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Discrimination of oilseed rape volatiles by the honeybee: combined chemical and biological approaches

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Abstract

Honeybees (*Apis mellifera* L.) were individually subjected to a classical conditioning procedure in order to obtain an olfactory conditioned proboscis extension response. To relate the behavioural response directly to antennal detection abilities, a technique was developped for coupling proboscis extension responses and electroantennogram recordings, with the stimulation being provided by the effluent of a gas chromatograph (GC). Bees were conditioned with a six-component mixture being part of oilseed rape (*Brassica napus* L.) floral volatiles, and tested with the individual components separated by GC. Responses of the conditioned bees were compared to those of unconditioned bees. No behavioural response was obtained in the control group, neither to the individual components nor to the mixture. Conditioning induced behavioural responses for three components, and an increase of electroantennogram responses for all components. A second experiment was conducted with an air entrainment extract of oilseed rape flower volatiles. Behavioural responses of conditioned and unconditioned bees were recorded. Responses obtained from conditioned bees tested with the air entrainment extract showed six groups of behaviourally active GC peaks. Unconditioned bees showed the same pattern of responses but at a lower level. The coupled technique described here appears to be a reliable tool for locating active components in a synthetic as well as in a natural mixture of floral volatiles. The effects of conditioning on odour discrimination and on its sensory correlates are discussed.

Introduction

Honeybee foragers visit plants to collect nectar and/or pollen for their own energetic needs and to provide the colony with food. Orientation to food sources is mainly mediated by floral volatiles, olfactory signals being particularly well learnt by honeybees compared to visual cues (Kriston, 1973; Menzel, 1984). Plant volatiles are complex chemical blends which may fluctuate according to species, varieties, or phenology (e.g. in sunflower: Etiévant, et al., 1984; Pham-Delègue et al., 1989). Now honeybees are able to select appropriate floral sources by adapting their behaviour to changing stimuli. Complex odour recognition might rely on the discrimination of a limited range of relevant components, as shown with free-flying bees visiting food sources scented with alfalfa volatiles (Waller et al., 1974) or sunflower volatile extracts (Pham-Delègue et al., 1986). To contribute to the understanding of natural odour recognition in the honeybee, we investigated honeybee responses to oilseed rape flower volatiles. Oilseed rape was chosen as a model since it is a crop plant intensively visited by honeybees which are attracted by its abundant production of nectar and pollen (Mesquida et al., 1988).

Previous work on sunflower volatile blends recognition showed that behaviourally active components could differ from the range of components detected at the antennal level (Thiéry et al., 1990). Vareschi (1971), and more recently Akers & Getz (1992, 1993), have categorized individual components according to the selectivity of receptor neurons and behavioural

discrimination abilities. However, in these attempts to correlate sensory and behavioural responses, both levels were investigated separately. To define biologically active components in floral mixtures, we designed a novel technique for the simultaneous monitoring of electrophysiological and behavioural responses. The odour stimulation was provided by the effluent from a gas chromatograph (GC), which led to a controlled and reproducible stimulation with the individual components of a mixture.

Proboscis extension responses together with electroantennograms were recorded from restrained individuals. Actually, the use of restrained bees enabled the simultaneous recording of behavioural and antennal responses to the same range of stimuli. The proboscis extension response was used since it occurs under natural conditions when the bee lands on a flower provided with nectar, and can be reproduced on restrained bees. Good correlations were demonstrated in the olfactory discrimination of either free-flying bees or restrained individuals (Pham-Delègue et al., 1993a; Mauelshagen & Greggers, 1993). Moreover, restrained bees can be subjected to a conditioning procedure (Takeda, 1961; Bitterman et al., 1983). As learning processes play an important role in the foraging behaviour of honeybees, we used a conditioning procedure to investigate the possible changes induced by learning on odour recognition at the sensory and behavioural levels.

In a first experiment, bees were conditioned to the mixture of six compounds identified previously as oilseed rape floral volatiles (Tollsten & Bergström, 1988; Blight et al., 1992). Then, we recorded simultaneously electroantennograms (EAG) and conditioned proboscis extension (CPE) responses to the individual compounds separated by the GC, to determine the biologically active components in the mixture. Responses of conditioned bees were compared to that of unconditioned ones.

