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THE OLFACTORY MEMORY OF THE HONEYBEE *APIS MELLIFERA*

II. BLOCKING BETWEEN ODORANTS IN BINARY MIXTURES

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Summary

Proboscis extension conditioning of honeybee workers was used to study the processing of odorants when bees were conditioned to binary mixtures. Responses to a set of pure floral odors and pheromones after conditioning have already been described. When bees are conditioned to certain mixtures of odorants, the response to both components is equal to that when they are tested alone. However, mixtures of an aliphatic aldehyde and an alcohol elicit asymmetric response patterns; that is, the response to the aldehyde is much stronger than that to the alcohol. A bee's response to the alcohol after it had been trained in an aldehyde background is significantly lower than when the bee is trained to respond to the same alcohol in the background of another odorant. Such response patterns are not necessarily caused by a behavioral decrement resulting from a compound-unique perceptual effect produced by the mixture. Furthermore, studies of blocking show that behavioral acquisition in response to one component can be hindered or blocked by pretraining with the other component. These results suggest that honeybees can perceive the individual components of some binary mixtures. The similarities in neural processing in olfactory systems of vertebrates and invertebrates mean that such studies could elucidate behavioral mechanisms of olfaction in a wide phylogenetic spectrum of animals.

Introduction

Situations in which animals depend on their olfactory sense for detection of ecologically relevant stimuli require solutions to several problems involved in the detection of odor signals. These signals vary in terms of the individual odorants that constitute the odor and because of temporal fluctuation in the signal caused by air turbulence and other stochastic processes (Baker *et al.* 1985; Kramer, 1986; Christensen *et al.* 1989; Getz, 1991; Hopfield, 1991). Odors that have behavioral relevance, either because of a learned association or because of innate response biases, must be properly detected while irrelevant odors are being filtered out of the signal.

In both vertebrates and invertebrates, the patterns of activity across large numbers of

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sensory axons produced by the initial transduction process are translated into a neural representation of a given odor (Mobbs, 1985; Shepherd, 1991; Kauer, 1991). Sensory receptors for non-pheromonal odorants can respond to many odorants and can show complex interaction effects (suppression and synergism) in response to odorants in mixtures (Ache, 1989; Atema *et al.* 1989). Detection of any one component might, therefore, be difficult. The mechanisms by which these neural representations of odors are generated and processed in both peripheral and central areas, and by which they are related to motor outputs, are only beginning to be understood (Homberg *et al.* 1989). Analyses of behavioral responses to odors are essential in order to provide a set of criteria that must be explained by any model of how the mechanism of olfactory representations are generated (Smith and Getz, 1994).

Because of their rich olfactory-mediated behavioral repertoires, insects provide an opportunity for understanding these basic behavioral mechanisms of olfaction. For example, worker honeybees (*Apis mellifera*) respond to odors in several behavioral contexts (Winston, 1987; Getz and Page, 1991). Pheromones emitted by queens and/or workers communicate a variety of messages and elicit fairly stereotypical responses in the proper context, all of which help to coordinate the activities in a colony. Workers also learn to respond to floral odors, which, prior to the learning experience, do not typically elicit such strong innate responses as do pheromones (von Frisch, 1967). In a natural foraging situation, workers learn the association of floral odors with the nectar and/or pollen rewards offered by the flowers, thus allowing for future identification of the types of flowers from which the rewards can be harvested.

The fact that honeybees learn to respond to odorants permits a detailed analysis of the behavioral outcomes of odorant processing. Previous work has described how pheromones and floral odorants are associated with a sucrose reward through learning paradigms (Smith and Menzel, 1989*a*,*b*; Smith, 1991). Some odorants are learned quickly whereas others are not. Once the association of an odorant with sucrose is learned, the behavioral response generalizes to other odorants that were not experienced in the conditioning procedure; that is, honeybees respond to several odorants after conditioning to one. These generalized responses may be based on several underlying mechanisms (Kalish, 1969). They are typically not as strong as the response to the odorant used as the conditioned stimulus, and the magnitude of the response to a novel odorant depends on structural similarity of the novel odorant to the conditioned odorant (Smith and Menzel, 1989*a*,*b*; Getz and Smith, 1990). Therefore, one basis for generalization probably lies in perceptual similarity of conditioning and test odorants.

The purpose of the work detailed below was to investigate the nature of interactions between odorants in binary mixtures, primarily using odorants that have been studied in previous behavioral analyses (Smith, 1991; Smith and Menzel, 1989*a*,*b*). The work investigates whether individual components can be perceived in a binary mixture and whether conditioning to one component can affect later conditioning to a new component added to the mixture. These experiments, albeit with a limited number of odorants, are a crucial first step for understanding how odorant processing takes place in a fairly realistic olfactory problem; that is, when biologically relevant odors occur in a potential ‘blizzard’ of olfactory information (Christensen *et al.* 1989).

Materials and methods

Proboscis extension conditioning was used to assay responses to odors. This procedure, which has been used in several studies of olfactory learning and memory in honeybees (Menzel *et al.* 1974; Menzel and Bitterman, 1983), utilizes honeybee workers restrained in harnesses in such a way that they can freely move their antennae and mouth parts (mandibles and proboscides). Workers were collected from the entrance to a colony during the early afternoon on the day prior to conditioning. Bees departing from the colony are mostly older workers that have a variety of behavioral specializations within the colony, which do not affect proboscis extension conditioning performance (Bhagavan *et al.* 1994).

