

# ODORANT INTENSITY AS A DETERMINANT FOR OLFACTORY CONDITIONING IN HONEYBEES: ROLES IN DISCRIMINATION, OVERSHADOWING AND MEMORY CONSOLIDATION

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## Summary

Stimulus intensity is an important determinant for perception, learning and behaviour. We studied the effects of odorant concentration on classical conditioning involving odorants and odorant-mechanosensory compounds using the proboscis-extension reflex in the honeybee.

Our results show that high concentrations of odorant (a) support better discrimination in a feature-positive task using rewarded odorant-mechanosensory compounds *versus* unrewarded mechanosensory stimuli, (b) have a stronger capacity to overshadow learning of a simultaneously trained mechanosensory stimulus, and (c) induce better memory consolidation. Furthermore, honeybees were trained discriminatively to two different concentrations of one odorant. Honeybees are not able to

solve this task when presented with rewarded low *versus* unrewarded high concentrations.

Taken together, our results suggest that high concentrations of odorant support stronger associations (are more 'salient') than low concentrations. Our results, however, do not indicate that honeybees can treat two different concentrations of one odorant as qualitatively different stimuli. These findings fill a gap in what is known about honeybee olfactory learning and are a first step in relating behaviour to recent advances in the physiological analysis of coding for odorant concentration in honeybees.

Key words: olfaction, mechanoreception, Pavlovian conditioning, stimulus intensity, honeybee, *Apis mellifera carnica*, learning, memory, salience.

## Introduction

The uncertainty in natural environments together with the high variability of environmental stimuli pose two fundamental problems for all animals.

First, animals must find valid predictors for important objects and events, such as food, mates, danger or shelter. In terms of classical conditioning, animals must thus determine which of the huge number of co-occurring stimuli are to be associated. To describe the ease with which different associations can be formed, psychologists developed the concept of stimulus 'salience' (e.g. Rescorla and Wagner, 1972; Sutton and Barto, 1990). This term refers to a stimulus-specific constant independent of previous experience. This constant is thought to determine the rate at which neutral conditioned stimuli (CSs) can enter into associations with rewarding (or punishing) unconditioned stimuli (USs) (see, however, Durlach, 1989; Spear *et al.* 1990, for a discussion of retrieval-based models). The first part of the present study deals with the putative effects of the intensity of a CS upon its salience.

Second, animals face the problem that a stimulus rarely occurs twice in an identical way. Thus, a valid predictor must

be recognized despite this lack of identity. One important concept that has been developed to analyze such processes is stimulus generalization (Pearce, 1987). Broad generalization, however, can also present animals with difficulties (Smith, 1993): they might erroneously respond to stimuli which, despite being similar to predictors, can themselves not serve predictive functions. Thus, there must be a trade-off between generalizing to non-identical presentations of learned stimuli on the one hand and discriminating between similar but different stimuli on the other hand. This issue is also important with respect to coding of, and learning about, different intensities of one stimulus. For olfaction, which is the stimulus modality used in the present study, Laurent (1996) identified the issue of whether the quality of an odorant is coded in an intensity-invariant manner. An issue related to this point is addressed by the second part of this study.

Qualitative intensity invariance does not have to be assumed: olfactory stimuli are coded as spatio-temporal activity patterns ('across-fibre patterns'). At the level of the primary olfactory neuropile (the olfactory bulb in vertebrates

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or the antennal lobe in insects), neural activity patterns show enhanced overall activity levels and slight qualitative changes with increasing odorant concentration (Cinelli *et al.* 1995; Joerges *et al.* 1996). It seems possible that such subtle changes in the activity pattern might result in a perceptual shift of odorant quality (Gross-Isserhoff and Lancet, 1988). With respect to olfactory associative learning in the honeybee, we thus investigated whether intensity information can be used to respond discriminatively to, for example, only low but not high concentrations of an odorant. That is, can animals treat two concentrations of one odorant as two qualitatively different stimuli?

We use the honeybee as a model system since it offers the rare opportunity to relate behaviour to findings from physiology and biochemistry (Joerges *et al.* 1996; Hammer, 1993; Laurent, 1996; Mauelshagen, 1993; Müller, 1996; Müller and Hildebrandt, 1995; Smith and Cobey, 1994). We use an olfactory classical conditioning paradigm of an appetitive reflex in restrained honeybees, the proboscis-extension reflex (Bitterman *et al.* 1983; Hammer and Menzel, 1995; Menzel and Müller, 1996). To vary stimulus intensity, odorant concentration was varied. The first three experiments investigate the roles of odorant intensity in discrimination, overshadowing and memory consolidation, and the final experiment involves differential conditioning to two concentrations of one odorant.

## Materials and methods

### Preparation of honeybees

Using standard methods (Bitterman *et al.* 1983), honeybees [*Apis mellifera carnica* (Pollm.)] were caught in the afternoon as they departed from an outdoor colony, were cooled to immobilize them and were fixed in metal harnesses which allowed movement of the antennae and mouthparts, including the proboscis. Some honeybees were obtained from a colony sited in an indoor flight room (van Praagh, 1972).

After recovery from cooling (approximately 10 min), honeybees were fed with two droplets (approximately 5 µl) of 25–30% sucrose solution. They were then kept overnight at room temperature (18–20 °C) in a dark and humid box. This procedure was used to ensure that all honeybees were in approximately the same motivational state at the start of the experiment.

10 min prior to experiments, honeybees were tested for the unconditioned response: extension of the proboscis immediately after one antenna was touched with a droplet of 20–30% sucrose solution. An unconditioned response (and later also a conditioned response) was scored if the tip of the proboscis crossed the line between the opened mandibles. Only honeybees that showed this unconditioned response were used for experiments.

In all experiments, we discarded honeybees that showed spontaneous responses towards the odorant stimuli subsequently used in conditioning experiments. This is because spontaneous responses might be indicative of memories

established during foraging prior to experiments (Gerber *et al.* 1997).

