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SINGLE UNIT ANALYSIS OF ODOUR QUALITY CODING BY THE OLFACTORY ANTENNAL RECEPTOR SYSTEM OF THE COLORADO BEETLE

BY

WEI-CHUN MA and J. H. VISSER

Department of Entomology, Agricultural University, Wageningen, The Netherlands

Peripheral events underlying plant odour quality discrimination in the Colorado beetle were investigated. Responses to saturated and unsaturated C-6 alcohols and aldehydes ("green leaf" volatiles) and to other plant compounds were recorded extracellularly. The olfactory receptors could respond by inhibition and/or excitation; inhibition was especially observed at the higher stimulus intensities. Variations in the response patterns were analyzed for a number of receptors constituting a representative sample, as indicated by rank correlation between mean spike responses and electroantennogram amplitudes for each odour stimulus. Application of cluster analysis showed that the olfactory receptors may be divided into two main groups, one reacting differentially to the complex of "green leaf" volatiles and their isomers, and another group showing a selective sensitivity to the aromatic compound methylsalicylate. Evidence is provided suggesting that individual olfactory sensilla can be innervated by more than one functional type of receptor unit. Responses to natural plant odours support the conclusion that plant odour quality is encoded by response patterning across a limited number of olfactory receptor types. The findings are discussed in relation to concepts of chemosensory coding and to the behaviour of the Colorado beetle.

Many authors have concluded from behaviour studies that the adult Colorado beetle, *Leptinotarsa decemlineata* Say, possesses a sense of smell which allows it to distinguish between the odour of its solanaceous hosts and that of non-solanaceous plants (Visser & Nielsen, 1977). It has been suggested that the so-called "green leaf" volatiles, consisting of saturated and unsaturated C-6 alcohols and aldehydes, play a significant rôle in the process of host odour recognition in this insect (Visser & Avé, 1978). In fact, plant odour constituents eliciting strong electroantennogram responses (EAGs) largely comprise this group of volatile chemicals (Visser, 1978).

The present work focusses attention on the problem of odour quality coding, that is, the manner in which quantitative and qualitative differences in the composition of plant odours are detected. Input patterns analyzed for single antennal receptor cells (units) suggest that the Colorado beetle has a considerable sensory capacity of plant odour discrimination.

MATERIALS AND METHODS

Young female Colorado beetles reared on potato foliage under long-day conditions were used throughout the experiments. Individuals with legs removed were attached dorsally to a cork base with a mixture of beeswax and colophonium. Extracellular recordings from olfactory receptors were made with electrolytically sharpened tungsten microelectrodes, using the method described by Boeckh (1962) with conventional apparatus for signal amplification and registration.

Air, purified through activated charcoal, was blown through two glass cartridges made of pasteur pipettes, one of which was loaded with a 6.0 × 0.5 cm strip of filter paper. The test chemicals were dissolved in paraffin oil (Merck, Uvasol) and pipetted in 25 μl quantities on to the filter paper strip. The common outlet of both cartridges (1.5 mm internal diameter) was positioned by micromanipulators at a distance of 5-8 mm from the tip of the recording microelectrode. The delivery of an odour puff of one second duration at a rate of 1 ml/sec was alternated with a constant flow of purified air. The alternation of flow was controlled by electromagnetic valves operated through a timer which also provided a 50 Hz signal as a marker of stimulus application.

The number of molecules per ml air leaving the outlet was determined from direct injection into a gas chromatograph fitted with an open stainless steel tube. The time course of the stimulus is shown in Fig. 2 as the signal of the flame ionization detector to cis-3-hexen-1-ol. Calibration curves for a number of compounds are given in Fig. 1. For the sake of convenience the stimulus intensities are referred to as the dilution step (v/v) in paraffin oil of the chemical inside the odour cartridge.

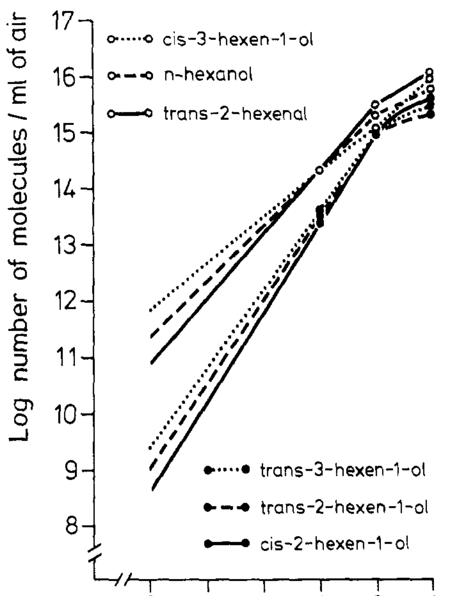

Fig. 1. Calibration of stimulus delivery system showing number of odour molecules per ml air for concentrations (v/v) of compounds in paraffin oil.

