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THE USE OF ELECTROMYOGRAM RECORDINGS TO QUANTIFY ODOURANT DISCRIMINATION IN THE HONEY BEE, *APIS MELLIFERA*

BRIAN H. SMITH* and RANDOLF MENZEL
Institut für Tierphysiologie der Freien Universität Berlin, Königin-Luise-Str. 28–30, D-1000 Berlin 33, F.R.G.

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Abstract—Proboscis extension conditioning of worker honey bees (*Apis mellifera carnica*) was employed to compare responses to several different conditioned odourants as well as to compare generalization responses to odourants to which they had not been exposed during conditioning (i.e. novel odourants). Quantification of the bees' responses was through analysis of the muscle potential evoked in the M-17 muscle, which operates during proboscis extension, retraction, and rhythmic movements of the glossa throughout the response (*J. Insect Physiol.* **33**, 501–507, 1987a). Electromyogram quantification allows for more accurate description of the graded nature of the response and demonstrates the usefulness of electromyogram recordings in the quantification of behaviour. Results show that, after an equivalent amount of conditioning, bees respond more strongly to some conditioned odourants than to others. Although responses occur to novel odourants, they depend on the combination of conditioned odourant and the novel odourant presented. Furthermore, generalization to novel odourants within the same chemical class as the conditioned odourants is usually, but not always, stronger than generalization to odourants of different classes.

Key Word Index: Electromyograms, honey bees, *Apis mellifera*, proboscis extension conditioning, odourant generalization

INTRODUCTION

A precise description of behavioural performance in the discrimination of stimuli is vital to an understanding of how a sensory system encodes and processes signals. Significant information is gained when subjects fail to discriminate stimuli that differ according to some defined parameter. Using proboscis extension conditioning (Kuwabara, 1957; Menzel *et al.*, 1974), Vareschi (1971) attempted to define the discriminatory capability of the honey bee's (*Apis mellifera*) olfactory system by conditioning bees to discriminate between different pairs of odourants. After using a large number of odourant pairs, it was evident that the bees learned the association between odourant and sugar water very quickly in most cases and failed to discriminate pairs of odourants in only a small number (4.5%) of cases. Thus the bee's peripheral olfactory receptors must be capable of encoding a large number of odourants, probably through a combination of cross fibre and labelled line firing patterns (Maes, 1984; Vareschi, 1971).

However, quantification of discrimination performance is limited by a researcher's ability to accurately describe the behaviour used to indicate discrimination. Recently, Rehder (1987a) described several response phases during proboscis extension in honey bees by recording the muscle potentials generated by the M-17 muscle, which extends from behind one eye to the ligular arm of the proboscis (Snodgrass, 1956). Characteristic firing patterns are correlated with feeding movements of the proboscis and can be divided into three distinct phases: extension, rhythmic licking, and retraction. The electromyogram recordings can be employed to accurately describe the variable nature of different aspects of the feeding motor programme under a range of stimulus conditions (Smith and Menzel, 1989). This technique offers the advantage that graded responses to a stimulus can be reliably quantified and used instead of visual registration of proboscis movement; the latter allows only a categorical (i.e. proboscis extended or not) distinction in classification of a bee's response.

We have used an olfactory *generalization* procedure to explore the reliability of electromyogram recordings in the quantification of graded response patterns during proboscis extension and to further explore the performance of the honey bee's olfactory system after olfactory conditioning. The experimental design is different from the discrimination conditioning procedure used by Vareschi (1971). In the discrimination procedure, exposure to an odourant is followed by feeding with sugar water; interspersed among such rewarded trials are unrewarded (i.e. no sugar water) trials with a different odourant. In the generalized procedure that we employ, worker honey bees are first conditioned to an odourant with several rewarded trials and then, during unrewarded trials, sequentially presented with a wide range of (novel) odourants, i.e. odourants to which the bees had not been exposed during conditioning. Our preliminary observations indicated that bees respond to the novel

*Send correspondence to: Brian H. Smith, ARL Division of Neurobiology, University of Arizona, 603 Gould-Simpson Science Building, Tucson, AZ 85721, U.S.A.

odourants, but that the tendency to respond depended on the combination of conditioning odourant and novel odourant. We therefore define generalization in this olfactory conditioning context as a measure of the *similarity* (not identity) of the neural signals encoding the stimuli. A non-zero, graded response to a novel odourant is not a failure in an absolute sense to discriminate it from the conditioning stimulus, but is instead indicative of a graded pattern of perceptual similarities among odourants.