In a second experiment, an air entrainment extract of oilseed rape flower volatiles (Blight et al., 1992) was used to stimulate the bees. Considering the great number of constitutive components of this natural blend, only behaviourally active components were investigated, using a GC-CPE coupled assay. Discrimination of individual components in the blend were compared between unconditioned and conditioned bees.

Materials and methods

Biological material. Italian worker bees, *Apis mellifera ligustica*, were used. The first experiment with the six-component mixture was conducted with known age individuals. Emerging bees collected from outdoor hives and caged in groups of about 50 individuals, were fed with sugar, water and pollen *ad libitum.* They were kept in an incubator (33 °C, 50% r.h., darkness) until 14–16 days old, the age at which the best learning performances in the CPE assay are obtained (Pham-Delègue et al., 1990). In the second experiment with the floral extract, worker bees of unknown age, identified as foragers by their pollen pellets, were collected on their way back to the hive to approach natural conditions.

Chemicals. The synthetic compounds used were linalool, 97%, 2-phenylethanol, 99+%, methyl salicylate, 99+%, benzyl alcohol, 99+%, (*E*)-2-hexenal, 99%, and 1-octen-3-ol, 98%. Solutions of the individual compounds (1 μg/10 μl) were made up in purified hexane and the mixture contained equal amounts (1 μg/10 μl) of the six compounds. The extract of floral volatiles was obtained from flowers of oilseed rape, *Brassica napus* L. (Topas variety), using a dynamic headspace technique (Blight, 1990).

Proboscis extension conditioning. The general conditioning procedure followed here has been described elsewhere (Pham-Delègue et al., 1993a). Before testing, caged bees were starved for three hours; foragers were fed after being collected and were then left to starve overnight. Bees were mounted individually in glass holders with only their mouthparts and antennae free. They were conditioned to the synthetic mixture, or natural extract, in five trials (6 s duration per trial, 15 min inter-trial intervals) in which the training odour was associated with a food reward (30% sucrose solution).

Coupled GC techniques. The first experiment was conducted using the synthetic six-component mixture as the stimulus source. Coupled GC-EAG-CPE recordings were carried out using the GC-EAG system described previously (Blight et al., 1979). General GC conditions are detailed in Wadhams et al. (1994). The effluent from the GC column was split approximately equally, with one part going to the flame ionisation detector (FID) of the GC while the other was directed over the bee antenna. EAG responses were obtained

using Ag-AgCl electrodes in glass capillaries, filled with saline. One antenna of the restrained bee was fixed, and the recording electrode was placed on the tip, with the indifferent electrode being inserted into a cut in the scape. FID, EAG and CPE responses were recorded simultaneously and stored on tape. The responses were sampled on a microcomputer and analysed using custom software (Marion-Poll & Tobin, 1992). At the end of the GC-EAG-CPE analysis, the initial conditioning mixture was again presented, and bees that did not exhibit a CPE response during this last trial were discarded. Data were obtained from six individuals previously conditioned to the six-component mixture as described above. In parallel, eight bees that were not subjected to the conditioning procedure were tested at the output of the GC, and coupled EAG and proboscis extension responses were recorded. At the end of the coupled assay, these bees were stimulated with the six-component mixture, and behavioural responses were noted. No discarding procedure was applied to the unconditioned bees.

In the second experiment, coupled GC-CPE recordings were carried out using the air entrainment extract. At the end of the coupled experiments, bees were tested with the initial extract. Two groups of bees, one unconditioned and the other conditioned to the extract, were considered. In the conditioned group, only bees that still responded to the extract at the end of the coupled procedure were kept, i.e. nine individuals. Their responses were compared with those of nine unconditioned individuals. FID and CPE responses were recorded as described above. CPE data were converted into bins that were later cumulated over 5 s to generate response profiles. Records from individual bees were time synchronized on the GC injection time.

