

Variation in complex olfactory stimuli and its influence on odour recognition

Geraldine A. Wright* and Brian H. Smith

Department of Entomology, Ohio State University, 318 West 12th Avenue, Columbus, OH 43235, USA

Natural olfactory stimuli are often complex and highly variable. The olfactory systems of animals are likely to have evolved to use specific features of olfactory stimuli for identification and discrimination. Here, we train honeybees to learn chemically defined odorant mixtures that systematically vary from trial to trial and then examine how they generalize to each odorant present in the mixture. An odorant that was present at a constant concentration in a mixture becomes more representative of the mixture than other variable odorants. We also show that both variation and intensity of a complex olfactory stimulus affect the rate of generalization by honeybees to subsequent olfactory stimuli. These results have implications for the way that all animals perceive and attend to features of olfactory stimuli.

Keywords: olfactory; honeybee; generalization; natural stimulus; odour recognition

1. INTRODUCTION

Olfactory stimuli are used by animals for many important tasks crucial to their fitness. Naturally occurring olfactory stimuli, such as floral perfumes or animal pheromones, are typically complex and often highly variable combinations of many chemical compounds (Dobson 1994; Laurent 2002; Pichersky & Gershenzon 2002). Each compound in an odour stimulus may differ from the others by several orders of magnitude in concentration (Dobson 1994; Levin *et al.* 2001). Differences in the ratios of concentration of these compounds can produce distinct perceptual changes that affect odour recognition (Laska & Hudson 1992; Olsson & Cain 2000). Odour recognition is made even more complex by the ephemeral nature of odour mixtures (Crimaldi *et al.* 2002). Odour stimuli emitted by the same type of 'object' often differ slightly from one encounter to the next, and this variability makes odour objects difficult to classify such that they can be recognized and distinguished.

The olfactory system of an animal must, therefore, form a memory representation for a class of objects, such as the odour of a flower species that presents nectar to a honeybee, which accounts for variability across objects and allows for identification of all objects that mean the same thing. Several behavioural mechanisms have been identified that may be involved in the extraction of features that can be used to classify stimuli. The features that olfactory stimuli have in common, such as the number and types of odorants present and their concentrations, are likely to be important in classifying stimuli (Chandra & Smith 1998; Hosler & Smith 2000; Wise *et al.* 2000; Laurent 2002; Wiltrout *et al.* 2003). Specific features that dominate a mixture, however, such as an odorant that is present at a higher concentration than other odorants, could perceptually overshadow other features. In addition, studies of olfactory blocking indicate that olfactory systems of a variety of animals are capable of perceptually isolating individual odorants or submixtures from a blend (Smith

1998; Hosler & Smith 2000; Giannaris *et al.* 2002) when the individual odorants were more reliably associated with reinforcement than the other odorants present.

Our study addresses how variability across experiences with an odour stimulus affects the subsequent identification of odour objects. Trial-to-trial variability could lead to consolidation of an olfactory memory that reflects an average of all experiences of odour stimuli. However, we predicted that the subsequent identification of odour objects, in our case odour mixtures, would reflect features that are most reliably associated with reinforcement. We conditioned honeybees to mixtures of odorants that varied qualitatively from trial to trial as they were reinforced with an appetitive reward, and then tested them with each odorant present in the mixture. Generalization from conditioning with a mixture to each of the test odorants was interpreted as a measure of how similar the test odorant was to the mixture.

2. MATERIAL AND METHODS

Worker honeybees (*Apis mellifera*) were collected from colonies at the Rothenbuhler Honeybee Laboratory, Columbus, OH, USA. Each subject was placed individually in a restraining harness as described by Smith (1998). All experiments used the proboscis extension response assay to evaluate the olfactory learning behaviour of individual subjects (see Smith (1998) for details). Odour stimuli were delivered by placing 5 µl of odour solution on a small strip of filter paper that was then placed in a modified, 1 ml tuberculin glass syringe attached to an air supply and controlled by a solenoid valve (see Smith (1998) for details).