Each individual was confined in a small glass vial that was transferred into an iced-water bath until the bee stopped moving. The bee was removed from the vial and fixed into a small harness using a strip of duct tape placed between the head and thorax. 30min later the bee was fed a 1.5mol l^{-1} sucrose–water solution until satiation, after which it was placed into a dark, humidified drawer overnight (this procedure minimized overnight mortality to approximately 10%). The following morning, bees were removed from the drawer and held on a bench top in the open for at least 2h and no more than 3h.

Odorants used for conditioning were citral, geraniol, hexanal, octanal, 1-hexanol and 1-octanol. Hexanal, octanal, 1-hexanol and 1-octanol are unbranched aliphatic hydrocarbons containing one oxygen molecule in an aldehyde or alcohol moiety. They have similar vapor pressures, so when equal amounts are combined in a mixture, approximately equal numbers of molecules would be delivered during a test or training trial. None of these compounds is known to be a component of honeybee pheromones. Citral and geraniol are monoterpenoid components of the honeybee Nasonov pheromone (Pickett *et al.* 1980), which is used during orientation and in marking unscented feeding dishes. Citral is a 60:40 mixture of its isomers neral and geranial.

Odor delivery was prepared by placing 3 μl of an odorant onto a small piece of filter paper. When odorant mixtures were used, 1.5 μl of each odorant was placed onto the filter paper. A 1.5mol l^{-1} sucrose–water solution was placed into a 1ml glass syringe to which a bevelled metal needle was attached. Sucrose solutions were always refrigerated overnight and prepared freshly every few days.

All conditioning procedures involved the use of forward-pairing conditioning trials: that is, the odor stimulus to which the subject is to be conditioned (S1, S2 or S3) precedes the sucrose unconditioned stimulus, to which the subject responds naturally. A conditioning trial involved first placing a subject into a slowly moving airstream that vented into a laboratory fume hood. In order to minimize background conditioning and sensitization effects, the subject was held in that position for 30–40s prior to presentation of conditioned stimuli. At that point, an odor, either from a single odorant or from a mixture of two odorants, was injected into the airstream over a 5s period. For most experiments, 15ml of odor-laden air was delivered through a 20ml plastic syringe. For experiments in which more precise measures of response were derived from video analysis, a relay board switched by a parallel port on a computer was used to control the presentation of odors to an accuracy of 55ms. Just before the end of odor delivery, a 1 μl

droplet of the sucrose–water solution was manually applied to the subject's antennae, which elicited proboscis extension in motivated subjects. The droplet was then applied to the proboscis and the subject was allowed to feed for 3s. Presentation of the sucrose was timed by an auditory signal from the computer. Each trial lasted 50s (odorant and sucrose delivery occurred only over a short interval during that time), after which the subject was returned to the holding tray and the next subject was placed into the airstream. The inter-trial interval was constant for each experiment (either 6 or 8min). After being used in an experimental procedure, subjects were never used again.

Two different types of response measures were recorded during test (extinction) trials. In all experiments, if a subject extended its proboscis after the onset, but before the end, of odor presentation, a response was registered. Otherwise, a non-response was registered. This allows for discrimination of response levels in many types of experiments (Menzel, 1990). However, more subtle differences in response cannot be discriminated with a two-state response measure (Smith and Menzel, 1989*b*). Therefore, a much more sensitive response measure was employed for some experiments, in which differences between groups were not expected to be reflected by the frequency of proboscis extension. During these experiments, responses of subjects were videotaped. A small red light-emitting diode was placed so that it appeared on the video but was not visible to the subject. This indicated the onset and offset of the computer-controlled odor stimulus. Each video frame contained a unique and sequential frame code that allowed for frame-by-frame analysis for determination of the frame in which the proboscis was extended and the frame in which it was retracted. It was then possible to calculate the duration of the response to a 33ms accuracy. Response duration was recorded as zero if no extension occurred.

Experimental design

Experiments were divided into either two or three different phases (Table 1). During the first phase(s), subjects received 4–12 acquisition trials depending on the experiment; inter-trial intervals (ITIs) were 6 or 8min. In one type of experiment, the change from the first to the second phase signaled a change in stimulus presentation during the conditioning trial (see blocking experiments below). The test phase was always the last phase of an experimental procedure. Tests during this phase always involved odor presentation without sucrose feeding; that is, extinction trials were performed with the odor-conditioned stimulus or with, for example, component odorants of a mixture. Multiple extinction trials were sometimes performed during the test phase in a pseudorandomized sequence and separated by 6 or 8min ITIs.

For mixture experiments (Table 1, group A), two odorants were presented simultaneously for acquisition trials during phase I, the only pre-test phase for this group. The test phase involved two sets of three extinction trials. Each set contained one trial with the mixture and trials for each of the separate components. The order of the test trials within each set was pseudorandom for each subject. The response to a given component (S2, for example) is affected by mixture training if that response level differs in a way that depends on the specific mixture configuration (e.g. the response to S2 after conditioning to S2+S1 *versus* S2+S3).

Table 1. *Summary of experimental designs*

Group	Phase I*	Phase II*	Test phase†
A	S1+S2→sucrose	–	S2, S1, S1+S2
B	S1→sucrose	S1+S2→sucrose	S2, S1
C	S3→sucrose	S1+S2→sucrose	S2, S1
D	sucrose→S1	S1+S2→sucrose	S2, S1
E	S1↔sucrose	S1+S2→sucrose	S2, S1

*Odorants separated by (+) indicate mixtures. Odorants or mixtures followed by a single-headed arrow indicate forward pairing with sucrose. A dash indicates no stimulus exposure when experiments involved only a single conditioning phase. Odorants or mixtures followed by a double-headed arrow indicate explicitly unpaired presentation of sucrose.