Data treatment. The effects of conditioning and of the nature of the components tested in the first experiment on the EAG responses (mV) were analysed using a Two-way ANOVA.

Results and discussion

In the first experiment when bees were tested with the six-component mixture using the GC-EAG-CPE technique, no proboscis extension response was obtained from any of the unconditioned bees to the individual components or to the mixture. Of the conditioned bees, only one did not respond to any of the individual compounds, although it still responded to the mixture at the end of the testing protocol. Under the GC conditions used in this study, linalool and 2-phenylethanol were only partly resolved. Five bees responded to the linalool/2-phenylethanol peak (Table 1). Two bees were found to respond to benzyl alcohol. These results are consistent with a previous study, using a standard stimulation device to record CPE responses, where bees were conditioned to the same six-component-mixture and tested to the individual components at different concentrations (Pham-Delègue et al., 1993a). EAG responses were elicited by all the GC peaks. EAG responses induced in conditioned bees were always higher (15 to 40%) than those obtained from unconditioned bees (Table 1), this difference being significant (Conditioning effect: $F = 11.12$, 1 df, $P = 0.0015$). This suggests that antennal sensitivity is increased by conditioning, a phenomenon already reported in bees conditioned to plant odours of fennel and violet (de Jong & Pham-Delègue, 1991), and in a parasitic wasp after an oviposition experience (Vet et al., 1990). However, further studies are required to determine whether these sensory changes related to conditioning are specific to the training odour or are the result of a general sensitisation process. Between components, no significant differences appeared in EAG responses (Component effect: $F = 1.90$, 4 df, $P = 0.12$), and the interaction between the conditioning and component effects was not significant ($F = 0.35$, 4 df, $P = 0.84$). The three components found to be the most effective at the behavioural level in the conditioned bees are not better detected at the antennal level. Therefore, no direct correlates between peripheral sensitivity and behavioural responses can be drawn. This had also been shown by Allan et al. (1987) with sting-gland components, and in a previous work using queen pheromonal blends (Pham-Delègue et al., 1993b).

For the second experiment, the cumulated response profiles of conditioned and unconditioned bees to the air entrainment extract of rape flowers in the coupled GC-CPE assay are shown in Figure 1. From these profiles, six major GC areas of CPE activity were located. The activity areas were defined from the response profile of the conditioned bees, every time an increase in responses occurred. Since bees eliciting a proboscis extension response may remain excited after the end of the stimulus delivery, we considered that responses recorded between areas III and IV, areas V and VI, and after VI, were related to the previous activity area. Five areas were common to the two groups of bees. In the conditioned group, all nine bees responded at least once during the GC stimulation. Their highest

Table 1. Behavioural and electrophysiological responses of worker honeybees to six synthetic compounds identified in oilseed rape volatiles

Compounds	Behavioural responses		EAG responses (mV ± SE)	
	Unconditioned	Conditioned	Unconditioned	Conditioned
linalool+ 2-phenylethanol[a]	0	5	0.29 ± 0.06	0.51 ± 0.09
methyl salicylate	0	1	0.23 ± 0.08	0.41 ± 0.04
benzyl alcohol	0	2	0.19 ± 0.06	0.29 ± 0.06
(*E*)-2-hexenal	0	0	0.19 ± 0.03	0.31 ± 0.07
1-octen-3-ol	0	0	0.23 ± 0.05	0.31 ± 0.07

[a] Linalool and 2-phenylethanol were only partly resolved on the GC column.