MATERIALS AND METHODS

Worker honey bees (*Apis mellifera carnica*) were collected from a colony held in a flight room as they left their nest on foraging trips after 5 p.m. on the day prior to an experiment. Shortly thereafter, they were cooled to 5°C and then were fastened into metal harnesses by a strip of tape (placed between the head and thorax) fastened to the sides of the harness (Menzel *et al.*, 1974). After feeding with a 1.5 M sugar–water solution until satiation (the proboscis was withdrawn by the bee), the bees were stored overnight at room temperature in a dark, humid location. This feeding procedure minimized differences in motivational state among bees on the following test day; differences in motivation might have led to differences in the tendency to generalize among odourants.

The next morning each bee was fed a small droplet of sugar water. The head was then immobilized by placing a drop of molten bee's wax between the head and the tape strip. Each bee was then placed onto a small plexiglass stage, and two copper wire electrodes were inserted into the head through small holes made in the cuticle (Rehder 1987a). One was inserted just behind one eye at the top, where the attachment point for an M-17 muscle is found (Snodgrass, 1956). The indifferent electrode was inserted into the contralateral eye.

Twenty such stages were plugged into a horizontal wheel in a computer-controlled training machine; this machine turned bees one at a time into a training position where they were presented with the conditioning stimuli. An associative conditioning procedure used in proboscis extension reflex conditioning was then begun (Menzel *et al.*, 1974; Bitterman *et al.*, 1983). Shortly after being turned into the training position, an odourant (conditioned stimulus) was blown across the bee's head and antennae for 3 s. Starting 2 s after the onset of the odourant, the bee was allowed to feed on 1.5 M sugar–water for 6 s from a piece of filter paper attached to an automatic feeding machine. All bees received eight such conditioning trials spaced at regular intervals (about 12 min). If a bee initially responded with proboscis extension to the odourant alone on at least three successive trials during the eight conditioning trials, then a test series composed mostly of unrewarded trials was begun with this bee. Any bees that did not meet the three response criterion were not used in a subsequent test series (see below). Furthermore, during conditioning it was frequently evident that the quality of the electromyogram recording from a fraction of the bees on a given day was too poor. These bees (about 10–15% per day) were not used in a subsequent test series.

Twenty-microlitre syringes were used to deliver the odourants. During the 3 s odourant delivery period a 12 ml vol of odourant-laden air was blown out of the syringe. Each syringe was loaded with odourant fresh each day by placing one microlitre of the odourant onto an approx. 0.5 cm^3 piece of filter paper. The filter paper was pinned to the plunger of the syringe in order to prevent excessive odourant build-up at the syringe opening. At least 5 min between each use of a syringe was allowed for equilibration of the concentration.

The test series consisted of unrewarded tests with 21 novel odourants (i.e. odourants to which the bee had not been conditioned) presented in a different randomized sequence for each bee. The same intertrial interval as above was used. During the 21 unrewarded trials some bees also received one unrewarded trial with their conditioning odourant during which time the electrolyogram response was recorded. Additionally, rewarded trials with the conditioned odourant were variably spaced every 2–5 trials. Air alone was not included in the test series because the bees very rarely respond to it once conditioned to an odourant. After completion of the series of unrewarded tests, the bees were never reused in the experiment.

An unrewarded test consisted of the following procedure. While an odourant was presented exactly as above, the bee's response to the odourant in terms of the muscle potential generated by the M-17 was recorded (Rehder, 1987a; Smith and Menzel, 1989). The muscle potential was monitored by a window discriminator, which recorded a spike every time the potential exceeded a set threshold above the baseline. The response was divided into 1024 20-ms time windows, and the number of spikes occurring within each time window was stored on a computer disk for later analysis.

