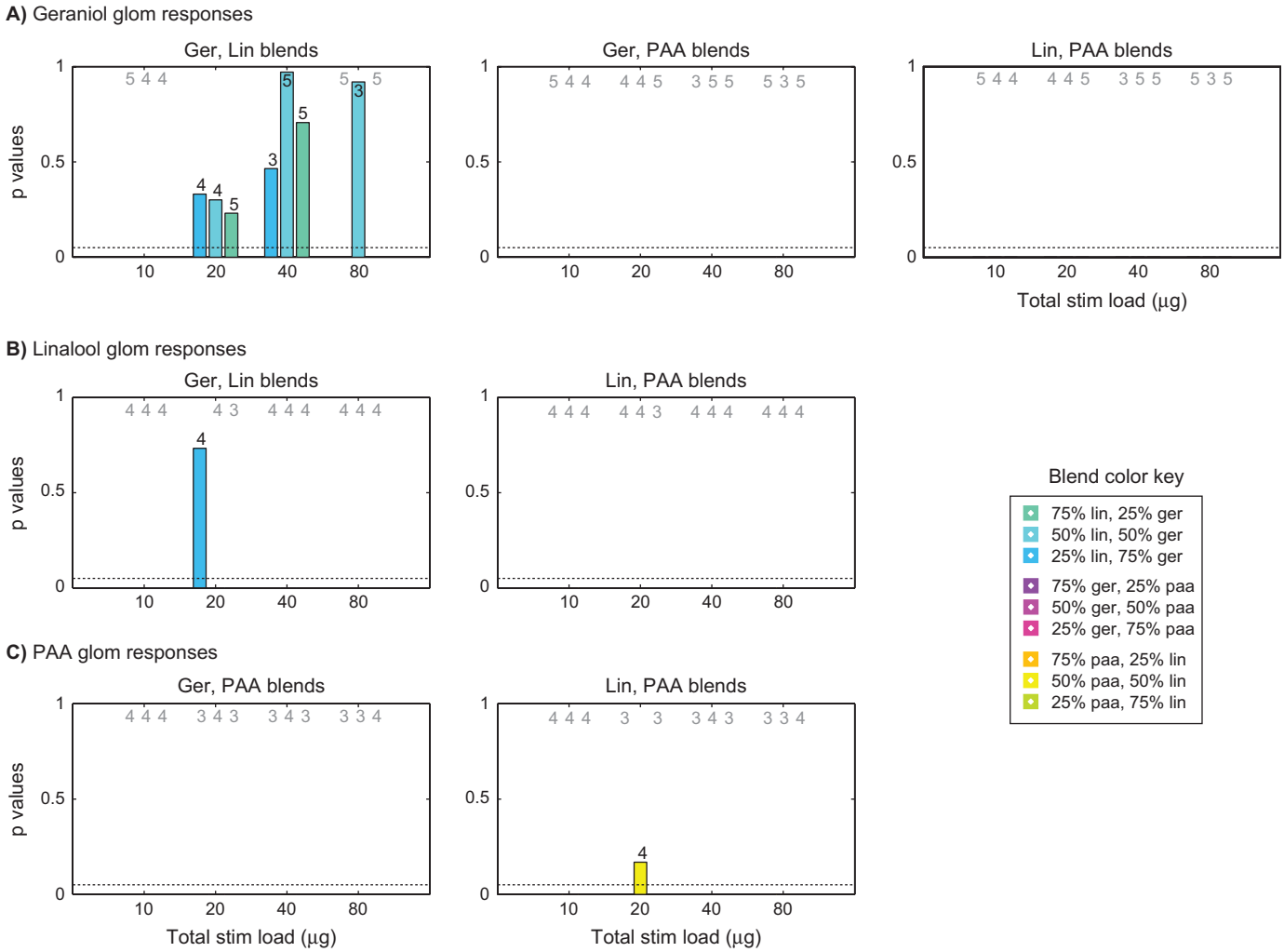


**Figure 4** Tests for synergy. Responses that satisfied the criterion for synergy were further assessed by Student's *t*-tests. **(A)** shows the results for the geraniol-best glomerulus, **(B)** linalool-best, and **(C)** the PAA-best glomerulus. The bars represent *P* values showing significance of the observed synergy where a *t*-test was performed. Where the responses did not satisfy the criterion for synergy, no bar was drawn. The dotted line indicates the 5% significance level. The numbers atop each bar are *n* values for the number of animals that provide data for that comparison. Where there was no test, and thus no bar, the *n* value is located at the top of the panels and shaded in gray. There were no *P* values that met the 5% significance level, and thus, no synergistic responses were significant.