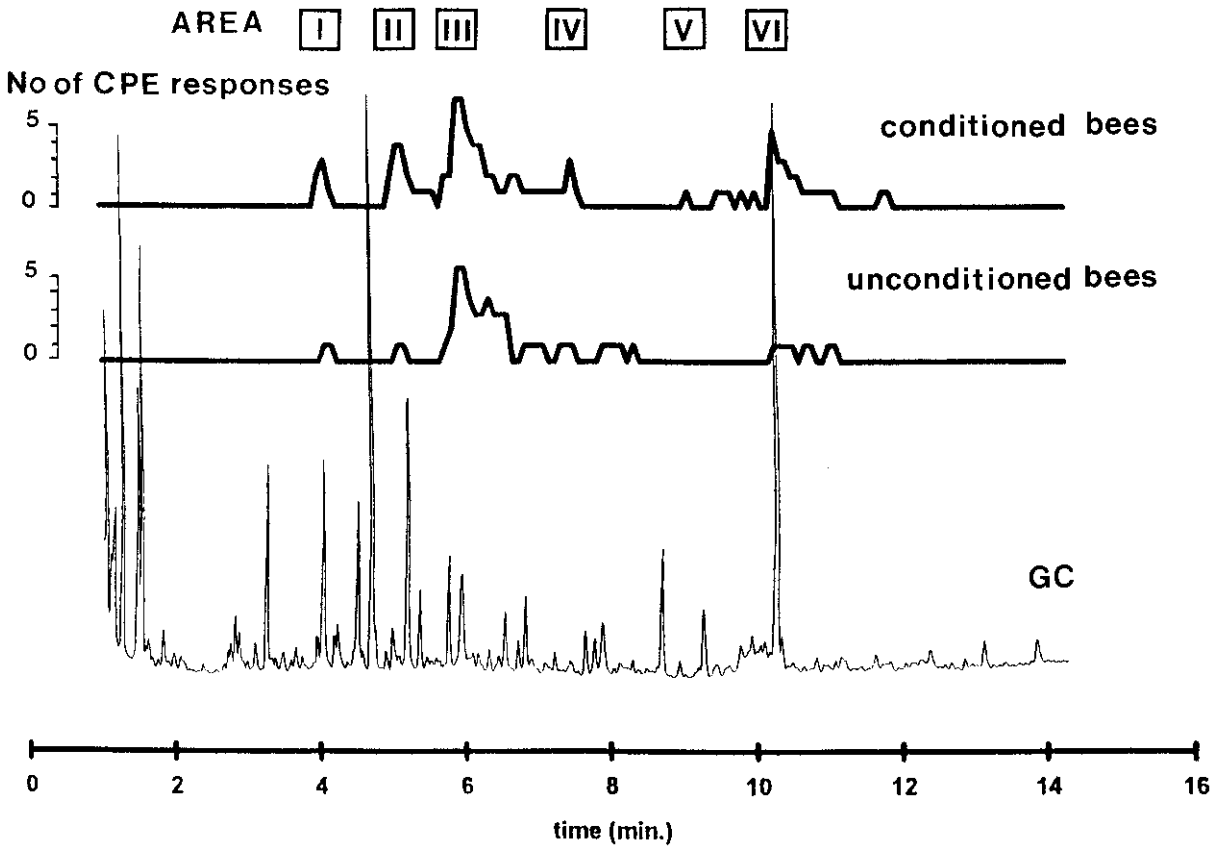

Figure 1. Coupled GC-CPE recordings showing the cumulated CPE responses of conditioned and unconditioned bees (nine individuals of each) to volatiles of a natural floral extract eluted from GC.

CPE activity was associated with areas III (seven individuals responding) and secondarily with area VI (five individuals responding). Area VI corresponded to the elution of a major GC peak. The remaining areas of activity each elicited weak to moderate CPE responses with one to four bees responding. In the unconditioned group, no bee responded to the mixture when presented at the end of the coupled procedure. Despite the fact that these bees were not conditioned to the mixture, of the nine bees tested, six actually responded to the individual components during GC stimulation. The highest CPE activity was again associated with area III (six bees responding). However, in contrast to

the conditioned bees, all other areas elicited only weak activity, or even no response to compounds in area V.