The data file for each bee was then analysed with a PASCAL programme that returned several parameters describing the bee's response. Of those parameters, only the total spike count for the response over the entire 20.48 s recording period will be used in the context of the present paper. This parameter reliably reflects length and vigour of a bee's response. (For a more detailed discussion of the different parameters from the electromyogram recording and their incorporation into the action pattern of the bees' feeding responses see Smith and Menzel, 1989).

Odourants used in this experiment were pure substances rather than mixtures, with the exception of citral, which is a 60:40 mixture of the isomers geranial and neral (Table 1). The rationale for choosing the substances was to present the bees with a series of compounds of varying degrees of similarity to any conditioned odourant. Thus, for example, when conditioned with an aliphatic aldehyde (Table 1) during the test series, the bee was presented with aliphatic aldehydes of different carbon chain lengths (i.e. chain lengths from five to eight) as well as substances with different oxygen moieties but the same chain lengths (e.g. 2° alcohols or 2° ketones).

Table 1. List of odourants which were used for conditioning and testing of bees; they are ordered into categories based on molecular structure

Aliphatic: aldehydes (ALD)	2° ketones (KET)	2° alcohols (ALC)
pentanal	2-pentanone	2-pentanol
hexanal	2-hexanone	2-hexanol
heptanal	2-heptanone	2-heptanol
octanal	2-octanone	2-octanol
	2-nonanone	2-nonanol
	Terpene:	
acetates (ACE)	alcohols (TALC)	aldehydes (CIT)
isopropyl	geraniol	citral
isobutyl	nerol	
isopentyl	farnesol	

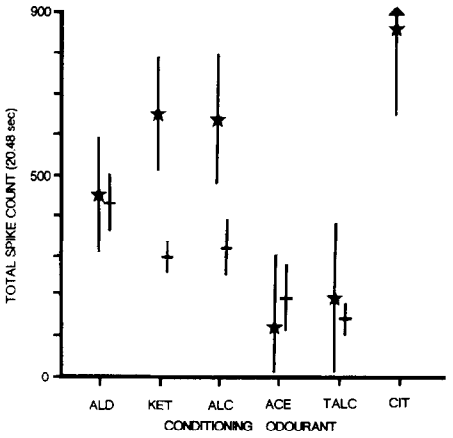

Fig. 1. Mean total spike counts and standard errors (vertical lines) for responses to a conditioning odourant (stars, displaced slightly to the left) or to a novel odourant (horizontal lines, displaced to the right) given that the bees had been conditioned to the odourants listed on the abscissa. Names (from Table 1) on the abscissa indicate statistically independent groups of bees conditioned to odourants from the indicated categories. The figure describes generalization from a conditioning odourant to other odourants within the same chemical class; i.e. it compares the unrewarded response to the odourant to which the bees had been conditioned to the unrewarded response to another odourant from the same chemical class. Numbers next to the means are the total number of measurements made from a minimun of 7 and a maximum of 15 bees; means and statistical measures were calculated by first averaging all responses from each individual bee together (see text). Differences among response to conditioning odorants is significant ($P < 0.01$, Kruskal–Wallis).

Because of widely varying distributions around the mean total spike count for each group, a non-parametric test (Kruskal–Wallis or Mann–Whitney-U tests) for statistical significance was performed with all sets of data (Sokal and Rohlf, 1982). Bees were tested with several novel odourants from the same chemical class. To obtain a single data point from each bee, which thereby ensured the independence among groups for statistical comparisons, multiple measurements (3–5) from the same bee were averaged together. This mean value was then used as the response of that bee to the novel odourant class.

RESULTS

There were no differences in the responses to the individual odourants within the same chemical class; i.e. no one aldehyde elicited a stronger response than the other aldehydes, and no acetate was more effective in eliciting a response than the other acetates, etc. Therefore, the data from tests with odourants from the same chemical class were lumped together.