Our odour stimuli were mixtures of pure monomolecular odorants at specific molarities created using hexane as a solvent. To examine the effect of qualitative variables on generalization from mixtures to the odorants present, we developed two types of odour mixtures: mixtures composed of perceptually 'similar' odorants, and mixtures composed of perceptually 'dissimilar' odorants (Stopfer *et al.* 1997; Laska *et al.* 1999; Daly & Smith 2000). The similar odours, 1-hexanol, 1-heptanol and 1-octanol (Sigma, 99.0% + purity), all aliphatic alcohols, differed only with respect to their carbon chain length. The dissimilar odours,

* Author for correspondence (wright.571@osu.edu).

1-hexanol, geraniol and 2-octanone (Sigma, 99% + purity) differed with respect to their alkyl groups and carbon chain lengths. All of the similar and dissimilar compounds are volatile, oxygenated hydrocarbons commonly encountered in floral perfumes (Knudsen *et al.* 1993).

To examine the effect of quantitative variables on generalization, we also varied the concentration of each odorant in the mixture. Individual odorants were presented at one of three possible levels: 0.0002 M (low), 0.02 M (intermediate) and 2.0 M (high). Each concentration was chosen based on its average detectability and discriminability. The low-level odorants were detectable above a solvent background both as conditioning stimuli and as stimuli in an electroantennogram (EAG) assay (Wright & Smith 2004). The high-level odorants were chosen based on their EAG responses relative to the other two odorant levels and the response of the antenna to undiluted odorant (Bhagavan & Smith 1997; Wright & Smith 2004).

(a) *The contribution of individual odorants*

The first experiment was designed to examine if perceptual qualities of mixtures are dominated by the odorants that were reliably associated with reinforcement. In our experiments, this was the odorant present with the least variability in concentration throughout conditioning. To accomplish this, we held one odorant of a mixture at a constant low or high concentration (either 0.0002 M or 2.0 M) while varying the other odorants from trial to trial at the low (0.0002 M) or the high (2.0 M) concentration. Thus, four different mixtures were used for each constant odour (e.g. for the low constant: \underline{L} :L:L; \underline{L} :H:L; \underline{L} :L:H; \underline{L} :H:H, where L is low concentration and H is high concentration, and the underlined letter is the constant odour). Each subject was trained with a pseudo-random array of these four mixtures over 16 trials and was exposed to each mixture four times during training.

Eight different combinations of training and testing conditions were used in separate groups of subjects. Mixtures were composed of either similar or dissimilar odours. Within each mixture composition, subjects were trained with the constant odorant at either the low or the high concentration. Finally, each of the four mixture groups was tested with either the low or the high concentration of each of the odorants.

An additional control group was also performed with the similar and dissimilar mixtures to test whether it was the average concentration during conditioning that affected generalization, or whether it was the variability in concentration. First, one mixture held all odorants at a constant, intermediate concentration (0.02 M) across all 16 acquisition trials. Second, a mixture was used in which 1-hexanol was maintained at a constant (intermediate) 0.02 M level, but the remaining odorants varied at either 0.0002 M or 2.0 M. In the latter mixture the average level of each odorant was 0.02 M, but the odorants varied in concentration from trial to trial. After conditioning, each subject was tested with the low concentration of each of the odorants of the mixture.

(b) *Variation versus mixture intensity*

This experiment was designed to test how variability in mixture intensity and the absolute level of mixture intensity affected generalization to low-concentration odorants. Intensity of a single mixture was gauged by summing the respective molarities of each of the odorants. Variability in the intensity of the mixture was generated by causing, as before, the concentration of *all* of the odorants of a mixture to vary from trial to trial during acquisition.

The mean intensity level of variable mixtures was calculated by averaging the intensity of each mixture over all the trials. The coefficient of variation (CV; $[s.d. \times 100]/\text{mean}$; Sokal & Rohlf 1995) was then calculated from the average intensities over all the trials. Both the similar and dissimilar odour mixtures were used.