Thus, both conditioned and unconditioned bees showed some CPE responses to the compounds eluting from the GC column, whilst the total extract was inactive on the unconditioned bees. This may be interpreted as spontaneous responses to some compounds, especially those eluting in area III. Further investigations are in progress to identify such compounds (Blight et al., unpubl.). Since the honeybee is a generalist insect that potentially visits different floral sources, we may assume that components eliciting spontaneous responses are common to several floral blends. An alternative hypothesis is that the unconditioned bees were not totally naive. Thus, the experiments were conducted with foragers of unknown olfactory experience, that may have previously encountered floral volatiles in the hive or on floral sources. However, the fact that no response to the oilseed rape extract was obtained suggests that the active components have been experienced in another floral blend context. To document this point, complementary experiments are needed with bees without foraging experience, i.e. reared under controlled conditions, as the caged bees used in the first experiment. Actually, data yet obtained (first experiment) with unconditioned caged bees tested with the six-component mixture support this second hypothesis, no response being elicited neither with the individual components nor with the mixture.

Nevertheless, the conditioning procedure increased the level of behavioural response to the individual components in the experiment with the extract, and induced new responses in the experiment with the six-component mixture. This may rely on a general process of changes in the olfactory detection threshold, as we found that EAG responses were increased after conditioning. However, an increase in the peripheral sensitivity cannot entirely account for the changes observed at the behavioural level, since the increase of antennal responses occurs for all components, whereas only a reduced number of components become behaviourally active after conditioning. Therefore, central nervous system processes are most probably involved in the behavioural salience of some components, as suggested by combined experimental and theoretical approaches to document complex odour recognition in the honeybee (Masson et al., 1993; Masson & Linster, 1996).

This study, using the coupled GC-EAG-CPE technique demonstrates the occurrence of key behavioural compounds in a synthetic mixture as well as in a much more complex floral volatile blend. Based on the observed CPE activity areas, studies are in progress to identify the active compounds in the mixture of oilseed rape flower volatiles. The coupled technique described here appears to be a reliable tool for locating active components in complex mixtures of volatiles and for investigating the effects of learning on the ability to discriminate complex odours.

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References

Akers, R. P & W. M. Getz, 1992. A test of identified response classes among olfactory receptor neurons in the honey-bee worker. Chemical Senses. 17: 191–209.

Akers, R. P. & W. M. Getz, 1993. Response of olfactory receptor neurons in honeybees to odorants and their binary mixtures. Journal of Comparative Physiology A 173: 169–185.

Allan, S. A., N. K. Slessor, M. L. Winston & G. G. S. King, 1987. The influence of age and task specialization on the production and perception of honeybee pheromones. Journal of Insect Physiology. 33: 917–922.

Bitterman, M. E., R. Menzel, A. Fietz & S. Schäfer, 1983. Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). Journal of Comparative Psychology 97: 107–119.

Blight, M. M., 1990. Techniques for isolation and characterization of volatile semiochemicals of phytophagous insects. In: A. R. McCaffery & I. D. Wilson (eds), Chromatography and Isolation of Insect Hormones and Pheromones. Plenum Press, New York, pp. 281–288.

Blight, M. M., A. J. Hick, J. A. Pickett, L. E. Smart, L. J. Wadhams & C. M. Woodcock, 1992. Volatile plant metabolites involved in host plant recognition by the cabbage seed weevil, *Ceutorrhynchus assimilis*. In: S. B. J. Menken, J. H. Visser & P. Harrewijn (eds), Proceedings of the 8th International Symposium on Insect-Plant Relationships. Kluwer Academic Publ., Wageningen, pp. 105–106.

Blight, M. M., L. J. Wadhams & M. J. Wenham, 1979. The stereoisomeric composition of the 4-methyl-3-heptanol produced by *Scolytus scolytus* and the preparation and biological activity of the four synthetic stereoisomers. Insect Biochemistry 9: 525–533.

Etiévant, P. X., M. Azar, M. H. Pham-Delègue & C. Masson, 1984. Isolation and identification of volatiles constituents of sunflower (*Helianthus annuus* L.). Journal of Agriculture and Food Chemistry 32: 503–509.

Jong, R. de & M. H. Pham-Delègue, 1991. Electroantennogram responses related to olfactory conditioning in the honey bee (*Apis mellifera ligustica*). Journal of Insect Physiology 37: 319–324.

Kriston, I., 1973. Die Bewertung von Duft- und Farbsignalen als Orientierungshilfen an der Futterquelle durch *Apis mellifera* L. Journal of Comparative Physiology 84: 77–94.