†Separation of odorants by commas indicates that separate extinction trials were performed with odorants during this phase. The presentation sequence was pseudorandomized and thus different for each subject. All subjects were tested at least with S2 and S1. For initial experiments, subjects in group A were also tested with S1+S2 (see text).

Blocking experiments used two odorants that do not elicit different responses after mixture training (citral and hexanal or geraniol and 1-hexanol). One group of subjects was exposed to six acquisition trials with S1 (phase I) and an additional six acquisition trials with a mixture of S1+S2 (phase II; Table 1, group B). Two types of control groups were run in parallel to blocking groups. One group received mixture training only (Table 1, group A); that is, no prior exposure to S1. A second control group received six initial acquisition trials to a third odor (S3, 1-octanol) followed by six acquisition trials to the S1+S2 mixture (Table 1, group C). If the response to S2 in the blocking treatment group is lower than that to S2 in both control groups, then prior training of S1 has blocked acquisition to S2.

A final experiment utilized four groups of subjects and tested whether the nature of pairing of S1 with sucrose in phase I is critical for the blocking effect. In this experiment, a blocking group was conditioned as above using 1-hexanol as S1 and geraniol as S2 (Table 1, group B). A second group replicated a critical reference group needed for blocking studies (Table 1, group A): that is, they received no conditioning exposure in phase I followed by mixture-only training in phase II. Responses to S2 should be lower in the blocking group than in the mixture-only training group. A third group received ‘backward pairing’ of S1 and sucrose in phase I (Table 1, group D). That is, sucrose was presented to subjects as described above. Just prior to the end of feeding, the S1 odorant was presented for a period that continued for several seconds beyond the termination of feeding. The fourth group received ‘explicitly unpaired’ presentations of S1 and sucrose (Table 1, group E). Sucrose feeding and the S1 odorant were presented on separate, pseudorandomly ordered trials (the order was the same for all subjects in this group). Subjects in the unpaired group received four exposures to each stimulus (S1 and sucrose) across eight trials. In the first three groups, trials during which S1 and sucrose were presented were interspersed with ‘placement only’ trials, in which subjects were placed into the conditioning platform without delivery of conditioning stimuli. Thus, all groups received an equivalent number of exposures to placement, odorant and sucrose in phase I;

they differed only in the way in which those stimuli were associated. All four groups received identical forward pairing of the S1/S2 mixture with sucrose across four trials in phase II. During the test trials, two extinction trials were performed with S2, followed by a single extinction trial with S1.

Statistical analyses

All statistical analyses were performed on the response pattern during extinction trials. When only proboscis extension was recorded, each subject could be classified according to whether or not it responded when tested with each of the odors. χ^2 analyses (calculated with SYSTAT version 5.0) were used to test whether distributions of responses were independent of the combination of treatment categories used for an experiment. When response duration was measured, differences between treatment groups were assessed using non-parametric analysis of variance (Kruskal–Wallis analysis in SYSTAT).

When conditioning honeybees in the proboscis extension procedure, there were always subjects that did not respond to conditioning stimuli (i.e. they never responded to odorant and/or to sucrose; Smith *et al.* 1991). The number of non-responders varied throughout the year depending, for example, on factors such as low motivational states, maintenance conditions or genotype (Bhagavan *et al.* 1994). In order to train more standardized, uniform groups of subjects, we established selection criteria prior to beginning the series of experiments reported below. Any subject that showed no response to the associated odorant during acquisition trials and/extinction tests was not used in statistical analyses. The percentage of such subjects never differed significantly among treatment groups conditioned in parallel: it never exceeded 25% and was frequently as low as 5%. Furthermore, this selection criterion never qualitatively altered the patterns of responses among groups conditioned in parallel. It only increased the mean level of response in extinction tests. All sample sizes given below reflect the number of subjects per group that met the response criterion.

Results

Interaction between two odorants in a mixture

Several experimental groups of subjects were conditioned to binary mixtures in the first experimental phase. They were later tested with the mixture and with each of its components in order to assess whether the response to either component was less than that to the mixture and to assess whether the odor-conditioning background affects the response to a given odorant. Several different mixtures had to screened in this initial study because perception of odorant mixtures could be affected by the particular mixture. It would be difficult to screen all possible binary combinations of large numbers of odorants. Instead, our strategy was to screen a few combinations of odors to discover any replicable effects that could then be studied in more detail.

Subjects acquired the conditioned response (proboscis extension) to all odor mixtures within a few trials during phase I of conditioning (Fig. 1). The first experiment involved two independent treatment groups that received 12 acquisition trials to a hexanal/citral mixture. The remaining two experiments involved several treatment groups, each using