### Conditioned and unconditioned stimuli

The olfactory conditioned stimuli (CSs) used were citral (97%, Fluka), geraniol (purchased pure from a local pharmacy), pure (+)-limonene (Sigma) and linalool (95–97%, Sigma). These monoterpenoids are components of floral odours; citral and geraniol are also components of the honeybee Nasonov pheromone (Pickett *et al.* 1980). The solvent was mineral oil (Sigma) in the first three experiments and paraffin oil (purchased at a local pharmacy) in the last experiment. Mineral oil is not effective as a chemosensory stimulus (Akers and Getz, 1992). For paraffin oil, we carried out the appropriate control experiment (see Fig. 4D).

A mechanosensory stimulus was also used in the first three experiments. This was an air puff stimulus and is described below in more detail.

The unconditioned, rewarding stimulus (US) used was a 25–30% sucrose solution delivered by touching one antenna with a droplet of this solution, a stimulation that elicits proboscis extension. The honeybees were then allowed to feed on the droplet for 2 s. The total procedure lasted for approximately 3 s.

### Conditioning and test trials

All conditioning and test trials lasted for 1 min. During rewarded trials, the conditioned stimuli had a duration of 2 s and were applied 45 s after the beginning of a trial. The sucrose reward was delivered upon CS offset and lasted for approximately 3 s. Honeybees were moved back to their resting positions remote from the experimental site after an additional 10 s. The inter-trial interval was 10 min, except where stated otherwise. During unrewarded and test trials, the reward was omitted.

### Odorant delivery

Two different odorant application devices (A and B) were used. Device A delivered a scented air puff, whereas device B delivered a continuous air flow which could be shunted through an odorant cartridge. Thus, stimulation with device A can be viewed as a compound stimulus with a chemosensory (odorant, O) and a mechanosensory (air puff, M) component. This compound stimulation was used in the first three experiments. In the final experiment, stimulation device B was used to remove the effects of the mechanosensory component by adaptation.

Device A consisted of a 60 ml syringe loaded with a small glass vial containing 300 µl of the odorant at its respective dilution. To ensure constant concentrations of the odorants in the gaseous phase, room temperature was maintained between 19 and 22 °C. The concentrations described below refer to the concentration in the liquid phase, not to the concentration in the gaseous phase. After each trial, the syringe was replaced, and at least 10 min was allowed to elapse before a syringe was re-used.

For stimulation, the plunger of the syringe was moved forwards automatically by a pneumatic apparatus, resulting in an air puff with a volume of 35 ml. The outlet of the syringe was positioned 2.5–3 cm in front of the honeybee, and the odorant was removed by an exhaust system mounted behind the honeybee. For the application of a mechanosensory stimulus alone, the glass vial inside the syringe was filled with solvent with no added odorant.

In device B, a valve system allowed a continuous air flow from one of six brass cartridges to be directed towards the honeybee; the air flowing through the other five cylinders was shunted directly to the exhaust system. These cartridges contained glass vials filled with odorant [three cylinders: 100 % (no solvent), 10 %, 1 % (v/v)] or solvent (one cylinder) or were empty (two cylinders). One of the empty cylinders was used to apply a continuous air stream. Since honeybees were placed at the experimental site 45 s prior to CS onset, the presence of a continuous air stream is likely to remove the effects of mechanosensory stimulation due to sensory adaptation. A 'blank' stimulus was applied by switching between the two empty cartridges.

For both devices, the syringes, cartridges and odorant dilutions were freshly prepared every day.

### Experimental procedures

#### Experiment 1: testing for detectability and discrimination

This experiment was conducted to test whether the odorant concentrations used were detectable to honeybees. A feature-positive discrimination task was employed, with the odorant as the discriminative positive feature. The concentration of the odorant (linalool) was varied from 1 % (v/v) to 0.001 % (v/v) (Table 1). Linalool was chosen because it is known not to be a component of honeybee pheromones and because screening experiments suggested that it has a standard salience and perceptual threshold.

Table 1. Odorant concentrations and discrimination protocol for experiment 1, investigating the detectability range and discrimination ability of honeybees

Odorant concentration (%, v/v)	CS+	CS–
1	O M	M
0.1	O M	M
0.01	O M	M
0.001	O M	M
Pseudodiscrimination control	M	M

Animals received seven rewarded and six unrewarded trials in alternating order and with an inter-trial interval of 10 min.

CS+ indicates the rewarded and CS– the unrewarded conditioned stimulus. The odorant used was linalool.

O, olfactory stimulus applied at the specified concentration; M mechanosensory stimulus (air puff).

Results from these experiments are presented in Fig. 1.

For feature-positive discrimination, a compound olfactory and mechanosensory stimulus (OM) was rewarded (CS+), whereas the mechanosensory stimulus alone (M) was not (CS–) (Table 1). Successful performance in such a task indicates that the concentration lies within the detectable range. Furthermore, the degree of discrimination can determine the effects of odorant concentration upon discrimination ability. Thus, the critical question is whether discrimination ability increases with odorant concentration.

Since differences in the response levels to CS+ versus CS– stimulation could be due to unintentional handling differences (holding the reward in front of the honeybee before its application, etc.), we included a pseudodiscrimination control group, in which honeybees were trained to a rewarded mechanical stimulus versus an unrewarded mechanical stimulus (see Table 1).

Training started with presentation of the rewarded stimulus and proceeded with alternating unrewarded and rewarded trials. Honeybees received seven rewarded and six unrewarded trials. Since spontaneous responders on the first rewarded trial were excluded from further experimentation (see above), statistical analyses were carried out on data from the last 12 trials only.

#### Experiment 2: testing for concentration-dependent overshadowing

To investigate whether the concentration of an odorant affects its salience as a conditioned stimulus, we used the 'overshadowing' paradigm (Pavlov, 1927). Overshadowing means that the response level to a certain stimulus (X) is higher when animals are conditioned to that stimulus alone than when they are conditioned to a compound stimulus XY. That is, the presence of stimulus Y overshadows learning about stimulus X in the XY compound. The degree to which stimulus Y can overshadow learning of stimulus X is generally thought to be directly proportional to the salience of stimulus Y (Rescorla and Wagner, 1972; Sutton and Barto, 1990).

We used an inter-modal compound stimulus consisting of a

Table 2. Odorant concentrations and experimental groups for experiment 2, investigating the effects of odorant concentration on overshadowing in honeybees

Odorant concentration (%, v/v)	Training stimuli	Test stimulus
1 (high)	O M	M
0.01 (low)	O M	M
Control	M	M

Animals received only one training and one test trial. The interval between training and test trials was 10 min. Four different odorants were used: linalool, limonene, citral and geraniol.