Different conditioning odourants elicited different levels of responding when tested as unrewarded trials during a test series, and the degree of generalization from a conditioning odourant to a novel odourant within the same chemical class depended on the class from which the odourants were derived (Fig. 1). The abscissa in Fig. 1 shows different groups of bees that had been conditioned to one of the odourants from Table 1. The ordinate represents mean responses of the bees in each group when they were tested either with the same odourant (conditioning odourant, star) or with a novel odourant from the same chemical class as the conditioning odourant (horizontal line). Aldehyde, ketone, alcohol, and citral conditioning odourants elicited uniformly stronger responses (more spikes) than acetate and monoterpene alcohol conditioning odourants. Furthermore, responses toward novel aldehydes, acetates, or monoterpene alcohols did not differ from responses toward the respective conditioning odourants. That is, for these classes of novel odourants responses averaged about 100% that of the conditioning odourants even though differences in responding among the conditioning odourants was evident. For ketones and alcohols, response to novel odourants were significantly less than responses toward conditioning odourants. Therefore, alteration of carbon chain length has more of an effect on perceptual similarity for alcohols and ketones than it does for the remaining groups.

Several different patterns of generalization from the conditioned odourants to novel odourants from diverse chemical classes were evident as well. The first pattern is represented by responses toward novel aldehydes, ketones, or alcohols [Fig. 2(a)–(c), resp.], where generalization was strongest toward novel odourants from the same chemical class as the conditioning odourant. That is, a higher mean number of spikes was generated in the M-17 muscle when the bee was exposed to a novel odourant from the same chemical class as the conditioning odourant, which indicates that the proboscis extension response was longer in duration. Responses to a novel odourant when bees were conditioned to an odourant from another chemical class were always significantly lower than when the two odourants were from the same class.

Some differences in the patterns of generalization are also evident [Fig. 2(c)]. As above, responses to novel alcohols were highest when the conditioning odourant was another alcohol. However, when conditioning took place with other odourants, responses are heterogenous. For example, higher generalization to a novel alcohol odourant took place when bees were conditioned with a ketone than when conditioning was with one of the remaining odourants,