The chemicals selected for study had a known EAG activity (Visser, 1978), and include compounds belonging to the complex of "green leaf" volatiles, viz. cis-3-hexen-1-ol, trans-2-hexen-1-ol, n-hexanol, trans-2-hexenal, hexanal and cis-3-hexenyl acetate, and their isomers cis-2-hexen-1-ol, trans-3-hexen-1-ol, hexanol-2 and hexanol-3. The monoterpene alcohol linalool, the aromatic compound methylsalicylate and the monoterpene α-pinene were also studied. All chemicals were commercially obtained and checked for purity by gas liquid chromatography. Most compounds had a purity of greater than 97%, except for hexanol-3 (96%) and α-pinene (95%).

The response magnitude of a receptor unit was evaluated as the number of nerve impulses generated in an interval of 6 sec including the period of maximum spike density. A correction was made for the level of spontaneous discharge by deducting the number of nerve impulses over a similar 6-sec period preceding stimulus delivery. In experiments involving serial stimulation an interstimulus time of at least 2.5 min was imposed, while all trials were arranged in a random order.

RESULTS

The olfactory receptor neurones associated with the sensilla basiconica on the antennae of the Colorado beetle displayed a spontaneous spike discharge which may vary considerably between individual receptors. The level of spontaneous activity appeared related to the type of receptor concerned. Thus, receptors which normally responded with an increase of neural activity showed constant spontaneous discharges which varied between 0.2 to about 5 impulses per sec (Fig. 2). Receptors responding with inhibition exclusively, and hence classified as inhibitory receptors, exhibited much higher levels of spontaneous activity of between 10 to 20 impulses per s. About 20% of a total number of approximately 100 receptors investigated were of the inhibitory type. They could be differentiated as receptors responding (1) to the C-6 alcohols only, and (2) to the aldehydes as well as the alcohols. These units had a response threshold of at least two log steps higher than the excitatory types of receptors. Typical complete cessation of spike activity such as shown in Fig. 2, was never recorded at stimulus intensities lower than 10^{-2}. Inhibition was occasionally observed to be followed by delayed excitation.

The inhibitory types of olfactory receptor are difficult to detect when the spontaneous level of activity is low, so that there may have been inhibition in those receptors that showed no discernable alteration in basal firing rate with any of the odour stimuli. This was the case for about half of the total number of recorded receptors. The significance of these non-responding receptors is not clear. Apart from the possibility of being hidden inhibitory receptors, it could be that none of the stimuli were of an adequate type. It is also possible, although less likely, that during the implantation of the microelectrode the functional properties of the receptor cell were affected.

In the following analysis attention has been concentrated on the excitatory type of olfactory receptor units. This type reacts to most of the odour stimuli by

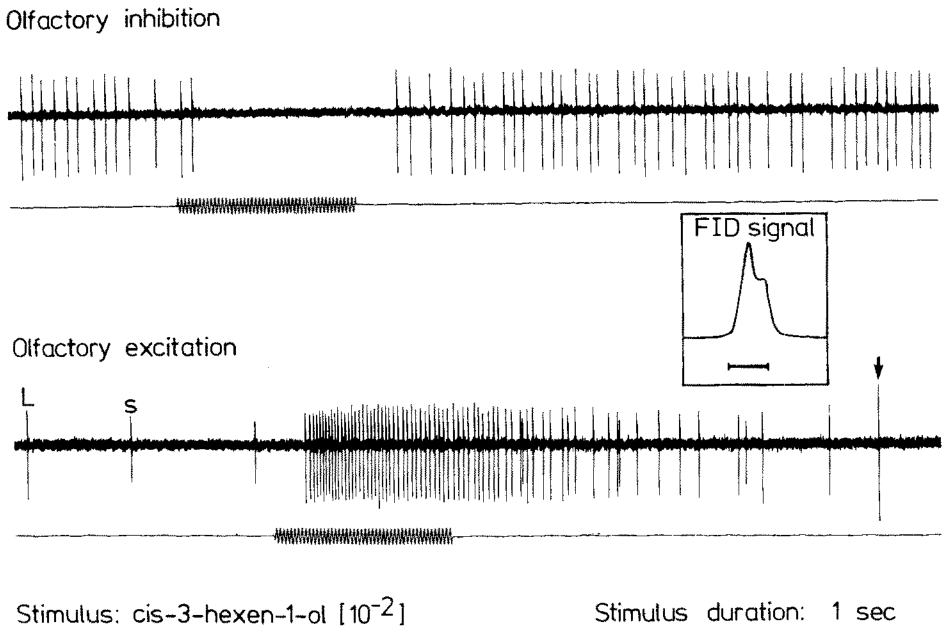

Fig. 2. Inhibitory and excitatory types of response of olfactory antennal receptor cells. Inset shows time course of stimulus as monitored by flame ionization detector (FID). Note firing of two receptor units ("L"and "s") in second trace; arrow points to coincidence of the two spike types. 50 Hz signal marks period of stimulus delivery.

excitation (Fig. 2), although instances of inhibition in the same unit may occur as well. The location of the recording sites was chosen randomly on the three terminal antennal segments for a sample of 25 units. Since each recorded unit was from a different beetle the group of receptors can be regarded as a truly random sample of the total receptor population present on these antennal segments. Virtually all the basiconic olfactory sensilla are concentrated on the five terminal segments (Schanz, 1953).