Two levels of variability were used in different treatment groups of subjects: constant (CV = 0) and variable (CV = 72 for the mid-level intensity condition and 75 for the high). Two levels of mean intensity were used: mid (0.03 M in the variable condition and 0.06 M in the constant condition) and high (2.0 M in the variable and 2.1 M in the constant). To produce an average concentration with integer levels of odorants in a mixture, our mixture intensities resulted in a range that varied from trial to trial. We used intermediate (0.02 M) and low (0.0002 M) concentrations of each odorant to produce a mean intensity of 0.03 M for the mid-level, and we used intermediate (0.02 M) and high (2.0 M) concentrations to produce a mean intensity of 2.0 M for the high level. We were unable to use the original odorant concentrations to produce a constant mean intensity at the high level with no variation. We therefore chose a concentration of 0.7 M for each odorant. The variable mixtures had a range of 0.0006–0.06 M for the low intensity and 0.02–4.02 M for the high intensity. After acquisition, subjects were tested with the low-level odorants of the mixtures as described in the previous experiment.

(c) *Statistical analyses*

For all of the experiments, the responses of subjects were scored as binary variables, and we used multivariate logistic regression to test all hypotheses (Agresti 1996).

3. RESULTS

In all of our experiments, we conditioned subjects to respond to a mixture of three odorants, and we then tested each of the odorants present in the mixture. The rate of generalization to the test odorants was interpreted as a measure of the perceptual similarity between the mixture and the test odorants. Differences in generalization across treatment conditions were interpreted as an index of the way that our experimental conditions affected consolidation of memory for the mixture.

(a) *A mixture was more similar to a constant odorant than to a variable odorant*

We evaluated whether the constant odorant was responded to with a greater probability than the other odorants by comparing the differences in mean response levels to the constant and variable odorants. The result depended on the mixture type (figure 1). For similar mixtures, responses to constant and variable odorants were not significantly different, regardless of whether the subjects had been trained to either the low-constant odorant or the high-constant odorant (logistic regression: $\chi^2_1 = 0.60$, $n = 178$, $p = 0.434$; figure 1*a,c*). For the mixtures of dissimilar odorants, the response depended on the level of the constant odour during conditioning and the level of the test odorants (logistic regression: $\chi^2_1 = 17.6$, $n = 226$, $p < 0.001$; figure 1*b,d*). Subjects generalized significantly more to the constant odorant than to the variable odorants, except for the condition in which subjects were tested with high levels of odorants after conditioning