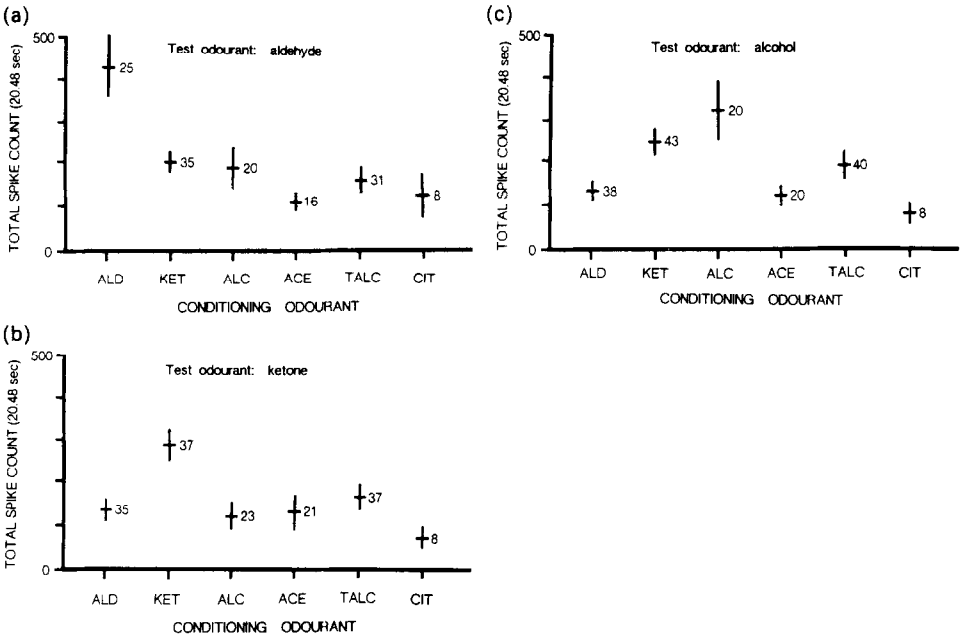

Fig. 2. Mean total spike counts (horizontal lines) and standard errors (vertical lines) for responses to a novel odourant given that the bees had been conditioned to the odourants listed on the abscissa. Names (from Table 1) on the abscissa indicate statistically independent groups of bees conditioned to odourants from the indicated categories. (a)–(c) Display on the ordinate responses to different novel odourants (Table 1), which are: aldehyde (a), ketone (b), and alcohol (c). For example, in (a) the mean indicated above ALD represents the response to a novel aldehyde after bees had been conditioned to an aldehyde, and the mean above KET indicates the response to an aldehyde after bees had been conditioned to a ketone, etc. Significant differences among means for the conditioning odourant groups are as follows: (a) ALD greater than all other means ($P < 0.01$); (b) KET greater than all other means ($P < 0.01$); (c) ALC greater than KET, which is greater than all other means ($P < 0.05$) (Kruskal–Wallis test; Sokal and Rohlf, 1982). Everything else as in Fig. 1.

not including the alcohol conditioning odourant group.

A second pattern of generalization occurred in responses to novel acetate odourants (Fig. 3), which elicited the same low level of response as an acetate conditioning odourant (Fig. 1). No significant differences existed in the responses to novel acetates across all conditioning odourants. The lack of significant differences among the conditioning odourants was due to the overall short responses toward acetates when bees were conditioned to them.

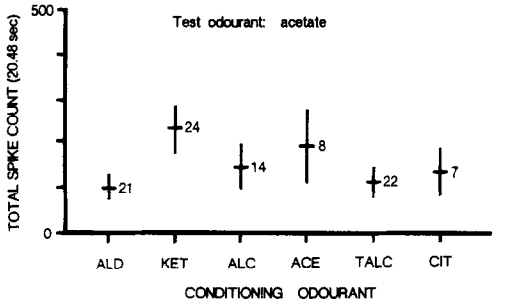

Fig. 3. Mean total spike counts (horizontal lines) and standard errors (vertical lines) for responses to novel acetate odourants given that the bees had been conditioned to the odourants listed on the abscissa. Names (from Table 1) on the abscissa indicate independent groups of bees conditioned to odourants from the indicated categories. No significant differences exist among the means ($P < 0.05$) (Kruskal–Wallis test; Sokal and Rohlf, 1982). Everything else as in Fig. 1.

Asymmetric responses occurred between certain odourant pairs as well. An asymmetric response was defined when the test response towards a novel odourant after conditioning to an odourant from a different chemical class was stronger than when the former odourant is conditioned and the latter tested. For example, after conditioning to a ketone, bees responded to a novel alcohol significantly more than when conditioned to other odourants [mean 245.9 spikes; Fig. 2(c)]. When conditioned to an alcohol, however, the response to a novel ketone was not different from the response after conditioning to other odourants [mean 129.6 spikes; Fig. 2(b)]. This latter response was 52.7% as strong as the response to an alcohol given ketone conditioning. Therefore, generalization from a ketone to an alcohol is greater than from an alcohol to a ketone.

A similar asymmetry exists between terpene alcohols and citral [Fig. 4(a) and (b)]. Strong responses toward novel terpene alcohols were elicited after conditioning with citral, but not when conditioning was with one of the other terpene alcohols. That is, honey bees generalized to a novel terpene alcohol much more readily from citral, an aldehyde, than they did from another terpene alcohol.