neurons, projection neurons (PNs), from the male-specific cluster of glomeruli, the macroglomerular complex (MGC), have been reported to have blend-specific properties and respond exclusively to a species-specific blend and not to its individual components (Christensen and Hildebrand 1987; Christensen et al. 1989, 1991, 1995; Anton and Hansson 1995; Wu et al. 1996; Anton et al. 1997; Hartlieb et al. 1997). Such interactions have, however, not been reported from studies of ORNs (Akers and O'Connell 1988, 1991; Almaas and Mustaparta 1990; Berg and Mustaparta 1995; Carlsson and Hansson 2003). Thus, blend-specific responses in the pheromone subsystem are likely elicited by interglomerular computation within the MGC. Responses to mixtures of plant-related compounds in insects have been less well studied and the results diverge. De Jong and Visser (1988) showed that extracellular responses in certain ORNs in the Colorado potato beetle were suppressed when stimulated with binary mixtures containing general green leaf volatiles. Likewise, suppressive interactions were observed in single cockroach ORNs (Getz and Akers 1997). However, Akers and Getz (1993) found that responses to binary mixtures of aromatics and octyls in the honeybee were often stronger than would be predicted.

In honeybees, a blocking paradigm was used to show that conditioning could alter the perception of blends (Hosler and Smith 2000). In their experiment, preconditioning to one odorant diminished the strength of association between a reward and another odorant when animals were conditioned to associate the reward with the blend of both odorants. In order to associate the unconditioned stimulus with components of a blend rather than only the blend in full, the components must be identifiable from the blend. This shows