O, olfactory stimulus applied at the specified concentration; M mechanosensory stimulus (air puff).

Results from these experiments are presented in Fig. 2.

Table 3. *Odorant concentrations and experimental groups for experiment 3, investigating the effects of odorant concentration on memory consolidation in honeybees*

Odorant concentration (% v/v)	Training stimuli	Test stimuli	Interval between training and test (min)
1 (high)	O M	O M	1
0.01 (low)	O M	O M	1
1 (high)	O M	O M	10
0.01 (low)	O M	O M	10

Animals received only one training and one test trial. The interval between training and test trials is indicated in the right-hand column. Four different odorants were used: linalool, limonene, citral and geraniol.

O, olfactory stimulus applied at the specified concentration; M mechanosensory stimulus (air puff).

Results from these experiments are presented in Fig. 3.

chemosensory and a mechanosensory stimulus (OM) or an exclusively mechanosensory stimulus (M) (Table 2). After a single conditioning trial with either OM or M, the response to M alone was tested. We used high and low odorant concentrations [1% and 0.01% (v/v)] of four different odorants (linalool, limonene, citral and geraniol) and investigated whether overshadowing was stronger for higher odorant concentrations.

#### *Experiment 3: testing for concentration-dependent consolidation*

Since preliminary results suggested that the salience of an odorant is influenced by its concentration, we investigated whether this effect might correspond to differences in memory consolidation. Therefore, we tested the response of a honeybee either 1 min or 10 min after a single conditioning trial. At both times, we determined the response levels to either high or low odorant concentration using any one of four different odorants (linalool, limonene, citral and geraniol) (Table 3). Memory consolidation is indicated by an increase in response levels between 1 min and 10 min after training. Thus, we investigated whether memory consolidation occurs to the same extent for high and low odorant concentrations.

#### *Experiment 4: testing for qualitative effects of different concentrations*

This experiment was designed to investigate whether honeybees can treat two concentrations of one odorant as two qualitatively different stimuli, and involved differential conditioning to any two of three concentrations of linalool [100%, 10%, 1% (v/v)]. Six experimental groups were conditioned as shown in Table 4.

If honeybees treat two concentrations of one odorant as two qualitatively different stimuli, they should be able to differentiate between them; i.e. they should be able to solve the high+/low- and the low+/high- tasks. If, however, these

Table 4. *Odorant concentrations and experimental groups for experiment 4, investigating the ability to discriminate between different concentrations of one odorant*

Group	CS+ at concentration (% v/v)	CS- at concentration (% v/v)
High-/low+	10	100
„	1	100
„	1	10
High+/low-	100	10
„	100	1
„	10	1
Detectability control	1	Solvent
Solvent control	Solvent	Blank

Animals received six rewarded and five unrewarded trials in alternating order and with an inter-trial interval of 10 min.

CS+ indicates the rewarded and CS- the unrewarded conditioned stimulus. The odorant used was linalool.

O, olfactory stimulus applied at the specified concentration; M mechanosensory stimulus (air puff).

Results from these experiments are presented in Fig. 4.

concentrations are processed as differentially salient variants of one odorant, differential performance in the low+/high-presentation should be disrupted.

Honeybees underwent six presentations of the respective rewarded concentration and five presentations of the unrewarded concentration. The first trial was always rewarded, and training continued as described for experiment 1. Statistical analyses were carried out on results from the last 10 trials only (see above).

In this experiment, device B was used for odorant application. Therefore, the mechanical component of the conditioned stimulus is unlikely to have an effect due to sensory adaptation. However, because there were differences between the two odorant delivery devices, and because a different solvent was used from that in previous experiments, we carried out two additional control experiments. In the first, we tested whether the new solvent (paraffin oil) was itself a salient olfactory stimulus ('solvent control'), by training honeybees in a solvent+ versus blank- task. Low response levels on such a task would be indicative of low saliences of the stimuli involved. The second control investigated whether the lowest odorant concentration used [1% (v/v)] with application device B was detectable by honeybees ('detectability control'). Therefore, the honeybees were trained differentially using 1% (v/v) linalool as the rewarded stimulus versus solvent alone as the unrewarded stimulus.

#### *Data analyses and statistics*

All data are presented as percentages of conditioned proboscis extension (%PE). For the discrimination experiments, two additional measures were introduced. One represents the total response level, whereas the other reflects

the degree of discrimination (discrimination index). The total response level is calculated as the cumulative sum of a honeybee's responses to the rewarded stimulus and to the unrewarded stimulus. The discrimination index is calculated as the cumulative sum of a honeybee's responses to the rewarded stimulus minus the cumulative sum of that honeybee's responses to the unrewarded stimulus.

$\chi^2$  tests were used to compare response frequencies between groups. Performance during discrimination training was analyzed within each group using Wilcoxon matched-pairs tests and between groups using Mann-Whitney *U*-tests. For multiple group comparisons of the total response level and the discrimination index, we used Kruskal-Wallis tests. Results of statistical analysis were regarded as not significant for  $P > 0.05$ .

In cases where several odorants were used, data take the form of a multi-way table. Thus, we carried out an additional analysis based on log-linear models. Three factors were used: (1) odorant; (2) response number; and (3) odorant concentration (overshadowing and consolidation experiments) or time between training and testing (consolidation experiment). To test for the significance of a term, we first tested for three-factor interactions. Then, we compared a model containing all two-factor interactions with a model omitting each one of these interactions in turn. An interaction was defined as having a significant impact if the differences in the  $\chi^2$  values of the models were significant.

All analyses were carried out using StatView for Macintosh, with the exception of the log-linear models, which were carried out using CSS Statistica.

## Results

### *Experiment 1: detectability of odorant concentrations*

As a first step in our analysis, we determined whether the odorant concentrations used can serve effectively as conditioning stimuli. Honeybees were able to detect the conditioning stimulus significantly at all odorant concentrations (Fig. 1A-D,  $P < 0.001$ ,  $T = 1$ ). The pseudodiscrimination control group (see Table 1) showed response levels on rewarded trials which were indistinguishable from the response levels on unrewarded trials (Fig. 1E,  $P > 0.05$ ,  $T = 6$ ). Thus, these results demonstrate that odorant concentrations as low as 0.001% (v/v) can be detected by honeybees and can function as conditioning stimuli.