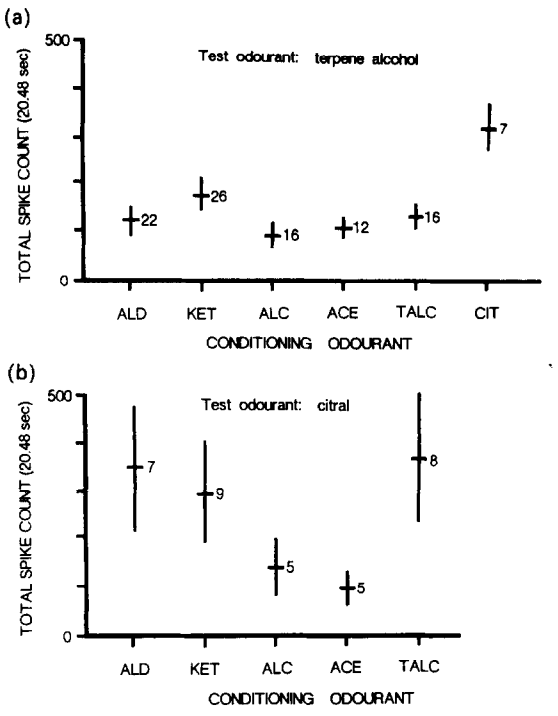

Fig. 4. Mean total spike counts (horizontal lines) and standard errors (vertical lines) for responses to novel odourants given that the bees had been conditioned to the odourants listed on the abscissa. (a) and (b) Display on the ordinate responses to different novel odourants (Table 1), which are: terpene alcohol (a) and citral (b). Names (from Table 1) on the abscissa indicate independent groups of bees conditioned to odourants from the indicated categories. No mean is presented above CIT on the abscissa in (b) because only a single odourant was tested from this category; thus there are no novel terpene aldehyde odourants given that bees were conditioned to citral. Significant differences among means for the conditoning odourant groups are as follows: (a) CIT greater than all other means ($P < 0.01$); (b) ALD, ALC, and TALC greater than all other means ($P < 0.01$) (Kruskal–Wallis test; Sokal and Rohlf, 1982). Everything else as in Fig. 1.

Strong responses to citral presented as a novel odourant were elicited by a broad spectrum of conditioning odourants, ranging from aliphatic aldehydes and ketones to terpene alcohols [Fig. 4(b)]. In fact, after conditioning with the terpene alcohols responses toward citral were significantly higher than responses towards terpene alcohol conditioning odourants themselves [Figs 1 and 4(b)].

DISCUSSION

Recordings of muscle potentials are a useful and practical means for quantification of graded responses patterns, and, therefore, greatly improve the collection of the kinds of data needed for the behavioural analysis of sensory coding. In the proboscis extension conditioning procedure, which has been successfully employed with flies and bees (Nelson, 1971; Menzel *et al.*, 1975; Maes and Bijpost, 1979; Holliday and Hirsch, 1986), the stardard means for registering the appearance of a response is visual confirmation of proboscis extension. However, exact quantification may at times be difficult because of partial or multiple extensions of the proboscis during tests. Electromyograms allow registration of several different aspects of the response that together allow for a more accurate quantification (Smith and Menzel, in press).

Some odourants are more salient conditioning stimuli for honey bees in that the conditioned response is stronger, as demonstrated by more spikes recorded in the electromyogram [compare the conditioned responses (stars) in Fig. 1]. When bees are tested with the odourant to which they are conditioned, all of the bees respond with proboscis extension; therefore, a discrimination among the conditioning stimuli treatments based solely on visual confirmation of proboscis extension would not have been possible. Differences in appetitive responses to conditioned odourants are most likely due to different lengths of time that the bees respond to a 3 s odourant exposure by extension and licking movements of the proboscis (Rehder, 1987a; Smith and Menzel, 1989) and most likely reflect differences in associative strength between the odourant and sugar water reward (Wagner, 1981).

Bees associate odourants with many different social and environmental stimuli (von Frisch, 1967; Seeley, 1985), including appetitive responses toward floral food sources. The bees used in our study came from a hive which had been kept for several months in a flight room away from floral stimuli normally experienced during foraging. Nevertheless, odourants to which they showed stronger conditioned responses in our study may have been those associated with stored food in their nest or with the bees' own pheromones (terpene odourants). However, an alternative hypothesis is that some odourants have been more reliably associated over evolutionary time with resources that are normally linked with a food reward. Therefore, although honey bees can associate a wide variety of odourants with a floral reward (Menzel, 1985), they may have become predisposed to do so with certain odourants much more quickly than others in order to more efficiently recognize resources (Koltermann, 1973).