The stimulus intensity was set at a standard concentration of 10^{-2} in paraffin oil. The choice of this relatively high intensity was based on the consideration that at the higher intensities the stimulus-response curves are more reliably separated from each other. An example of stimulus-response relations for different odour stimuli is given in Fig. 3. The lowest threshold values measured for single receptor cells were at 10^{-6} concentration, determined for cis-3-hexen-1-ol and trans-2-hexen-1-ol. This would correspond to threshold values of approximately 10^{12} and 10^{9} molecules per ml air, respectively (Fig. 1).

For each test chemical, the average response magnitude was calculated from three to four trials for the entire sample of olfactory receptors. The data were analyzed using a multivariate statistical method for sorting into groups with a high similarity among spectral characteristics (Blackith & Reyment, 1971). Fig. 4 shows

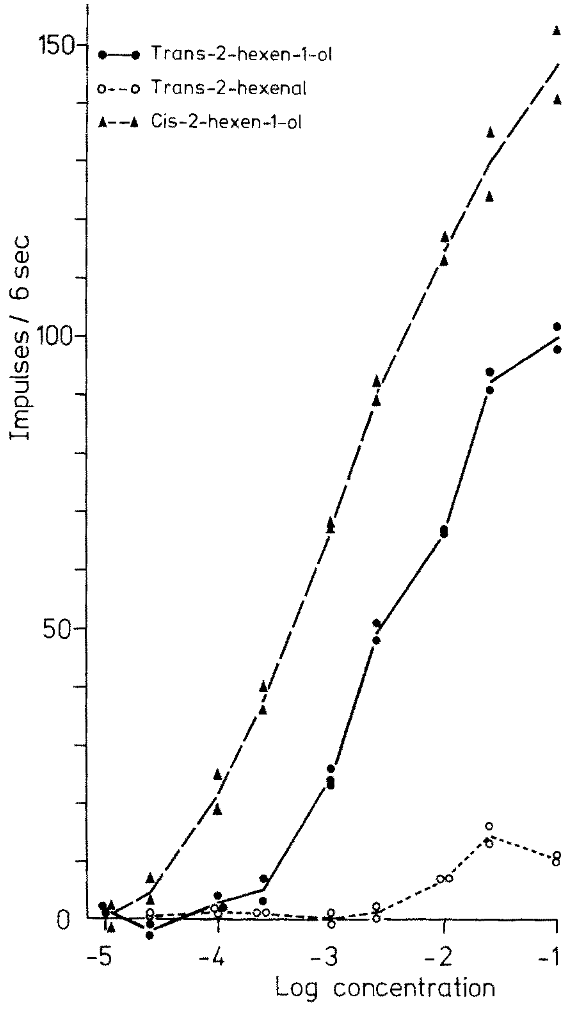

Fig. 3. Stimulus-response functions for three odour compounds for one single olfactory receptor unit stimulated at various intensities. Each point represents one trial.

the result of an agglomerative analysis, by way of a two-dimensional hierarchically branched figure, indicating a reasonable grouping when taking into account the degree of overlap of the various spectra. In Fig. 5, distinguishable groups have been visualized by presenting the average response spectrum per group. The grouping obtained can be considered as carrying a predictive value for the existence of separate receptor types, designated here as types A, B, C, D and E. To facilitate a comparison of the various types the response values in Fig. 5 have been computed as percentages of the highest value measured.

According to the respective response profiles, receptor type E is separated from the other four by having a selective sensitivity for the aromatic compound methylsalicylate. The other types are insensitive to this chemical, but show a

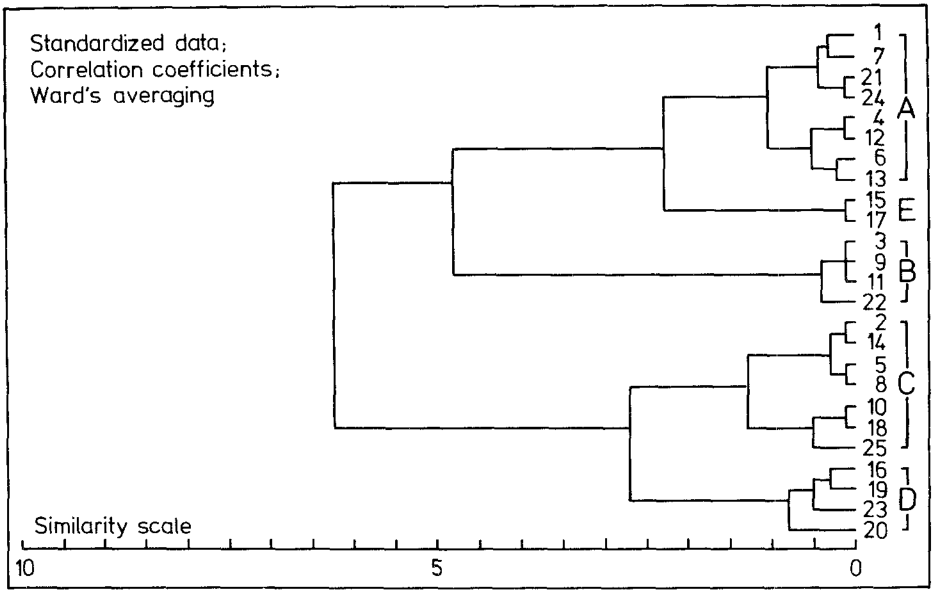

Fig. 4. Ward average cluster analysis based on correlation coefficients among 25 individual response spectra. Similarity scale indicates increasing similarity from left to right.