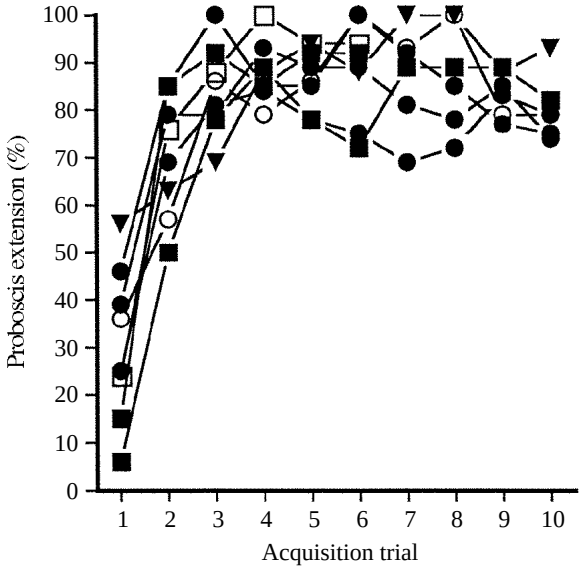

Fig. 1. Acquisition curves for conditioning to mixtures of odorants in eight independent treatment groups of subjects. Responses on acquisition trial 1 show spontaneous responses to citral/hexanal (●, *N*=18, 16, 19); citral/geraniol (▼, *N*=16); geraniol/1-hexanol (○, *N*=18); hexanal/1-hexanol (■, *N*=18 and 13); octanal/1-octanol (□, *N*=17). χ^2 analysis revealed a significant difference in spontaneous responding among groups: χ^2=14.66, *P*<0.05, 7 d.f.

10 or 6 acquisition trials, respectively. The mixtures used to condition these groups differed (see Fig. 1). In all experiments, 5–56% of the subjects responded spontaneously to odor presentation on the first trial.

It is interesting to note that mixtures containing Nasonov pheromone components (citral or geraniol) elicited significantly higher spontaneous response levels than did mixtures that did not contain pheromone components (Fig. 1). The three lowest spontaneous response levels were from groups conditioned to mixtures of odorants that are not pheromone components. The five highest spontaneous rates were from groups conditioned to odor mixtures that contained at least one component of the honeybee Nasonov pheromone. The response levels in all groups increased within two or three trials to an asymptote of 70–100%.

In the first repetition of this experiment, two groups of subjects were conditioned in 12 trials to a mixture of hexanal (S1) and citral (S2). During the extinction trials in the test phase, subjects responded equally often to the mixture and to each of the components. Response levels to the three odors for each conditioning group were: hexanal (86%, 84%); citral (75%, 84%); mixture (86%, 84%). Differences among responses to odors (2 d.f. in each group) for this particular mixture configuration were not significant in either group (χ^2=0.57, *N*=28, *P*>0.05; χ^2=1.46, *N*=32, *P*>0.05). That is, the response to either of the components was not significantly less than the response to the mixture.

This result may not necessarily generalize to other odorant mixtures or to conditioning situations that involve fewer conditioning trials. Therefore, the next four groups of

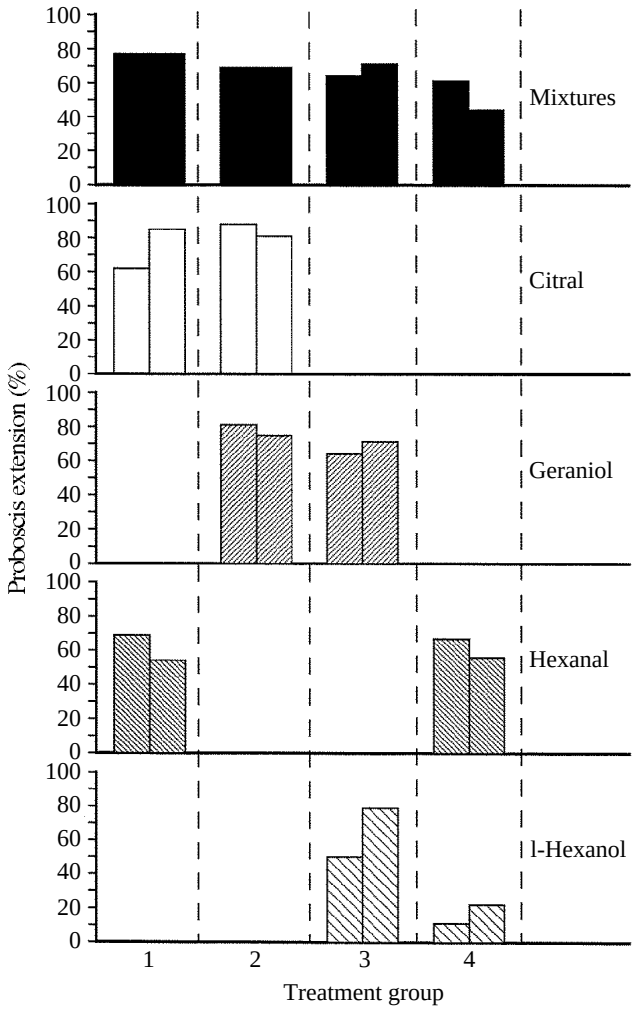

Fig. 2

subjects were conditioned to different binary combinations of four odorants to test whether certain configurations affected the level of behavioral acquisition to a given odorant. The test thus compared responses to different components within a group of subjects (as above), but also compared response levels to the same odorant presented during training in different odor backgrounds across subject groups.

Three of four binary mixtures caused no significant difference in response levels to mixtures compared with responses to the components (Fig. 2). When subjects were trained to mixtures of citral/hexanal, citral/geraniol or geraniol/1-hexanol (first three columns in Fig. 2), response levels were the same to mixtures and to components. However, subjects behaved differently in response to components after conditioning to a mixture of hexanal/1-hexanol (right-hand column in Fig. 2). Response levels to 1-hexanol were significantly lower than to hexanal or to the mixture. The latter two odorants elicited equivalent response levels.