### *Effects of odorant concentration on discrimination*

Fig. 1F shows that higher odorant concentrations result in significantly better discrimination index values than lower concentrations ( $P < 0.001$ ,  $H = 18.0$ , d.f.=3). Total response levels, however, were not influenced by odorant concentration (Fig. 1G,  $P > 0.05$ ,  $H = 2.5$ , d.f.=3).

### *Experiment 2: effects of odorant concentration on overshadowing*

We investigated whether the degree to which an odorant can

overshadow learning of a mechanical stimulus depends on odorant intensity (see Table 2).

Overshadowing between olfactory and mechanosensory stimuli occurs at high but not at low odorant concentrations, as is indicated by significantly different response levels between the three experimental groups (Fig. 2A,  $P < 0.01$ ,  $\chi^2 = 13.3$ , d.f.=2).

Analysis of these data using log-linear models revealed a significant interaction between odorant concentration and response levels ( $P < 0.01$ ,  $\chi^2 = 10.3$ , d.f.=1). There were no other significant three- or two-factor interactions, indicating that there is a concentration-dependent but not an odorant-specific effect on performance (see Fig. 2B).

The concentration range for odorant effectiveness was investigated only for linalool (see above). Thus, any variation in the degree of overshadowing at low concentrations of other odorants could be due to the use of subthreshold concentrations of these odorants. However, the next experiment, which was conducted in parallel, demonstrates that, after training to an OM compound, response levels towards that compound after 10 min (see open bars in Fig. 3A) were greater than those for the mechanosensory component alone (Fig. 2A) for both high [1% (v/v)] ( $P < 0.001$ ,  $\chi^2 = 72.8$ , d.f.=1) and low [0.01% (v/v)] ( $P < 0.01$ ,  $\chi^2 = 10.5$ , d.f.=1) odorant concentrations, demonstrating that the concentrations used were indeed detectable.

### *Experiment 3: effects of odour concentration on memory consolidation*

Given that salience differences exist between odorant concentrations, we investigated whether memory consolidation for low odorant concentrations was also reduced relative to higher odorant concentrations (see Table 3).

For high odorant concentration [1% (v/v)], response levels increase between 1 and 10 min following training (Fig. 3A,  $P < 0.05$ ,  $\chi^2 = 4.1$ , d.f.=1), whereas response levels remain the same for low odorant concentration [0.01% (v/v)] (Fig. 3A,  $P > 0.05$ ,  $\chi^2 < 0.01$ , d.f.=1). Therefore, honeybees show stronger memory consolidation for high than for low odorant concentrations.

Analysis of these data using log-linear models revealed that the only significant two-factor interaction was that between the time interval between training and testing and the response level for the high odorant concentration (Fig. 3B,  $P < 0.05$ ,  $\chi^2 = 4.1$ , d.f.=1). No significant two-factor interactions were found for the low odorant concentration groups, and in neither group were there any significant three-factor interactions, again indicating that there is no significant odorant-specific effect on performance.

Interestingly, honeybees show identical response levels 1 min after conditioning, regardless of the odorant concentration (Fig. 3A,  $P > 0.05$ ,  $\chi^2 = 0.13$ , d.f.=1), but by 10 min following training the high odorant concentration group shows higher response levels than the low odorant concentration group (Fig. 3A,  $P < 0.05$ ,  $\chi^2 = 6.64$ , d.f.=1). Again, analysis using log-linear models confirms the conclusions drawn from the pooled data. There is a significant two-factor