differential sensitivity to the array of "green leaf" volatiles and their isomers. Among these receptors, type A possesses the broadest spectrum by responding strongly to both the cis and trans isomers of 2-hexen-1-ol and 3-hexen-1-ol, as well as to n-hexanol. This type responds to the positional isomers n-hexanol, hexanol-2 and hexanol-3 in a characteristically decreasing order of magnitude; it does not show a significant sensitivity to the C-6 aldehydes. It is interesting to note that one of the above-mentioned inhibitory types of receptor forms in fact a mirror-image of the type A unit, except for the lower response threshold of the latter.

Receptor types B, C and D have more narrowly tuned reaction spectra; for example, both B and C have a low sensitivity to the cis form, but a high responsiveness to the trans-isomeric form of the unsaturated C-6 alcohols. On the other hand, type D shows a preferential sensitivity to cis-3-hexen-1-ol only. Another salient feature of the type D receptor is te time course of the response to cis-3-hexen-1-old. After a one second stimulus at concentrations of 10^{-2} or higher these receptors continue firing almost tonically and require as long as 5-10 minutes before reaching baseline level. This slow decrement of the excited state occurred in spite of the constant flow of purified air over the preparation. In the other receptor types similar phasic-tonic discharges returned to resting activity much more rapidly within one or, at most, two minutes.

The reaction patterns further show that a sensitivity to the C-6 aldehydes trans-2-hexenal and hexanal is confined to receptor type C only. Sensitivity peaks for the saturated alcohols hexanol-2 and hexanol-3 are localized in type B, while a responsiveness to cis-3-hexenyl acetate is linked exclusively to receptor cell type D.

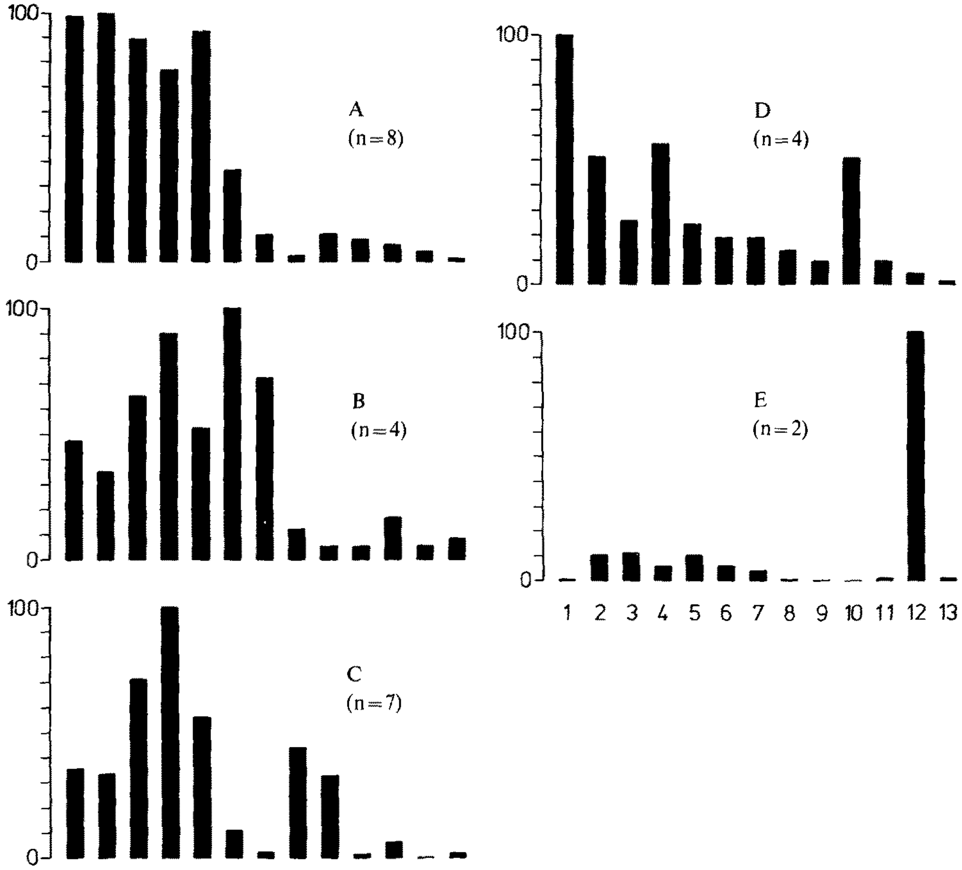

Fig. 5. Relative reaction spectra of receptor types A-E derived from grouping indicated in Fig. 4. The odour stimuli numbered: (1) cis-3-hexen-1-ol; (2) cis-2-hexen-1-ol; (3) trans-3-hexen-1-ol; (4) trans-2-hexen-1-ol; (5) n-hexanol; (6) hexanol-2; (7) hexanol-3; (8) trans-2-hexenal; (9) hexanal; (10) cis-3-hexenyl acetate; (11) linalool; (12) methylsalicylate; (13) α-pinene.