Fig. 2. Response levels from four independent treatment groups of subjects (corresponding to four columns separated by dashed lines). Six extinction tests were performed with each subject, two each with the mixture indicated (S1/S2; A) and with each of the components (S1 and S2; B–E). Responses were categorized as response or no response. Pairs of bars that touch each other indicate replicate response probabilities for subjects in the two extinction trials with each odor or mixture. In each of the four columns there are three such sets of bars, which indicate response levels to the mixture (A) and to each of the components (two sets of bars in B–D) within a subject group. For example, the leftmost column shows results from a group trained to a mixture of citral and hexanal. Each graph in B–E shows response levels to the same odorant presented during conditioning in different odor backgrounds across subject groups. For each graph, statistical comparison of response levels for each treatment group, derived from the first replicate (left-hand column in each pair) extinction tests, yields the following χ^2 (1 d.f.) values: A, 0.93, NS; B, 2.64, NS; C, 1.10, NS; D, 0.02, NS; E, 5.89, $P<0.05$. Identical comparison of the second replicate (right-hand column of each pair) extinction tests yields the following χ^2 (1 d.f.) values: A, 4.42, NS; B, 0.05, NS; C, 0.05, NS; D, 0.01, NS; E, 10.04, $P<0.01$. χ^2 comparisons (2 d.f.) of first extinction test responses to the mixture and to each component within subject groups (i.e. within columns) are as follows: citral/hexanal, 0.72, NS; citral/geraniol, 1.77, NS; geraniol/1-hexanol, 0.79, NS; hexanal/1-hexanol, 13.56, $P<0.01$. Sample sizes are constant within each column and are, from left to right: 18, 16, 18, 18. NS, not significant.

Comparison of responses to particular odorants across subject groups (i.e. within rows, Fig. 2) clearly shows an effect of odorant background on response levels (Fig. 2B–E). There were no significant differences in response levels among mixtures (Fig. 2A). For citral, geraniol and hexanal, response levels were unaffected by the odor backgrounds in which they were conditioned (Fig. 2B–D). Response levels to citral were not significantly different when bees were conditioned in a backgrounds of geraniol or hexanal. The same conclusion holds for geraniol when conditioning took place in backgrounds of citral or 1-hexanol, and for hexanal in backgrounds of citral or 1-hexanol.

In contrast, a mixture effect was evident for 1-hexanol (Fig. 2E). When bees were conditioned to 1-hexanol in a background of geraniol, the response level to 1-hexanol was significantly higher than when 1-hexanol was conditioned in a background of hexanal.

In an attempt to replicate the response pattern for hexanal and 1-hexanol, two additional groups of subjects were conditioned. They were tested only once each with the mixture and each of the components. One group was conditioned to a mixture of hexanal and 1-hexanol. Response levels during testing with hexanal, 1-hexanol or the mixture were: 62%, 23% and 50% ($\chi^2=5.13$, $N=13$, $P=0.07$, 2 d.f.), respectively. The pattern of response levels was the same as before (Fig. 2, right-hand column), but in this case the probability value was just above the level required for significance. A second group was conditioned to a mixture of octanal and 1-octanol, which have the same functional groups as hexanal and 1-hexanol, respectively, but contain carbon chains of different lengths. In this subject group, levels of response to octanal, 1-octanol or the mixture were identical in pattern to those to hexanal/1-hexanol mixtures reported above and were: 82%, 41% and 76% ($\chi^2=7.59$, $N=17$, $P<0.05$, 2 d.f.), respectively.

For several binary mixtures, there was no significant difference in response levels to the mixture or to the components and there was no significant effect of conditioning

background. However, consistent decrements and background effects could be detected for binary mixtures of an aliphatic aldehyde and a 1-alcohol of equal chain length, which might indicate that the mixture is perceptually more similar to the aldehyde than to the alcohol.

Blocking by prior reinforced experience with S1

Odor components could affect perception of binary mixtures through salience. One component, by virtue of being perceptually more salient, could hinder acquisition of a response to a second, less salient component. Indeed, Smith (1991) has shown that hexanal is a more salient stimulus than a short-chain alcohol similar to 1-hexanol, when tested under the same conditions that were used for the experiments above. If salience affects perception of components of binary mixtures, then it should be possible to induce a decrement of response to one component in mixtures that normally show no such decrement (i.e. citral/hexanal or geraniol/1-hexanol) by increasing the salience of one component prior to mixture conditioning. Such a change can be induced by prior conditioning to one component in a blocking paradigm (Table 1, group B).

Acquisition responses during phases I and II in the blocking and control groups were indistinguishable from those shown in Fig. 1. Acquisition was rapid and, in all groups in the blocking experiments described below, reached an asymptote of 60–80% of subjects responding within a few trials. Addition of a new component never significantly decreased responses in phase II of blocking groups. High response levels to S1 were registered through the test phase in all groups.

Responses to S2 during the test phase differed between blocking treatments and mixture treatments that were run in parallel to the blocking group. When hexanal was S1 and citral was S2 (Fig. 3A), a significant decrement was observed in response to citral (S2) in the blocking group compared with the responses to citral after conditioning to a hexanal/citral mixture in a mixture-only treatment group (i.e. Table 1, group A). When citral was S1 and hexanal was S2 (Fig. 3B), there was a significant, but less pronounced, decrement in response to hexanal (S2) relative to the mixture-only treatment group. Therefore, a significant blocking effect was observed in both groups.