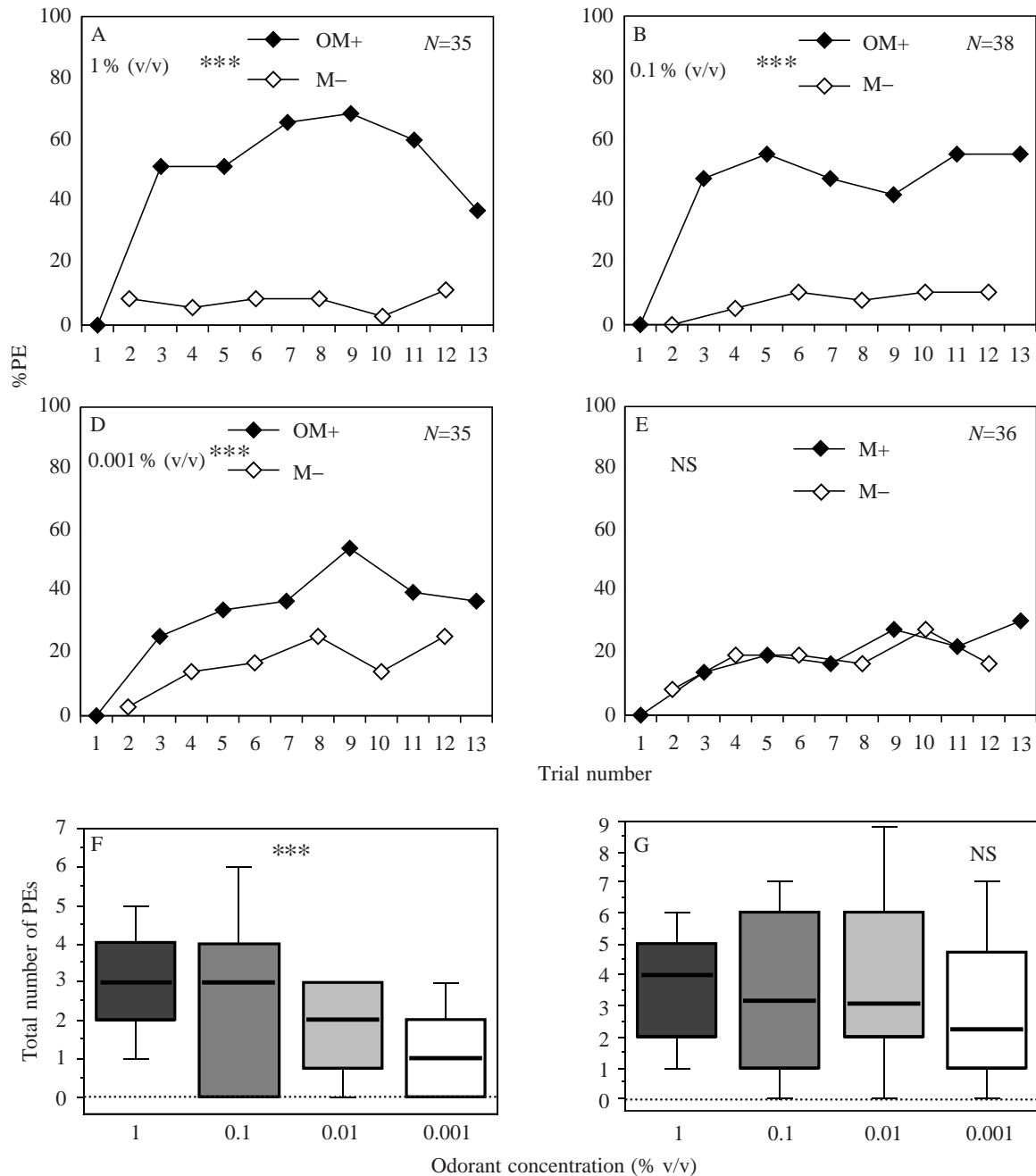


Fig. 1. Performance of honeybees in a feature-positive discrimination task with a rewarded olfactory-mechanosensory compound (OM+) and an unrewarded mechanosensory component (M-). The experimental groups are described in Table 1. Proboscis extension (as percentage responses, %PE) for groups trained with odorant concentrations ranging from 1 to 0.001% (v/v) are presented in A–D, and the results from the pseudodiscrimination control experiment in E. The degree of discrimination (F) and the total response levels (G) are shown *versus* odorant concentration. For each honeybee, the total response level is calculated as the cumulative sum of its responses to the rewarded stimulus and the unrewarded stimulus. The degree of discrimination is described by the discrimination index, which is calculated as the cumulative sum of a honeybee's responses to the rewarded stimulus minus the cumulative sum of its responses to the unrewarded stimulus. Box plots present the median as the bold line, box boundaries indicate the 25% and 75% quartiles, respectively, and the small horizontal bars represent 10% and 90% quartiles. In A–D, the performance of honeybees towards OM+ *versus* M- was compared within groups, as was performance towards M+ *versus* M- in the pseudodiscrimination control (E) (Wilcoxon matched-pairs test); in F,G, the discrimination indices or the total response levels, respectively, were compared across groups (Kruskal–Wallis test). PE, conditioned proboscis extension; N, sample size; NS, not significant; \*\*\* $P < 0.001$ .

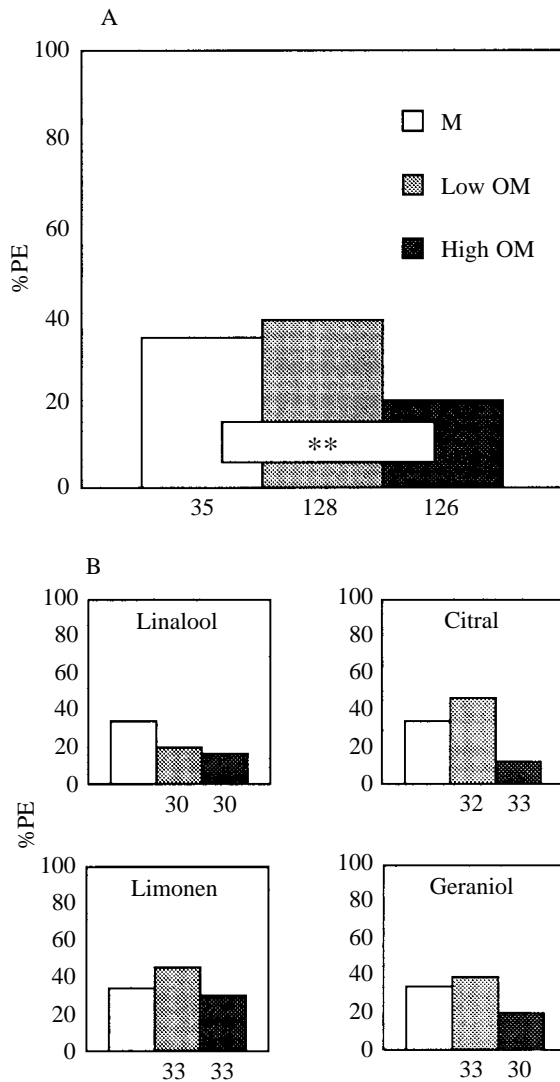


Fig. 2. Performance of honeybees in the overshadowing experiment (see Table 2 for details of experimental groups). Values represent proboscis extension (as percentage responses, %PE) in response to a mechanosensory stimulus (M) after training to either an olfactory-mechanosensory compound (OM) [dark bars for high and light bars for low odorant concentration, using 1% (v/v) or 0.01% (v/v) dilutions, respectively] or to the mechanosensory stimulus alone (M, open bars). (A) Pooled data with all odorants combined. (B) Data for each odorant given separately. Sample sizes are indicated below each column. In B, the results for the control group (M) are presented for comparison (for sample size, see A). Data in A were tested across groups using a  $\chi^2$ -test, and in B using a log-linear model (for details and statistical results, see text). PE, conditioned proboscis extension; \*\* $P < 0.01$ .

interaction between odorant concentration and response levels 10 min after conditioning (Fig. 3B,  $P < 0.05$ ,  $\chi^2 = 6.6$ , d.f. = 1). No other interactions were significant.

#### Experiment 4: no qualitative effects of different odorant concentrations

We investigated whether honeybees can treat two

concentrations of one odorant as qualitatively different stimuli by attempting to train them to respond discriminatively to two concentrations [any two of 100% or 10% or 1% (v/v)] of the same odorant (see Table 4 for experimental groups).