In order to estimate whether the sample studied was large enough to be considered as representative for the total receptor population on the antenna the average response values of the total sample to each odour stimulus were plotted against corresponding values of EAG responses. The reasoning behind this procedure is that the EAG represents the summated evoked receptor potentials from the majority of olfactory cells present in the antenna (Schneider, 1957; Boeckh *et al.*, 1965), while each receptor potential is related to the triggering of the impulse discharge of the afferent nerve (Boeckh *et al.*, 1965; Kaissling, 1971).

As shown in Fig. 6 a reasonable degree of correlation between the single unit responses and the corresponding EAG's was observed with no exception for all of the thirteen odour stimuli investigated. The correlation computed as Spearman's coefficient of rank correlation was determined at $r_s = 0.9135$. It should be noted that the stimulus intensities shown in the ordinate of Fig. 6 should in fact be ten times lower. This is because in the EAG measurements the odour stimulus was injected into a glass tube carrying a continuous air stream rather than applied directly onto the antenna as in the single unit study. As a result the intensity of the odour stimulus in the EAG study can be calculated as being diluted ten times in the air passing over the antenna. In the left half of Fig. 6 the stimulus intensity indicated for the EAG data therefore is a hundred times lower than the concentration shown in the abscissa. Even so, the rank correlation remained highly significant ($r_s = 0.8242$, $P<0.01$). Consequently, the assumption that the sample of receptor units investigated is reasonably representative for the total antennal receptor population seems justified.

Depending on the depth of penetration of the sensillum base by the recording microelectrode the nervous activity from several neighbouring receptor cells can be picked up. Such multiple unit recordings can be analyzed from amplitude discrimination of the nerve impulses and offers the considerable advantage of permitting a direct comparison of receptor units without having to consider experimental recording conditions as a source of variability. In order to exclude any risk of confusion of the origin of the nerve impulses only those recordings were considered which allowed a clear distinction between the activity of not more than

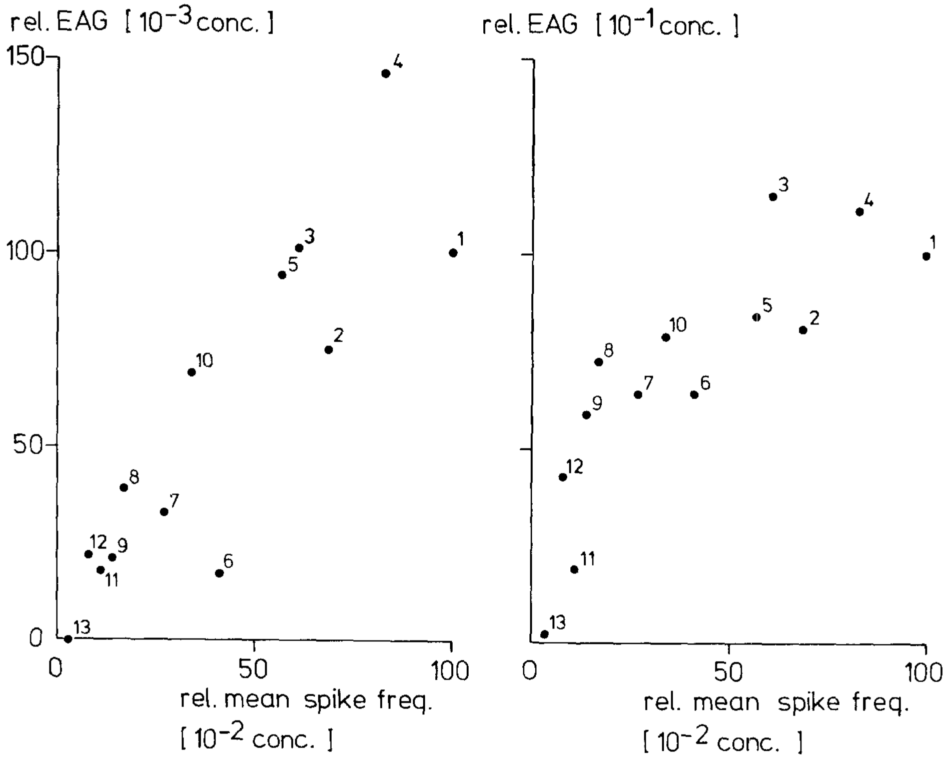

Fig. 6. Scatter diagram for average total evoked activity of nerve impulses and electroantennogram response as determined for series of odour stimuli listed in Fig. 5.

two units. In such recordings the unit firing with the largest spike was designated as the "L" cell, while its counterpart was named the "s" cell (Fig. 2).