Our odourant generalization data do not support the idea that bees use a simple decision rule to govern responses to novel odourants, such as, "in the absence of unrewarded trial information respond to all novel odourants equally". In most cases, novel odourants that are structurally similar to the conditioning odourant elicit stronger appetitive response than less similar odourants, which indicates that certain pairs of compounds are perceptually more similar than others. Although it is difficult to infer olfactory receptor response specificities directly from chemical structures of odourants, generalization among odourants categorized as we have done in Table 1 may reflect limitations in signal encoding by the primary olfactory receptors on the antennae. For example, due to the large number of potential olfactory stimuli that a bee may encounter, it would be impossible for each odourant to stimulate a set of primary olfactory sensilla that are specific for that compound only. Although a specialized neural subsystem exists for recognition of pheromones in many

insects (Christenson and Hildebrand, 1987), in most cases the central nervous system must discriminate patterns of receptor responses originating from a set of broadly tuned receptor cells. In fact, work with chemoreception in lobsters has shown that olfactory receptors may be so broadly tuned that each sensillum may exhibit almost a unique response spectrum for a given set of odourants (Atema *et al.*, 1988).

Vareschi (1971) found in honey bees several different olfactory receptor types that he was able to group into seven response spectra based on responses to test odourants. The responses of cells within any one spectrum to a set of odourants overlapped to a greater or lesser extent; most receptors responded to several, but not all, of the odourants defining a spectrum. There was no such overlap between spectra. Therefore, odourants can be ranked according to similarity based on the sets of olfactory receptors they stimulate. Odourants stimulating completely independent spectra of receptors would be perceptually the least similar, while the perceptual similarity of odourants stimulating a group of receptors from the same spectrum would depend on the degree of overlap of the responding receptors. In the latter case, discrimination among the odourants may be enhanced by higher-order neural processing (Getz and Chapmann, 1987).

It is most likely that honey bees could be trained to discriminate among the odourants used in our experiment if a discrimination conditioning procedure were used. Even in the few (4.5%) cases in Vareschi's work where no discrimination was evident, bees could discriminate those odourant pairs under longer or harsher conditioning regimes. Therefore, the gradient in generalization response recorded in the electromyogram analysis probably indicates initial perceptual *similarity* of the odourants to the bees. Such perceptual difficulties leading to generalization can be overcome by further differential experience with the odourant pair.

The theoretical and empirical information outlined above taken together with our data on odourant generalization enables us to make some preliminary predictions to guide further work with the set of odourants in Table 1. For example (1) when novel odourants from the same chemical class as the conditioning stimulus elicit the same level of responding as the stimulus (e.g. aldehydes), then the response spectra of olfactory receptors which respond to odourants from this class should overlap much more so than when novel odourants elicit a lower response level than the conditioning stimulus. (2) In instances where asymmetries in generalization result (e.g. novel terpene alcohol tests, or between alcohols and ketones), either the sets of receptors that respond to these odourants may asymmetrically overlap or the judgement process is biased in some way. In the first case, a greater percentage of the receptors responding to one odourant may be encompassed by the set of receptors responding to another odourant than vice versa (e.g. imagine a Venn diagram with two overlapping circles of unequal diameters). In the most extreme asymmetry, the set of receptors which respond to one odourant may make up a subset of the receptors which respond to the other. However, in a more complicated sense asymmetric responses may also indicate nonlinear changes in the response of a receptor to slight alterations in the chemical structure of an odourant.

If higher order judgements are involved, asymmetric responding may reflect less obvious decision rules governed by neural processing in more central areas in the brain (Erber *et al.*, 1980). Asymmetries can undoubtedly arise for several reasons, and the asymmetric response pattern documented between geraniol and citral may be somewhat more difficult to explain by invoking peripheral processing differences. Smith (unpublished) has shown that after a single conditioning trial with geraniol, honey bees respond much more strongly to citral than to the conditioning stimulus after memory consolidation has taken place. The data from our work with these compounds may, therefore, indicate that the bee's brain somehow differentially weights the different substances as it processes the information. For example, more receptors may be dedicated to detecting a given odourant, or a much stronger association may exist between the input fibres for a given odourant and fibres which are involved in activating the proboscis extension motor program in the suboesophageal ganglion (Rehder, 1987b). This weighting may reflect innate, imprinted, and learned preferences.

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