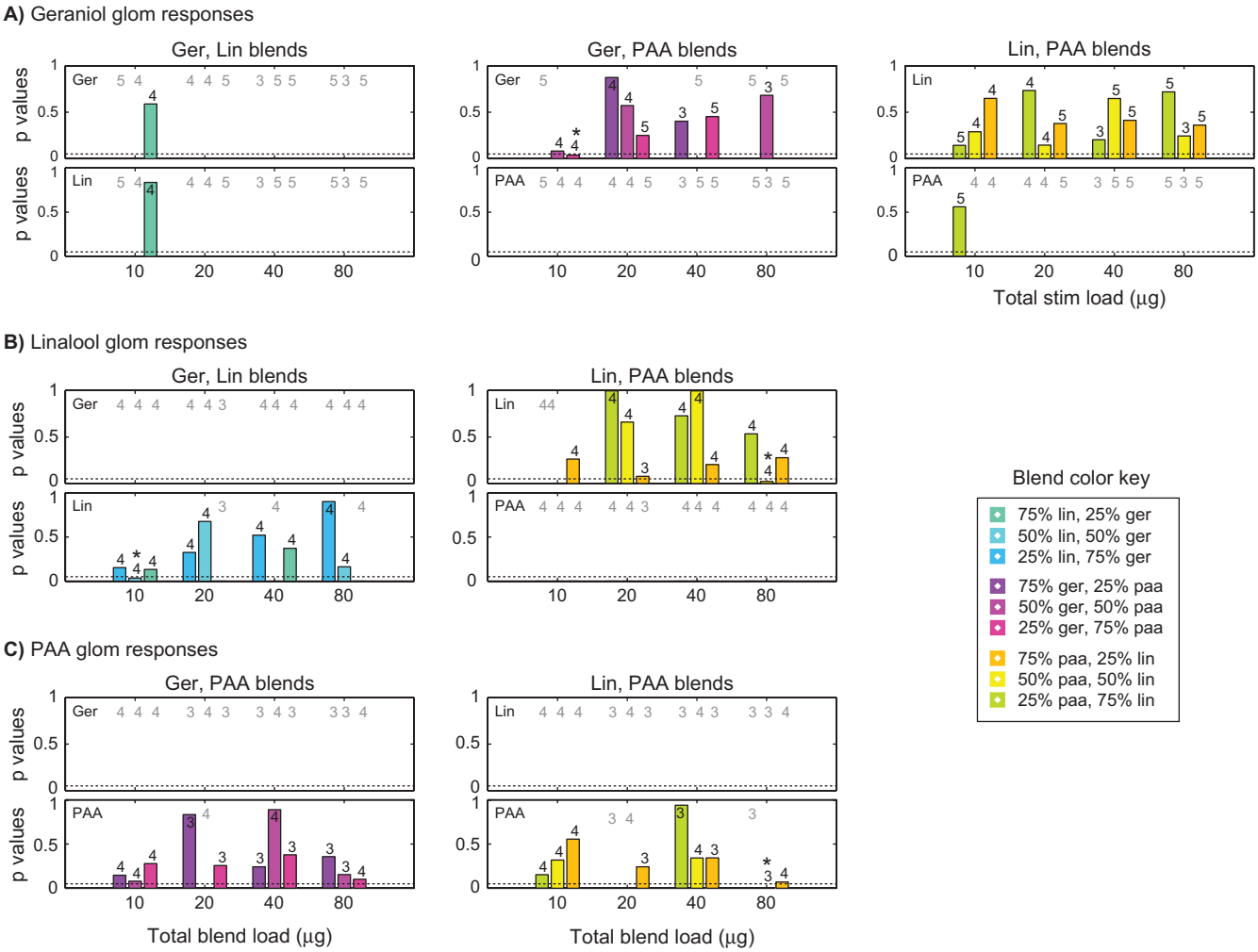


**Figure 5** Tests for suppression. Responses satisfying the criterion for suppression are tested for significance using Student's *t*-tests, with *P* values showing significance of the suppression plotted as bars. **(A)** shows the results for the geraniol-best glomerulus, **(B)** linalool-best, and **(C)** the PAA-best glomerulus. Responses not meeting the criterion for suppression have no bars. The numbers on each bar are *n* values as in Figure 4. Importantly, significant results (at the 5% level) are marked with asterisks to distinguish these results from responses with no bars. There were only 4 significant cases of suppression. However, when the Bonferroni correction is applied, none of the responses are significant (see text).

that mixture interactions can be altered by prior experience, so that components are recognized within a mixture. Therefore, it may be possible that an animal can exploit both configural and elemental coding paradigms depending on what it has learned from its environment. Associative learning of odors alters the synapses that drive PNs of the AL (Yu et al. 2004). With such plasticity at this level, it may be possible for animals to alter the role of these neurons between configural and elemental coding. For such a system, it would be necessary to preserve elemental information up to this stage of processing, as indicated by our data.

The infrequent and often weak (at moderate concentrations) mixture interactions observed at the ORN level may be necessary in a plastic system. That is, information should be reliably transferred to second-order neurons where collateral processes or efferent feedback may alter responses to specifically important mixtures after previous experiences. The more frequently observed blend interactions in pheromone-sensitive ORNs could be explained by the fact that the pheromone subsystem in moths is far less plastic (Hartlieb et al. 1999).

In summary, we did not find any significant mixture interactions between common plant compounds at biologically realistic concentrations at the AL input level. That is, the second component of a mixture, regardless if it is excitatory or neutral, does not alter the response to the mixture in an unpredictable manner. However, we find it likely that mixture interactions occur downstream of the ORNs. These interactions may be different in naive and experienced animals. In order to preserve the encoding of quality up to the second-order neurons, blend interactions in ORN should be weak or negligible.

We plan to study responses to the same odor panel used in the present experiment in both PN selective imaging (Carlsson et al. 2005) and in differential conditioning in order to elucidate if mixture interactions occur downstream of ORNs and how this would influence perception.

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