In Fig. 7 the results of two of such simultaneous recordings, each obtained from a

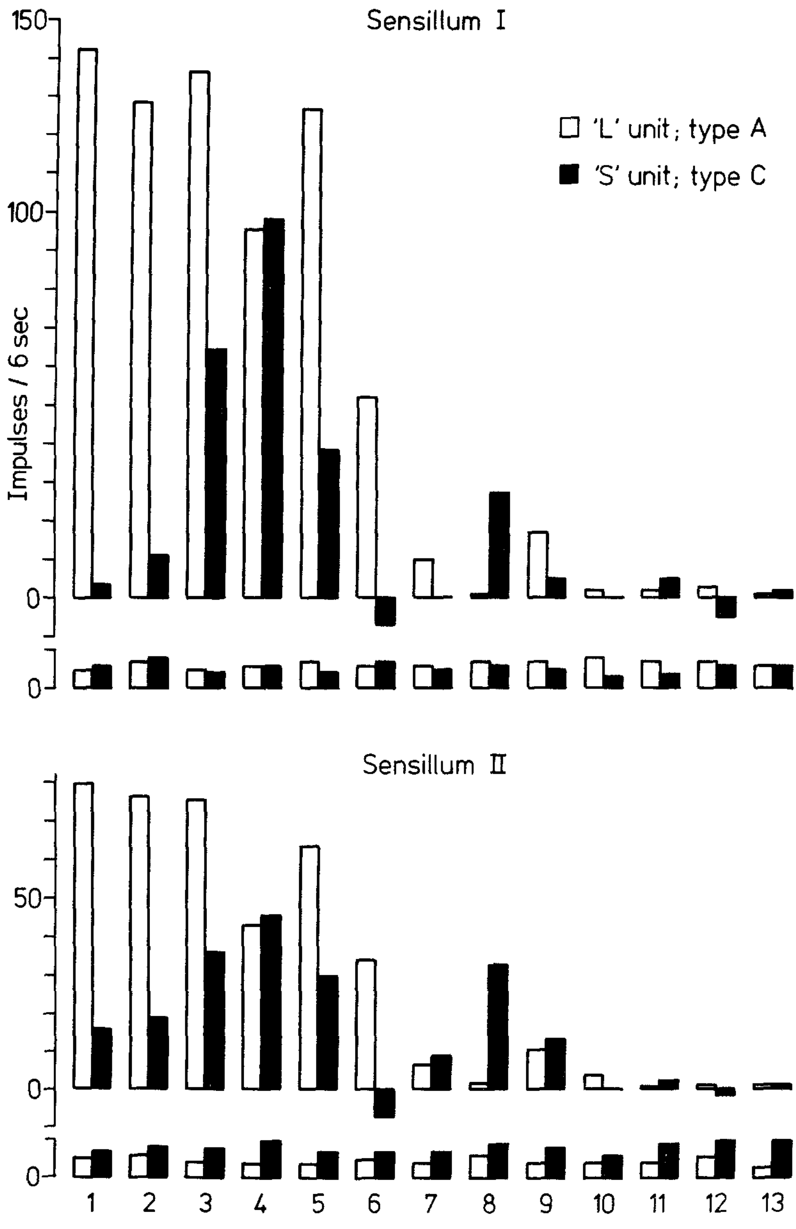

Fig. 7. Reaction spectra of two pairs of simultaneously recorded receptor units. Numbering of the odour stimuli same as in Fig. 5. Level of spontaneous neural activity preceding stimulus delivery is indicated under each set of spectra.

different beetle, are compared. The sensilla concerned were located on opposite sides of the annular groove of the terminal segment of the antenna. Although the general level of excitability appears considerably higher for the first than for the second sensillum, the response profiles of the paired units nonetheless remain qualitatively similar. In both cases one unit of each pair conformed to type A of the classification shown in Fig. 5 whereas the other corresponded to type C. A most significant feature, however, is the fact that between the two sensilla more or less similar ratios are observed for the "L" and "s" receptor responses.

It seems reasonable to conclude that a single sensillum can be innervated by receptor cells belonging to different types. Several combinations seem possible. Thus, the simultaneous neural activity was recorded from two cells which according to their reaction pattern, were identified as a type D unit showing a high sensitivity to cis-3-hexen-1-ol, and a type E unit with a specific sensitivity to methylsalicylate. In fact the latter receptor was inhibited by stimuli other than this aromatic compound. This specific pair of receptors was investigated for its responses to natural plant odours. To this end, equal pieces of fresh leaves of seven different plant species were crushed onto the test filter paper strip placed inside the odour cartridge of the stimulus delivery system, and the responses of the receptors to the emitted odour recorded immediately afterwards. The plant species tested included Solanaceae as well as non-Solanaceae (Fig. 8). The results show that each leaf odour evokes a certain ratio of activity between the two types of olfactory receptor units. The response ratios elicited by the three *Solanum* species were distinctly different from the proportions found for the other plants. Interestingly, only the odour emanating from crushed tomato leaves elicited a high neural activity in the type E unit. Since this type was specifically responsive to methylsalicylate it seems possible that the receptor had reacted to a significant above-threshold occurrence of this or some closely related chemical in the leaf odour of tomato plants (cf. van Straten, 1977). Thus, even with the combination of only two receptor types, a certain level of discriminatory capacity would be achieved. This pair of units would, for example, be able to discriminate the odour of crushed leaves of tomato from that of other Solanaceae, or, distinguish between the odour of Solanaceae and that of certain non-Solanaceae.