As shown in Fig. 4A, honeybees cannot solve the low+/high- task (see Table 4). Response levels to the unrewarded high odorant concentrations are significantly greater than those to the rewarded low concentrations [ $P < 0.001$ ,  $T = 9$ ; see also negative discrimination index values for the 1% (v/v) group in Fig. 4E]. None of the three groups (see Table 4) whose performance was pooled in Fig. 4A was successful at low+/high- discrimination (data not shown). The high+/low- task, however, was solved successfully with more frequent responses to the rewarded high than to the unrewarded low odorant concentration (Fig. 4B,  $P < 0.001$ ,  $T = 6$ ). This was also true for each of the three high+/low- groups which were pooled in Fig. 4B (see Table 4) considered separately (data not shown). Taken together, our results do not support the hypothesis that honeybees are able to treat two concentrations of one odorant as qualitatively different stimuli.

Total response levels increase with increasing concentration of the rewarded stimulus (Fig. 4F,  $P < 0.001$ ,  $H = 24.9$ , d.f. = 2). This variation in total response levels results in negative discrimination index values for the lowest rewarded odorant concentration [1% (v/v)] (Fig. 4E,  $P < 0.001$ ,  $H = 18.87$ , d.f. = 2). Note also that response levels to low odorant concentrations are higher in the high+/low- trials than in the low+/high- trials (compare Fig. 4A with Fig. 4B,  $P < 0.05$ ,  $U = 2428$ ).

The odorant delivery device used in experiment 4 was different from that used in the other experiments (see Materials and methods). However, Fig. 4C shows that honeybees were successful in a discrimination task using a rewarded olfactory versus an unrewarded solvent stimulus even at concentrations of 1% (v/v) ( $P < 0.001$ ,  $T = 1$ ), so the results shown in Fig. 4A,B cannot be accounted for by the inability of the honeybees to detect the lowest odorant concentration delivered by a different device. The relatively low response to the unrewarded solvent stimulation (compare Fig. 4C with Fig. 1A–D) implies that mechanosensory input was adapted out by this device.

A further control experiment was carried out to test whether the solvent (paraffin oil) used in experiment 4 acted as a chemosensory stimulant itself. Fig. 4D shows that, despite a reasonably large sample size ( $N = 23$ ), response levels towards the solvent were so low that they excluded statistical analysis.

## Discussion

### Effect of odorant concentration on associative strength

In recent theories of associative learning (e.g. Rescorla and Wagner, 1972; Sutton and Barto, 1990), the concept of 'saliency' refers to a constant and experience-independent feature of a conditioned stimulus which determines the rate at which it can enter into associations with a reward. That is, these theories suggest that memory formation depends on stimulus saliency (however, see Durlach, 1989; Spear *et al.* 1990, for discussions of alternative views). The present study showed

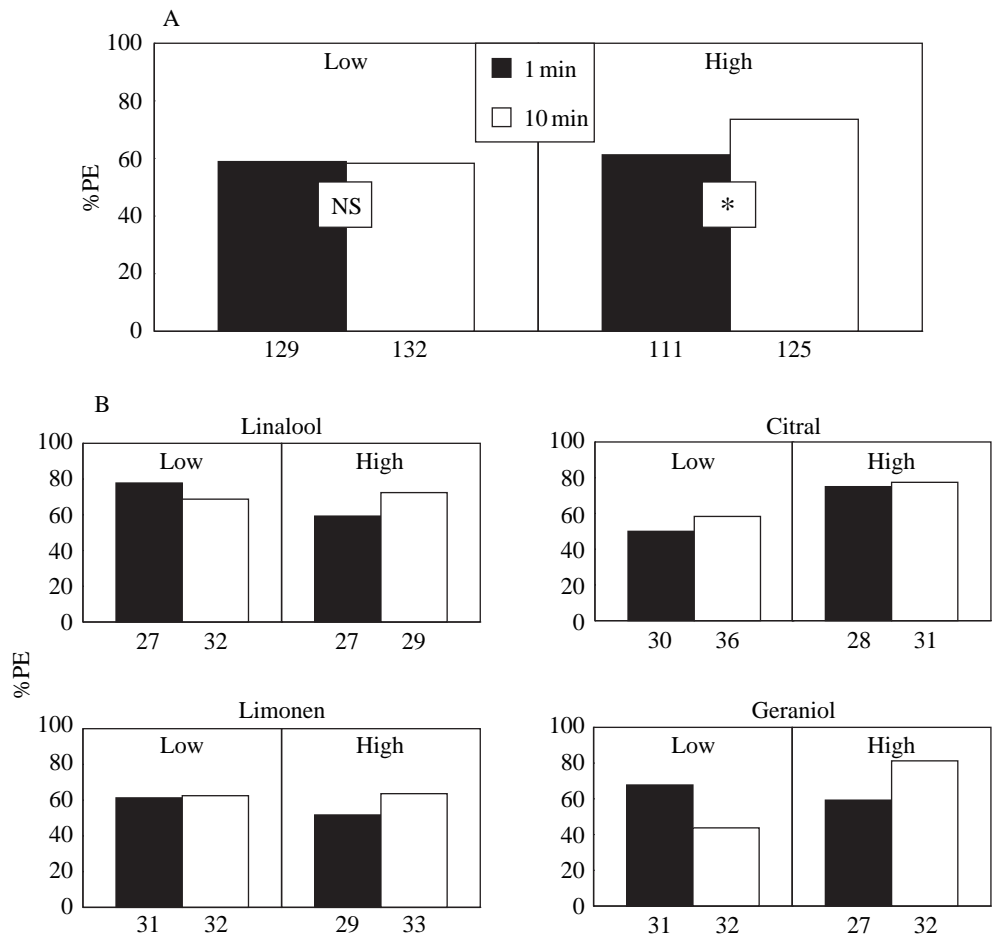


Fig. 3. Performance of honeybees in a memory consolidation experiment (see Table 3 for details of experimental groups). Values represent proboscis extension (as percentage responses, %PE) in response to either low (left) or high (right) odorant concentrations, using 0.01% (v/v) or 1% (v/v) dilutions, respectively. Testing was carried out either 1 min (filled bars) or 10 min (open bars) after training. (A) Pooled data for all odorants. (B) Data for each odorant given separately. Sample sizes are given below each column. Data in A were tested between groups using  $\chi^2$ -tests and in B using a log-linear model (for details and statistical results, see text). PE, conditioned proboscis extension; NS, not significant; \* $P < 0.05$ .

that overshadowing of learning about simultaneously presented mechanosensory stimuli by olfactory stimuli increased with increasing odorant concentration (Fig. 2A). Therefore, odorants at high concentrations support stronger associations (i.e. are more 'salient') than at lower concentrations. Indeed, because the critical test did not involve the odorants themselves but rather the mechanosensory stimulus alone, one cannot argue that different response levels in the experimental groups were caused by different activation levels of otherwise equally strong associations (for another argument in favour of this conclusion, see below). An effect of odorant intensity on salience is also consistent with the additional observation that higher odorant concentrations have a stronger capacity to act as discriminative stimuli (Fig. 1F).