An experiment was performed to replicate the results with hexanal as S1 and citral as S2 in a blocking group (Table 1, group B) and to incorporate an additional control group that received exposure to odor and the unconditioned stimulus equivalent to that in the blocking group. The control group of subjects, conditioned in parallel to the blocking group, received six phase I acquisition trials with 1-octanol (S3; Table 1, group C). The second phase was identical for the two groups (i.e. six trials with the citral/hexanal mixture). A true blocking effect should be specific to the former group but not to the latter (Rescorla and Holland, 1982; Rescorla, 1988).

Fig. 3C clearly shows a blocking effect. Response levels to S2 (citral) were significantly lower in the blocking group than in the control group, which had undergone preconditioning to 1-octanol in phase I. Preconditioning to hexanal was sufficient to block, or at least to hinder, acquisition to S2 in phase II.

If blocking is a general phenomenon of odorant processing, then it should not be limited to this particular mixture. An additional replication was carried out using a mixture of 1-hexanol/geraniol, the components of which elicit equal response levels after

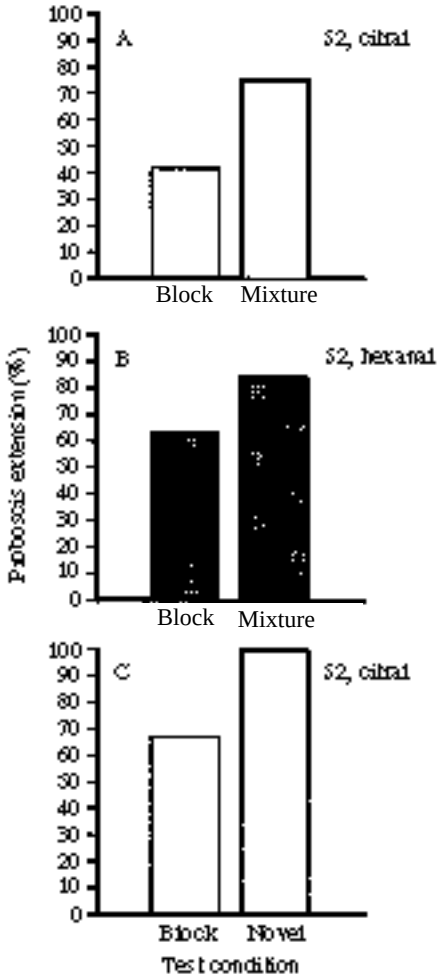

Fig. 3. Extinction responses to S2 (citral in A, C; hexanal in B) in groups given either a blocking treatment (Table 1, group B), mixture-only conditioning trials (Table 1, group A) or pretraining with the novel odorant 1-octanol (S3 in Table 1, group C). Three different experiments (corresponding to groups A, B and C) were performed in which two treatment groups were run in parallel. Bars in these graphs represent extinction responses of different subject groups to S2. The left-hand bar always shows response levels of groups that received blocking treatment; the right-hand bar shows response levels in groups that received no pretraining prior to mixture training or pretraining with S3. χ^2 comparison of the pairs of bars (1 d.f.) in each graph are: A, 5.96, $P<0.01$; B, 3.56, $P<0.05$; C, 7.20, $P<0.01$. Samples sizes for left and right columns in A–C are: 24, 28; 27, 32; 18, 18, respectively.

training in a mixture (Fig. 2). All three treatment and control groups were run in parallel (Table 1, groups A–C). Significant differences were observed among groups in response to S2 (Fig. 4). Subjects in the blocking group responded to S2 significantly less often than did subjects in the two control groups, which confirms the blocking results reported above.

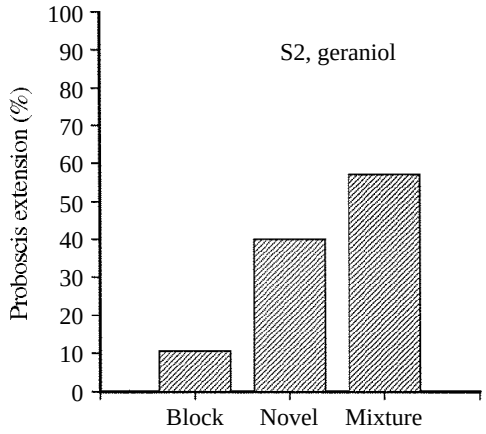

Fig. 4. Response levels to the S2 (geraniol) odorant in three subject groups conditioned in parallel and which received blocking treatment with S1, pretraining with a novel odorant S3, or no pretraining prior to mixture training. There is a significant difference between treatments (χ^2=9.49, P<0.01, 2 d.f.). Sample sizes for each column are from left to right: 15, 19 and 21.

The durations of S2 responses, determined from videotape, confirm the blocking results. The mean (± S.E.M.) response durations for the novel (N=14), block (N=19) and mixture-only (N=20) groups were: 1.88±0.68s, 0.51±0.30s and 4.27±0.98s, respectively. The means are significantly different based on non-parametric analysis of groups (Kruskal–Wallis; H=11.99, P<0.01). These means contain not only response durations for those subjects that responded to odor, but also mean durations for those individuals that did not respond (i.e. duration 0s). Differences among means may simply reflect different proportions of responders and non-responders. If, however, the analysis is run using only those subjects that responded to odor during the extinction trial, the differences are still maintained (H=8.66, P=0.01). In this case, mean durations for the novel (N=6), block (N=3) and mixture-only (N=12) groups were: 4.38±0.84s, 3.25±0.83s and 7.38±0.89s, respectively. Therefore, the build-up of response strength is reflected not only in response probability but also in duration, which is more sensitive to individual variation.