DISCUSSION

Plant odours encountered by insects in nature provide a complex of olfactory information, the perception of which involves an array of peripheral factors. These have been summarized by Dethier (1977) as: the number and kinds of specifically different receptors, band width of each receptor, character of each tuning curve, absolute threshold and rate of adaptation, and synergism and inhibition. Because of possible interactions between stimuli at the peripheral level the analysis of chemosensory input becomes even more complicated. Any analysis using pure compounds therefore only provides a simplified picture of the actual situation. Dethier (1977) rightly stated that such an approach "may provide an exaggerated picture in the sense that they overemphasize the rôle of labeled lines in (gustatory) perception or a diminished one in the sense that by implication they oversimplify complexities of peripheral and central integration". However, because of the extreme complexities involved in the perception of natural odours or tastes, it may be profitable first to analyze the perceptual capacity of the sensory system using the combination of single receptors and pure compounds. Once the basic properties have been established, one may then endeavour to modulate the picture with increasingly complex stimulus conditions. Such an approach to the analysis of chemosensory coding seems particularly suitable for insects, where it may often be possible to relate responses from single receptor cells to quantifiable, graded behaviour output, as has been demonstrated, for example, for the gustatory perception in caterpillars (Ma, 1972).

The present work concerns such a pilot study of the perceptual coding of plant odours at the primary receptor level in the Colorado beetle. Peripheral effects of lateral interactions and mixed stimuli have not yet been examined, so that the picture presented obviously must be a simplified one. The antennal olfactory receptor system offers the considerable advantage of a prior selection of relevant chemical stimuli on the basis of electroantennogram responses. In the Colorado

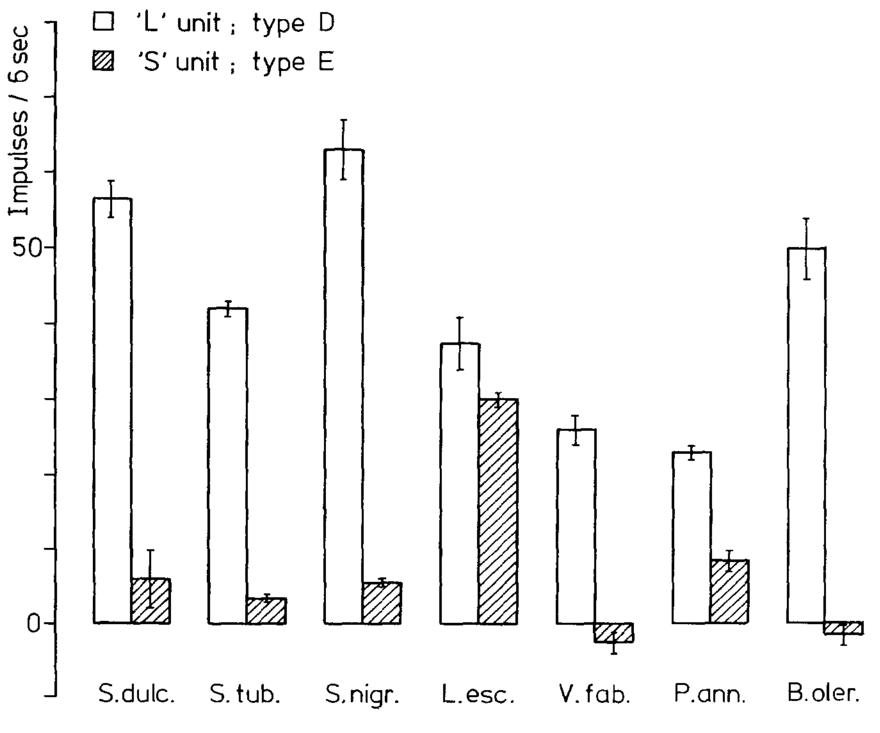

Fig. 8. Response magnitudes of two simultaneously recorded receptor units when stimulated with odour of following plant spp: *Solanum dulcamara* (S. dulc.); *S. tuberosum* (S. tub.); *S. nigrum* (S. nigr.); *Lycopersicon esculentum* (L. esc.); *Vicia faba* (V. fab.); *Poa annua* (P. ann.); and *Brassica oleracea* (B. oler.). Spontaneous discharge level 1-3 imp./s in "L" unit and 5-9 imp./s in "s" unit. Mean values from two trials per plant species.

beetle such studies so far indicate that the antennal receptor system has a strong filtering effect, with effective plant volatiles being largely restricted to "green leaf" volatiles (Visser, 1978). The results of the present study show that the Colorado beetle has a well-developed sensory capacity of quality discrimination of odour complexes that include "green leaf" volatiles, and possibly other chemicals as well.