Marion-Poll, F. & T. R. Tobin, 1992. Temporal coding of pheromone pulses and trains in *Manduca sexta*. Journal of Comparative Physiology A 171: 505–512.

Masson, C., M. H. Pham-Delègue, C. Fonta, J. Gascuel, G. Arnold, G. Nicolas & M. Kerszberg, 1993. Recent advances in the concepts of adaptation to natural odour signals in the honeybee, *Apis mellifera* L. Apidologie 24: 169–194.

Masson, C. & C. Linster, 1996. Towards a cognitive understanding of odor discrimination: combining experimental and theoretical approaches. Behavioural Processes 35: 63–82.

Mauelshagen, J. & U. Greggers, 1993. Experimental access to associative learning in honeybees. Apidologie 24: 249–266.

Menzel, R., 1984. Short-term memory in bees. In D. L. Alkon & J. Farley (eds.), Primary neural substrates of learning and behavioral change. London: Cambridge University Press pp. 259–274.

Mesquida, J., R. Marilleau, M. H. Pham-Delègue & M. Renard, 1988. A study of rapeseed (*Brassica napus* L. var. *oleifera* Metzger) flower nectar secretions. Apidologie. 19: 307–318.

Pham-Delègue, M. H., C. Masson, P. Etiévant & M. Azar, 1986. Selective olfactory choices of the honeybee among sunflower aromas: a study by combined olfactory conditioning and chemical analysis. Journal of Chemical Ecology 12: 781–793.

Pham-Delègue, M. H., P. X. Etiévant, E. Guichard & C. Masson, 1989. Sunflower volatiles involved in honey bee discrimination among genotypes and flowering stages. Journal of Chemical Ecology 15: 329–343.

Pham-Delègue, M. H., R. de Jong & C. Masson, 1990. Age-dependency of the conditioned proboscis extension response in honeybees. Comptes Rendus de l'Académie des Sciences, Paris, Serie III, 310: 527–532.

Pham-Delègue, M. H., O. Bailez, M. M. Blight, C. Masson, A. L. Picard-Nizou & L. J. Wadhams, 1993a. Behavioural discrimination of oilseed rape volatiles by the honeybee *Apis mellifera* L. Chemical Senses, 18: 483–494.

Pham-Delègue, M. H., J. Trouiller, C. M. Caillaud, B. Roger & C. Masson, 1993b. Effect of queen pheromone on worker bees of different ages: behavioural and electrophysiological responses. Apidologie 24: 267–281.

Takeda, K., 1961. Classical conditioned response in the honeybee. Journal of Insect Physiology 6: 168–179.

Thiéry, D., J. M. Bluet, M. H. Pham-Delègue, P. Etiévant & C. Masson, 1990. Sunflower aroma detection by the honeybee: Study by coupling gas chromatography and electroantennography. Journal of Chemical Ecology 16: 701–711.

Tollsten, L. & G. Bergström,1988. Headspace volatiles of whole plants and macerated plant parts of *Brassica* and *sinapis*. Phytochemistry 27: 4013–4018.

Vareschi, E., 1971. Duftunterscheidung bei der Honigbiene:Einzelzell-Ableitungen und Verhaltensreaktionen. Zeitschrift für vergleichende Physiologie 75: 143–173.

Vet, L. E. M., R. de Jong, W. A. Van Giessen & J. H. Visser, 1990. A learning-related variation in electroantennogram responses of a parasitic wasp. Physiological Entomology 15: 243–247.

Wadhams, L. J., M. M. Blight, V. Kerguelen, M. Le Métayer, F. Marion-Poll, C. Masson, M. H. Pham-Delègue & C. M. Woodcock, 1994. Discrimination of oilseed rape volatiles by honey bee: novel combined gas chromatographic-electrophysiological-behavioural assay. Journal of Chemical Ecology 20: 3221–3231.

Waller, G. D., G. M. Loper & R. L. Berdel, 1974. Olfactory discrimination by honeybees of terpenes identified from volatiles of alfalfa flowers. Journal of Apicultural Research 13: 191–197.