Dependence of blocking on type of S1 pre-exposure

The final experiment shows the specificity of the blocking effect to the type of pairing in phase I (Fig. 5). The response to S2 (geraniol) during the first of two extinction tests was uniformly high in groups that received no prior conditioning to S1 (mixture-only), backward pairing of S1 with sucrose or explicitly unpaired exposure to the two conditioning stimuli during phase I. The blocking group responded less to S2. This pattern of responses is maintained through the second extinction trial, although response levels to S2 in the backward pairing group were slightly lower in the first trial. Furthermore, these response patterns cannot be due to differing numbers of subjects having learned the association of S1 with sucrose during phase II, because virtually all

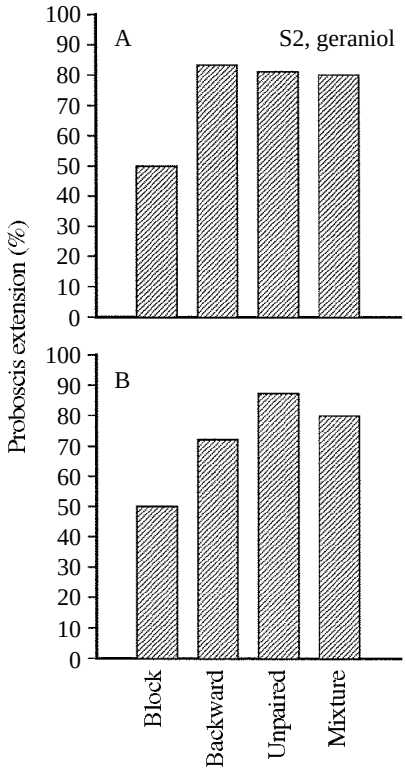

Fig. 5. Response levels to the S2 (geraniol) odorant in two different extinction trials (A, test 1; B, test 2) in each of four groups that differed in terms of the association of S1 with sucrose in phase I. Two groups (block and mixture) replicated earlier treatment groups (Figs 3, 4). One additional group received backward pairing of S1 and sucrose in phase I, and the remaining group received explicitly unpaired association of S1 and sucrose in phase I. All groups received identical pairing of the S1/S2 mixture with sucrose in phase II. There are significant differences between treatments in both A (χ^2=8.19, P<0.05, 3 d.f.) and B (χ^2=7.97, P<0.05, 3 d.f.). Sample sizes for each column are the same for A and B and are from left to right: 24, 18, 16 and 20.

subjects used in the data analyses responded to S1 during the third extinction trial (non-responders to S1 were selected out; see Materials and methods).

Discussion

Smith (1991) showed different levels of response to several odorants that were either identical, or similar, to those used in this study. After conditioning with a single forward pairing trial to citral, geraniol, hexanal or 2-hexanol, subjects responded less to the alcohols (geraniol and 2-hexanol) than to their corresponding aldehydes (citral and hexanal, respectively). These data could be explained as failures to learn (acquire) the information because of differences in information processing at one or more levels in the nervous system, beginning with the antennae. That is, associative strength to hexanal

might be built up faster than to 1- or 2-hexanol because more olfactory sensilla respond to hexanal. However, generalization assays indicated that the low response levels to geraniol may not have been due to a failure to acquire the association, but rather to failure to express learned information in the behavior assay. Therefore, olfactory learning abilities and/or deficits in the honeybee probably result from an interaction of the mechanisms involved in the acquisition (storage) of information and those involved in the retrieval of the information from long-term memory storage. An understanding of compound-stimulus processing can now contribute to an understanding of these mechanisms and, thus, to how information is processed in the honeybee's memory.

On the basis of earlier data, it was predicted that hexanal, because of its higher salience, would permit stronger acquisition than 1-hexanol (similar to 2-hexanol) when used for conditioning in a mixture. This prediction was confirmed. A lack of such an effect with other mixtures might be due to a variety of causes. For example, classification of responses into two discrete categories is not as sensitive as video-recording the response (Smith *et al.* 1991), or using electromyogram recordings (Rehder, 1987; Smith and Menzel, 1989*a*,*b*). Categorization of the response may simply produce a ceiling effect, above which no finer differences in response levels can be detected. Further analyses of responses to components of conditioned mixtures in which these response measures are employed should provide a more sensitive test of 'overshadowing' effects. Alternatively, mixtures that did not elicit asymmetric response patterns between components all contained components of the honeybee's Nasonov pheromone (Pickett *et al.* 1980). If these components are processed through a labeled-line subsystem, as are pheromone components in other insects (Boeckh *et al.* 1984; Christensen *et al.* 1989), then they may be more easily perceived in a binary mixture. More experimentation with a variety of floral odorants and known honeybee pheromone components would be necessary to test this hypothesis.

There are two broad classes of models that provide explanations for the decrement in the conditioned response to 1-hexanol when bees are conditioned to it in a hexanal background, relative to when they are conditioned to it in a geraniol background. The first regards generalization decrement (Rescorla and Holland, 1982; Macintosh, 1983). Assuming the two components and their mixture lie along one or more perceptual dimensions, then there will be a gradient of associative strength that tapers off along a dimension on either side of the mixture, so the response level to the components would depend upon where they lie along the dimension relative to the mixture. This model would explain both of the response patterns in Fig. 2; that is, both symmetrical and asymmetric levels of response to mixture components. For example, relatively low levels of response to a component that would otherwise elicit significant learned responses (e.g. 1-hexanol) would arise because the mixture is perceptually more similar to one component (hexanal) than to the other (1-hexanol).