Olfactory receptors responding to "green leaf" volatiles have also been described in *Locusta* antennae (Boeckh, 1967; 1974), and their properties worked out in great detail by Kafka (1970). Large differences have been noted between the individual receptor cells in *Locusta* with respect to the rank order of effective compounds, to the general sensitivity, and to the shape and the general steepness of the stimulus-response curves (Kafka, 1970, quoted in Kaissling, 1971). It would be interesting to investigate whether these differences can be clarified by the existence of "green odour" receptor types, with differential sensitivity spectra, similar to those which have been demonstrated in the present study for the Colorado beetle.

The range of chemical volatiles studied does not necessarily include all active constituents of the total complex of plant volatiles which may be perceived by the Colorado beetle. In fact, many recorded receptor neurones showed no significant change of resting activity to any of the odour stimuli applied. The question whether these receptors are specifically tuned to as yet unidentified components of the host plant odour has to await detailed chemical analysis of the odour composition. Insofar as the presently proposed system is concerned, it remains possible that with an extended sample additional receptor types may be found. However, the results of the rank correlation study (Fig. 6) suggest that a representative section of the total receptor population has been covered. The rank correlation is of special importance with regard to weakly EAG-active odour stimuli. Specific olfactory units that are relatively scarcely represented in the total receptor population obviously have a smaller chance of being selected in a limited random sample. Thus, the type E receptors constitute only 8% of the sample studied compared with 32% of type A (Fig. 5). Therefore, with regard to the total information coding, variations in the quantitative representation of the various receptor types have to be taken into account as well.

A basic and concrete example for understanding the mechanism of odour quality coding is given by the results shown in Figs. 7 and 8. These results, if extrapolated to the numerous olfactory receptor cells in the antenna, illustrate how each plant odour evokes a definite response pattern across the various receptor types, some of which are excited, others remaining indifferent, and still others inhibited. Thus, only a definite response patterning may convey the message for attractiveness of a plant odour. This idea is supported by behaviour observations which show that even a slight increase of the concentration of one "green leaf" volatile can reduce the attractiveness of the host plant odour for the Colorado beetle (Visser & Avé, 1978).

Odours from some non-host plants may evoke a similar response patterning to those emanating from host plants. Since the "green leaf" volatiles are common in nature the chance that the odour composition of a non-host plant resembles the attractive odour of the host plant cannot be regarded as negligible. Moreover, comparable response patterns in one and the same set of receptors may be produced by different stimuli at different intensities. Therefore, it is not surprising that, apart from the solanaceous plants, some non-solanaceous species are also able to attract the Colorado beetle, as has been shown in wind-tunnel tests (de Wilde *et al.*, 1969; de Wilde, 1976; Visser & Nielsen, 1976). Thus, the idea of the total pattern or "Gestalt", as the significant parameter, as expounded by Dethier (1974) and reviewed by Städler (1976) for insect chemoperception, applies to olfaction in the Colorado beetle as well. It will be interesting to investigate the manner in which the primary input converges onto the higher order neurones in the deutocerebrum, as has been studied recently in other insects (*e.g.* Boeckh, 1974). This would yield information on whether the across-fibre patterning concept would actually extend to the level of the central nervous system. The results of the present work emphasize the potential of graded input patterning as a sensory basis for host odour discrimination in the Colorado beetle.

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RÉSUMÉ

ANALYSE CELLULAIRE DU CODAGE DE LA QUALITÉ DES ODEURS PAR LE SYSTÈME DE RÉCEPTEURS ANTENNAIRES OLFACTIFS CHEZ LE DORYPHORE

On a étudié la capacité de distinguer la qualité des odeurs végétales chez le doryphore. On a enregistré extracellulairement les réactions de cellules de récepteur olfactif séparées à des C-6 alcools et des aldéhydes saturés et insaturés (substances volatiles de la "feuille verte") et à d'autres composés végétaux. Les récepteurs olfactifs pouvaient réagir par inhibition et/ou par excitation; on a particulièrement observé l'inhibition aux intensités élevées de stimulus. Les variations des types de réactions ont été analysées pour un certain nombre de récepteurs, qui constituent un échantillon représentatif, sur la base de la corrélation de rang entre les réactions de pointe moyennes et les amplitudes de l'électroantennogramme pour chaque odeur-stimulus. L'application d'une analyse en grappe a montré que les récepteurs olfactifs peuvent être divisés en deux groupes principaux, un groupe qui réagit de façon différentielle au complexe des substances volatiles de la "feuille verte" et de leurs isomères et un autre groupe qui a une sensibilité sélective à l'égard du composé aromatique méthylsalicylate. Le premier groupe a été subdivisé en différents types de récepteurs avec des réactions qui se chevauchent. Il semble que les sensilles olfactives individuelles peuvent être innervées par plus d'un type fonctionnel de cellule réceptrice. Les réactions aux odeurs végétales naturelles enregistrées appuient la conclusion que la qualité d'une odeur végétale est codée par un modèle de réaction pour un nombre limité de types de récepteurs olfactifs. On discute les résultats par rapport aux concepts du codage chimiosensoriel et par rapport au comportement du doryphore.

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