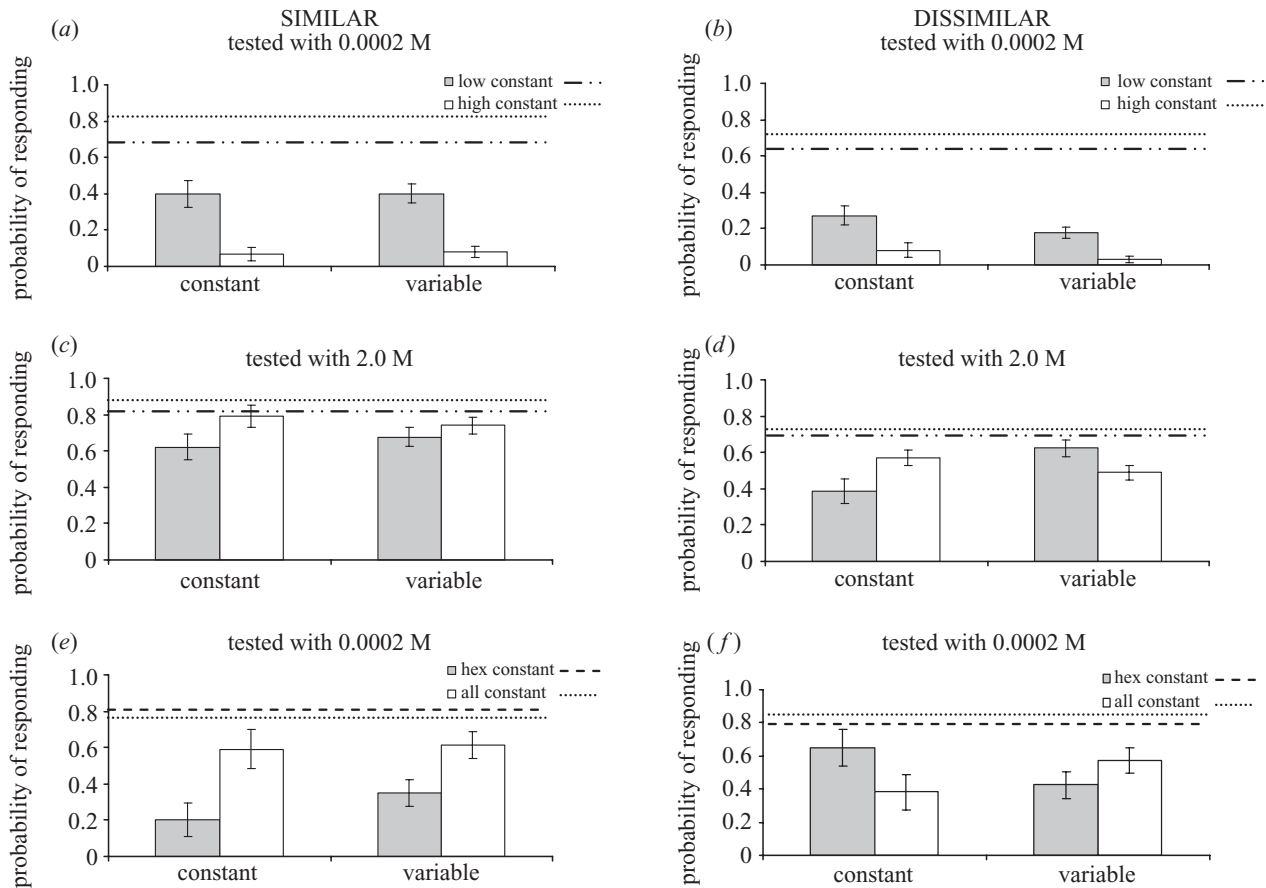


Figure 1. Generalization from odour mixtures to each odorant present in the mixture. (a,c) Subjects responded with equal probability to all the odorants of similar mixtures. (b,d) Subjects conditioned with dissimilar mixtures responded to the constant odorant if they were tested with the same concentration as the constant odorant. (e,f) The average concentration of an odorant during training affected generalization less than variability for subjects conditioned with dissimilar mixtures. The dotted and dashed lines represent the probability of responding to each mixture on the 16th trial of conditioning.

with a low-constant odorant (logistic regression: $\chi^2_1 = 6.91$, $n = 226$, $p < 0.01$; figure 1d). In this case, subjects generalized significantly more to the variable odorants.

The control group revealed that the variability in an odorant's concentration influenced generalization more than the odorant's average intensity. Subjects conditioned to mixtures of similar odorants generalized to each test odorant with equal probability regardless of variability in concentration (logistic regression: $\chi^2_1 = 1.39$, $n = 42$, $p = 0.237$; figure 1e). For the mixtures of dissimilar odorants, the response to a constant odorant was greater than the response to the variable odorants (logistic regression: $\chi^2_1 = 4.62$, $n = 41$, $p = 0.03$; figure 1f).

(b) Constant odorant concentration and mixture type influenced generalization

The average rate of generalization from mixtures to all of the test odorants depended on the difference in the concentration of the constant odorant during initial training and the concentration of the test (figure 1). For example, when tested with low-concentration odorants (figure 1a,b), subjects trained with the constant odorant at the low concentration (shaded bars) responded significantly more than subjects conditioned to mixtures that contained a high-constant odorant (logistic regression: $\chi^2_1 = 7.06$, $n = 217$, $p < 0.01$). Furthermore, the average response

levels to all of the test odorants were greater for the similar mixtures than for the dissimilar mixtures (logistic regression: $\chi^2_1 = 17.0$, $n = 217$, $p < 0.001$).

Generalization to high concentration test odorants increased for both types of odorants, though dissimilar odorants had a lower rate of generalization (logistic regression: $\chi^2_1 = 16.6$, $n = 187$, $p < 0.001$; figure 1c,d). Subjects generalized significantly more to a high-constant odorant when conditioned to a mixture that contained a high-constant odorant than when conditioned to a mixture with a constant odorant at a low concentration (logistic regression: $\chi^2_1 = 139$, $n = 187$, $p < 0.001$). The opposite pattern was observed for variable odorants.