Generalization decrement could occur if, for example, hexanal were to act as an inhibitor of sensory receptors that respond to 1-hexanol. Inhibition of sensory receptors is a well-described phenomenon (Akers and Getz, 1992; O'Connell, 1986; Kaissling *et al.* 1989). For example, hexanal may excite a population of receptor cells that is largely independent of receptors excited by 1-hexanol. If hexanal could also inhibit the 1-hexanol

receptor population by out-competing 1-hexanol for the receptor site without depolarizing the receptor cell membrane, then 1-hexanol might not be detected because its receptor population would not respond when presented with a mixture of the two odorants.

Other explanations for asymmetric response patterns are based on models that invoke processes of competition for attention to the conditioned stimulus (CS; Macintosh, 1983), change in the type of attention to the CS (Pearce and Hall, 1980), depreciation in the effectiveness of the reinforcer (Rescorla and Holland, 1982), several of these processes acting together (Wagner, 1981) or retrieval deficits (Spear *et al.* 1990). Unlike the perceptually based model referred to above, these models assume that individual odorants in binary mixtures can be independently perceived and processed, such that any mixture-unique property of the compound stimulus would be minimal.

There is empirical evidence that this assumption holds true in the insect olfactory system for some odorant combinations. Sensory cell recordings in insects have shown that receptor populations distinguish between alcohols and aldehydes at the peripheral level of processing. Sass (1978) has shown, from recordings of cockroach sensory receptors made during exposure to food odors, that receptors for short-chain alcohols (e.g. 1-hexanol) distinguish between the alcohols. In the honeybee, both specialist and more generalist receptor types can be identified for several odorants (Akers and Getz, 1992). Vareschi (1971) identified receptors for 1-octanol in the honeybee but could not find receptors responsive to octanal. Drones can be trained to respond to the types of short-chain aldehydes used in the present study (Bhagavan *et al.* 1994), so receptors for octanal must exist but represent a set of receptors different from those that respond to short-chain alcohols. Further support for this assumption, at least for certain odorant mixtures, comes from Getz and Smith (1990), who found that mixture-unique perceptual effects from binary mixtures might not be strong in honeybees.

It cannot be assumed *a priori* that odorant mixtures necessarily produce strong mixture-unique perceptual properties. Instead, the extent to which configurational properties dominate perception may vary depending on the particular odorants used. In the extreme, the effect of background on 1-hexanol conditioning might occur because one component is more effective at competing for the animal's attention or for effectiveness of the sucrose reinforcer, i.e. it is a more salient conditioning stimulus. The observation that associative strength is built up more rapidly in response to conditioning to hexanal (Smith, 1991) might support this interpretation.

The fact that blocking occurs in the olfactory system supports the contention that the latter class of models must be invoked to obtain a complete explanation of olfactory processing. More experiments are now needed to determine the effectiveness of blocking under different conditions. It appears from Figs 3–5, for example, that blocking treatments did not completely suppress responses to the blocked odorant; that is, acquisition to S2 may only be retarded and not completely blocked. Further experiments that appreciate or depreciate the value of the reinforcer, that vary the number of initial conditioning trials and that are designed to aid or hinder retrieval processes are now necessary to determine the exact nature of the blocking effect. Because the blocking phenomenon probably arose independently in invertebrates and vertebrates, these types

of studies are necessary to document the degree to which the mechanisms underlying blocking are the same or different in these divergent animal lineages.

Studies of compound stimulus processing in the honeybee using mixtures of colored visual cues and odors (Couvillon and Bitterman, 1982; Couvillon *et al.* 1983) or mechanosensory cues and odors (Menzel, 1990) failed to find blocking. In the former study, results were explained in terms of generalization decrement. However, interpretations of the lack of blocking in studies of color-odor compound stimuli, or in studies of compound stimuli consisting of odors and mechanosensory stimulation, may be confounded by the strong overshadowing effect that odors have over colors and mechanosensory stimuli. That is, under normal conditions, the two stimuli are not processed in an equivalent manner. Abramson and Bitterman (1986) found that pre-exposing honeybees to an aversive reinforcer retarded subsequent acquisition of the association of that reinforcer with a compound stimulus. Such latent inhibition could be due to a blocking effect that the background stimulation might have on a newly introduced compound stimulus.

Blocking has also been shown for olfactory processing in the snail *Limax maximus* (Sahley *et al.* 1981). Therefore, if the olfactory systems of gastropods and arthropods are structurally homologous, it would seem that the neural mechanism that gives rise to blocking in the invertebrate olfactory system might be phylogenetically ancient. Alternatively, if, as between vertebrates and invertebrates, the olfactory processing neuropils of the two groups arose independently, then blocking might be such an important ecological process relating to olfactory processing that it has evolved independently in two groups of invertebrates. Further comparative studies would be necessary to test hypotheses related to these observations.

In conclusion, the evidence reported above points to the need for more experimental analyses of olfactory processing in the honeybee, which shows analogies to learning processes such as blocking in vertebrates. Further work must now clarify the role of generalization decrement, attention to the conditioned stimulus and/or unconditioned stimulus and retrieval effects in olfactory processing. In general, comparative studies of olfactory processing in other invertebrates and in vertebrates can be expected to lead to insights into the generality and the adaptive role of these mechanisms.

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