In addition, it is likely that the biological mechanism underlying the psychological concept of 'salience' is related to memory consolidation. This conclusion is based on the observation that response levels between 1 and 10 min following training to an olfactory stimulus increase for high but not for low odorant concentrations (Fig. 3A). This suggests that increasing odorant intensity leads to enhanced internal processing which supports memory over time.

Regarding memory consolidation in olfactory learning of honeybees, it is known that response levels follow a biphasic time course with a 'dip' approximately 2–3 min after training

(Menzel, 1990). Responses in the short-term range (up to 2–3 min) seem to be largely due to non-associative memories (sensitization), whereas associative memories seem to underlie responses over the longer term (Hammer and Menzel, 1995; Menzel, 1990). From the present study, it is tempting to speculate that odorant concentration might be a determinant for associative but not, or to a lesser extent, for non-associative memories.

#### *Honeybees do not discriminate between different concentrations of one odorant*

We found no evidence for discrimination between different concentrations of one odorant: honeybees that respond to a rewarded low odorant concentration will also respond to an unrewarded high concentration (Fig. 4A). If the situation is reversed (rewarded high *versus* unrewarded low odorant concentration), however, honeybees can 'discriminate' (Fig. 4B). Thus, response levels to the high odorant concentration are always higher than response levels to a lower concentration, regardless of reward status. These findings can be explained if high and low concentrations are processed as differentially salient variants of the same odorant quality. From this perspective, the 'discrimination' task presented to the honeybee could be described more accurately as a partial reinforcement task, in which the odorant quality is rewarded



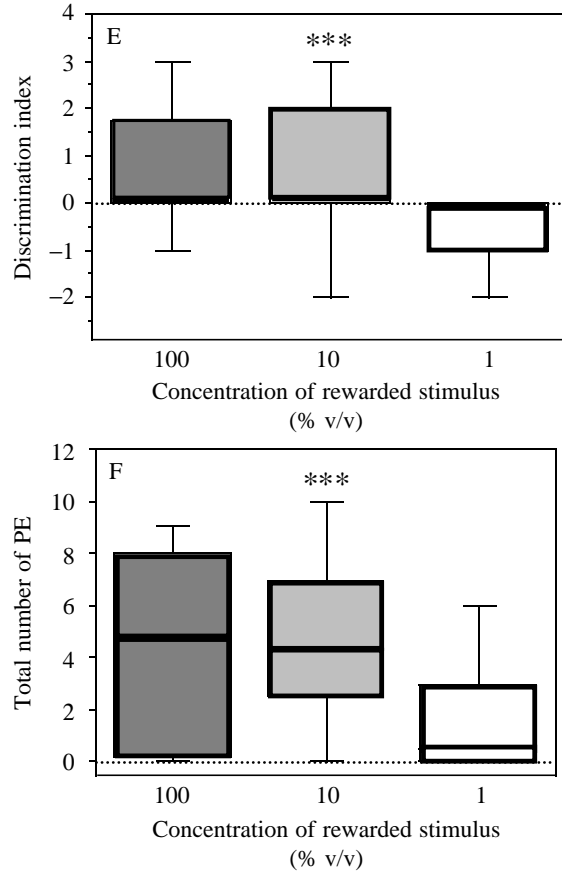
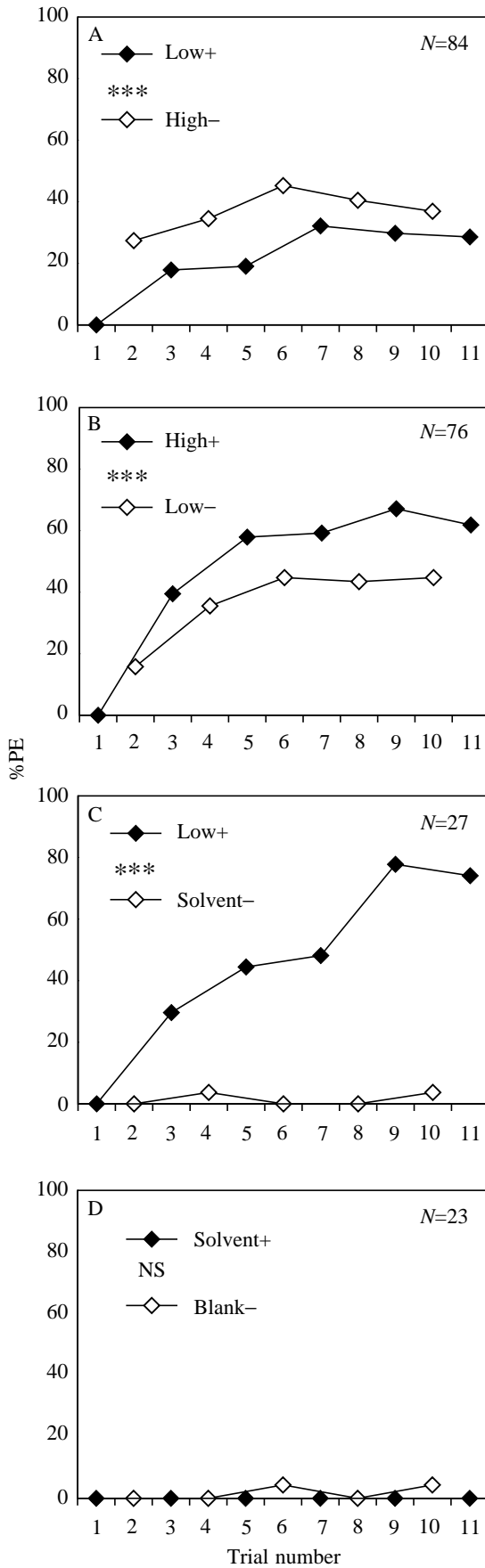