(c) Mixture variability increases generalization to odorants

The average rate of generalization to all the test odorants was a function of mixture intensity and variability in mixture intensity. In all four cases, the variable mixtures had greater rates of generalization to test odorants than constant mixtures (logistic regression: $\chi^2_1 = 59.7$, $n = 229$, $p < 0.001$; figure 2). Furthermore, when subjects were conditioned to mid-level (0.03–0.06 M) intensity mixtures, they responded with a greater probability to the test odorants than subjects conditioned with the high-level (2.0–2.1 M) intensity mixtures (logistic regression: $\chi^2_1 = 97.9$, $n = 229$, $p < 0.001$).

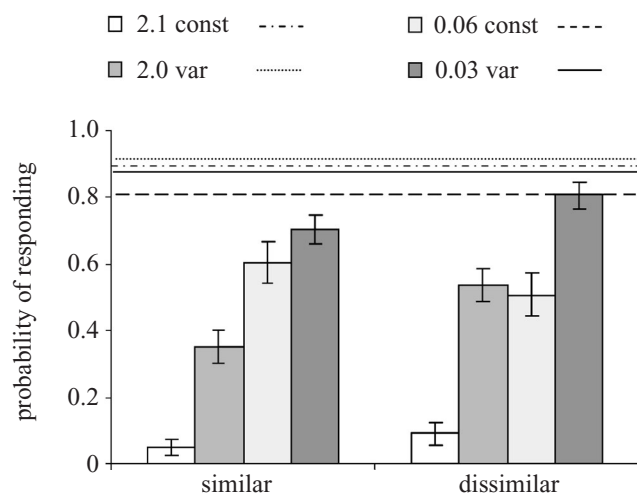


Figure 2. The average response to all the odorants at the low concentration during the test with 0.0002 M odorants (for each group, the leftmost bar is 2.1 M and the rightmost bar is 0.03 M). When subjects were conditioned with high-intensity mixtures (2.0 M and 2.1 M) they responded less than subjects conditioned with low-intensity mixtures (0.03 M and 0.06 M). Subjects conditioned with highly variable mixtures (var) responded with a higher probability than subjects conditioned with no variation present in the mixture (const). The dotted and dashed lines represent the probability of responding to each mixture on the 16th trial of conditioning.

The effect of variability depended on the intensity of the mixture (logistic regression: $\chi^2_1 = 12.6$, $n = 229$, $p < 0.001$; figure 2). The increase in generalization from constant to variable mixture treatments was greater at low than at high intensities. Generalization was also slightly higher for dissimilar mixtures (logistic regression: $\chi^2_1 = 4.86$, $n = 229$, $p = 0.028$; figure 2b), but the relative patterns across treatments were the same.

4. DISCUSSION

Our results indicate that quantitative variation in a complex, olfactory stimulus during conditioning affected generalization to subsequently experienced odours in two ways. First, individual odorants that were at a constant concentration in a mixture became more associated with the mixture's identity, whereas variable odorants become less representative. Second, in mixtures where all the odorants were variable, generalization to all subsequent olfactory stimuli was greater than it was for mixtures in which all odorant concentrations were constant. Conditioning with high-intensity stimuli decreased generalization to low-concentration test odorants, as has been shown previously (Marfaing *et al.* 1989; Bhagavan & Smith 1997; Pelz *et al.* 1997; Sakura *et al.* 2002; Cleland & Narla 2003; Wright & Smith 2004). Variability modified the effect of stimulus intensity by increasing generalization to the low-concentration test odorants.

(a) *A constant odour influences odour object classification*

When a subject experienced an odour mixture in our experiments, what it learned about the odour was affected by quantitative variation in each of the odorants present

in the mixture during conditioning. We hypothesize that the increase in the contribution of the constant odorant to a mixture's identity was a function of its more reliable association with reinforcement. Blocking protocols have also demonstrated that the odorants present in a mixture, which are the most reliably associated with reinforcement, gain salience over the other odorants present (Smith 1998; Hosler & Smith 2000). Blocking has been shown to occur when an odorant with a pre-conditioned association with reinforcement is experienced subsequently in a binary mixture. The pre-conditioned odorant often overshadows the presence of another odorant such that the latter is not learned as well as it would have been if no pre-conditioning had taken place (Rescorla & Holland 1982; Rescorla 1988; Pearce 1994). The increase in salience of the pre-conditioned odorant is hypothesized to be mediated by lateral inhibition in the olfactory bulb or antennal lobe, the first synaptic relay for sensory input from the olfactory receptor neurons (Linster & Smith 1997; Urban 2002).