Fig. 4. Performance of honeybees in a discrimination task in which they were presented with different rewarded or unrewarded concentrations of the same odorant (see Table 4 for details of experimental groups). In A–D, all values represent proboscis extension (as percentage responses; %PE). (A) Pooled results from the three groups trained in a discrimination task using rewarded low *versus* unrewarded high concentrations (see Table 4 for the exact concentrations). (B) Pooled results from the three groups trained in a discrimination task using rewarded high *versus* unrewarded low concentrations (see Table 4 for the exact concentrations). (C) Results from the detectability control and (D) from the solvent control. The degree of discrimination as indicated by the discrimination index (E) and the total response levels (F) are shown *versus* the concentration of the respective rewarded stimulus. For the computation of the discrimination index and the total response levels, see the legend to Fig. 1. Values in E and F are represented by their medians and quartiles as in Fig. 1. In A–D, the performance of honeybees towards rewarded *versus* unrewarded stimuli was compared within groups (Wilcoxon matched-pairs test); in E,F, the discrimination indices or the total response levels, respectively, were compared across groups (Kruskal–Wallis test). PE, conditioned proboscis extension; *N*, sample size; NS, not significant; \*\*\* $P < 0.001$ .

on any other trial. Since stimulus salience increases with odorant intensity, overall response levels will also increase if the high concentration is rewarded (Fig. 4F). If the high concentration is unrewarded, the net effect of conditioning decreases.

Direct evidence for an effect of odorant concentration on salience is found by comparing the responses of honeybees to the low concentration in the high+/low- versus the low+/high- condition (Table 4; Fig. 4A,B). Although the stimulation intensity under both conditions was the same and although the low concentration was rewarded in the low+/high- condition, honeybees responded *less* to the low concentration in that group. This result can be explained only if the odorant associations established in the low+/high- group are less strong than those established in the high+/low- group.

Thus, honeybees can recognize an olfactory quality irrespective of its quantitative features. On an ecological level, this ability seems understandable, considering the ever-varying intensities of natural stimuli. A honeybee which did not revisit a rewarding flower simply on the grounds of odour intensity variation would probably have a rather poor foraging efficiency. Therefore, the inability of honeybees to discriminate between two concentrations of one odorant does not represent a 'failure' of the olfactory system but rather indicates its highly adaptive organization.

Interestingly, Joerges *et al.* (1996) found that the spatio-temporal pattern of neural excitation in the antennal lobe of the honeybee is qualitatively almost (but not completely) identical for low and high odorant concentrations. However, the intensity of the signals measured using Ca<sup>2+</sup>-sensitive fluorescent dyes varied strongly with increasing odorant concentration. Similar results were obtained by Cinelli *et al.* (1995) for the salamander (*Ambystoma tigrinum*) olfactory bulb. Our results do not support the hypothesis that the subtle qualitative differences in excitation patterns observed in honeybees can be utilized to respond discriminatively to different odorant concentrations. Joerges *et al.* (1996) also argued that odorants are coded in a concentration-invariant way at the level of the antennal lobe.

Contrary to our findings, Bhagavan and Smith (1997) recently reported successful low+/high- discrimination in a proboscis-extension reflex conditioning paradigm (see also Gross-Isserhoff and Lancet, 1988). The effect occurred for one particular odorant only (1-hexanol), but not for a second odorant under investigation (geraniol), and was restricted to one, particularly large, difference in odorant concentration. Further research is warranted to determine the generality of this effect across olfactory stimuli and its putative biological role.

#### *Integration of olfactory and mechanosensory input*

The present study, extending the initial work carried out by Menzel (1990), is the first to investigate systematically the effects of compound olfactory-mechanosensory stimuli in conditioning of the honeybee proboscis-extension reflex. So far, conditioning stimulus modality in this system has, with

very few exceptions (Gerber and Smith, 1995; Masuhr and Menzel, 1972), been limited to olfaction. Thus, our study provides an opportunity to investigate how behaviour is jointly organized by those two different sensory modalities. The demonstration of overshadowing between chemo- and mechanosensory stimuli extends previous studies on intramodal olfactory overshadowing (Smith, 1996) and adds to the growing evidence that invertebrate and vertebrate learning at the behavioural level follow rather similar rules (for further details of overshadowing in invertebrates, see Bitterman, 1996; Sahley *et al.* 1981).

On a theoretical level (e.g. Rescorla and Wagner, 1972; Sutton and Barto, 1990; Pearce, 1994), overshadowing is explained by the components of a mixture competing for the effects of one common reinforcing signal ('competition rules') (however, see Durlach, 1989; Spear *et al.* 1990, for discussions of retrieval-based theories, and Pearce, 1994; Smith, 1996, for similarity-based alternative explanations). Physiologically, a neuronal substrate for such a reinforcing signal has been characterized for olfactory proboscis-extension conditioning in honeybees (Hammer, 1993): the identified neurone VUM<sub>mx1</sub> mediates the reinforcing properties of the sucrose reward. Output from this neurone converges with olfactory processing at the first olfactory relay station (the antennal lobe), at more central and at premotor neuropiles (the lip of the mushroom bodies and the lateral protocerebral lobe, respectively). Output from mechanosensory afferents, however, are not known to converge directly upon VUM<sub>mx1</sub>: the first relay station for mechanosensory antennal input is the dorsal lobe (Homberg *et al.* 1989; Mobbs, 1985). The antennal and dorsal lobes are interconnected (Flanagan and Mercer, 1989), and it might be due to these interconnections that 'olfactory' projection neurones from the antennal lobe to the mushroom bodies often respond not only to chemo- but also to mechanosensory stimulation (Homberg *et al.* 1989), a situation that also occurs for mushroom body output neurones (Erber, 1978). Thus, it seems probable that, at the level of the antennal lobe, olfactory-mechanosensory compounds are coded as a common across-fibre pattern. All convergence points of olfactory pathways with VUM<sub>mx1</sub> may therefore also be convergence sites with mechanosensory inputs. This could explain why, at the behavioural level, compounds of olfactory and mechanosensory stimuli have properties similar to those of odorant mixtures. This is interesting, given that compounds of olfactory and visual stimuli seem to obey rather different rules (Gerber and Smith, 1995).

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