(b) *Variation increases generalization*

Theories of generalization propose that animals must generalize from a specific instance to subsequent experiences because no two presentations of stimuli can be exactly alike (Pavlov 1927; Kalish 1970; Shepard 1987; Pearce 1994). From a behavioural standpoint, generalization arises from two different mechanisms. First, animals might generalize across stimuli that they can easily discriminate because they have experienced the same reinforcement for each stimulus (Shepard 1987; Pearce 2002). Second, generalization could also arise from a failure of the sensory system to distinguish among stimuli (Shepard 1987; Pearce 1994).

When generalization occurs to different stimuli that have been classified together according to their common association with reinforcement (Kalish 1970; Shepard 1987), features that are common to the reinforced stimuli are used to classify novel stimuli. For the conditions in which all the odorants in a mixture varied in concentration, subjects generalized more to the test odorants than subjects who experienced no variation during training. Thus, increasing variation in the stimulus also increased generalization to all subsequent odorant stimuli. These results support the hypothesis that our subjects were generalizing to a class of odour 'objects' and that the extent of the generalization was modified by variability in the stimulus. We show that the features used to classify odour stimuli in the absence of variation are different from the features used to classify stimuli when variability is present.

Differences in generalization from the conditioning to the test odorants in our study may also have arisen due to the type of odorants present in the mixtures. Subjects conditioned to similar mixtures had higher mean rates of generalization than mixtures of dissimilar odorants, and they showed equal generalization to all of the odorants regardless of the level of variability in concentration. This may have occurred because the odorants present in the similar mixtures were perceptually similar and therefore more difficult for bees to differentiate (Stopfer *et al.* 1997; Laska *et al.* 1999). Each odorant may have appeared less variable, causing the effects of variability in the similar mixtures to be dampened.

(c) *Stimulus intensity modulates the effect of variability*

Furthermore, a subject's ability to generalize to low-level odorants was also affected by the intensity of the stimulus during training. If subjects had been trained to a high-intensity stimulus, their response to the low-concentration test odorants significantly decreased. This change in sensitivity from high to low could be facilitated by either of two mechanisms: adaptive gain control or adaptation of the neurons in the antennae and/or antennal lobe.

Adaptive gain control adapts a sensory system to variability in a stimulus by amplifying the stimulus in inverse proportion to its range of intensity (Fairhall *et al.* 2001). When the range is narrow, the sensory system broadens the intensity-response function to carry as much information as possible about the stimulus within that range. In our study, this mechanism could be tuning the olfactory system to odorants that are present at a specific concentration dependent upon the subject's exposure to olfactory stimuli of a given intensity and the level of variability present during conditioning.

Alternatively, adaptation of the neurons of the antennal lobe would also change the sensitivity of the olfactory system (Potter & Chorover 1976; Mair 1982; Devaud *et al.* 2001). If the neurons in the antennal lobe were adapted, exposure to high-intensity stimuli would change the response range such that the olfactory system would respond only to high-intensity stimuli and not to low-intensity stimuli. Adaptation, however, could not account for the change in the response due to the variability of the conditioning stimulus. Which of these two mechanisms caused a reduction in the response to low-concentration odorants after conditioning with high-intensity stimuli remains unclear from our data.

5. CONCLUSIONS

Quantitative variation of the odorants present in an olfactory stimulus affects how animals form olfactory memories. Decreasing the variation of an individual odorant in an odour mixture causes it to gain salience; increasing the variation in *all* the odorants produces greater generalization. These results indicate that consolidated odour memory depends on variation in stimuli across experiences. This odour memory does not simply reflect the across-trial average values of odorants in a mixture. Rather, the memory seems to be more inclusive of stimuli that were a part of the mixture as a result of variation. Future studies of sensory processing in the antennal lobe may provide insight into the neural mechanisms underlying these results.

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