Effect of Conditioning on Discrimination of Oilseed Rape Volatiles by the Honeybee: Use of a Combined Gas Chromatography–Proboscis Extension Behavioural Assay

M. Le Métayer, F. Marion-Poll[1], J.C. Sandoz, M.H. Pham-Delègue, M.M. Blight[2], L.J. Wadhams[2], C. Masson and C.M. Woodcock[2]

Laboratoire de Neurobiologie Comparée des Invertébrés, INRA-CNRS (URA 1190), BP 23, 91440 Bures-sur-Yvette, [1]Laboratoire de Phytopharmacie, INRA, Route de St Cyr, 78026 Versailles Cédex, France and [2]IACR Rothamsted, Department of Biological & Ecological Chemistry, Harpenden, Hertfordshire AL5 2JQ, UK

Correspondence to be sent to: M H. Pham-Delègue, Laboratoire de Neurobiologie Comparée des Invertébrés, INRA-CNRS (URA 1190), BP 23, 91440 Bures-sur-Yvette, France

Abstract

The coupled gas chromatography–proboscis extension assay technique was used on restrained worker bees to study responses to components of an extract of oilseed rape floral volatiles. Bees were stimulated with the effluent from the gas chromatograph after either paired or unpaired conditioning to the extract, or after a control treatment. Proboscis extension activity was elicited in six areas of the chromatogram. However, the number of bees responding in two of these areas were too low to be considered in the present study. One significant area of activity was associated with the major component, (*E*,*E*)-α-farnesene, whilst the others were associated with several minor components. Although all three groups of bees, irrespective of the treatments applied, showed some responses to the components eluting from the GC column, only bees subjected to paired conditioning consistently responded when re-tested to the mixture. In addition, paired conditioning increased the responsiveness of individuals in terms of the number of bees responding at least once to the effluent from the gas chromatograph. This work confirmed the occurrence of key compounds in floral volatile mixtures. Possible synergistic/inhibitory effects between components, relating to olfactory experience, are discussed. **Chem. Senses 22: 391–398, 1997.**

Introduction

Orientation to floral sources by foraging honeybees is enhanced by a conditioning process in which foragers recognize flower characteristics associated with the nectar and pollen (von Frisch, 1919; Lindauer, 1976; Masson, 1983). Honeybee–plant interactions are cued by floral colour and shape and by volatile semiochemicals, with odours being most important for food source recognition (Kriston, 1973). Floral odours are often complex mixtures, e.g. in the case of the sunflower, >100 volatile components were detected (Etiévant *et al.*, 1984). Plant volatile blends

are to some extent species-specific, although even within species, their composition fluctuates qualitatively or quantitatively during plant phenology (e.g. in sunflower, Pham-Delègue *et al.*, 1989) or according to pedoclimatic conditions (e.g. in soybean, Robacker *et al.*, 1982). Bees have been shown to respond behaviourally to a discrete part of a complex odour. This was ~10% of the total extract of sunflower aroma (Pham-Delègue *et al.*, 1986). These data on honeybee ability to utilize a limited range of the components of a complex olfactory blend as orientation cues were obtained from observation of the behaviour of free-flying bees foraging on scented artificial food sources. In addition, common characteristics were found in the olfactory responses and learning performance of free-flying bees and of restrained bees subjected to conditioned proboscis extension (CPE) protocols (Mauelshagen and Greggers, 1993).

The recording of CPE responses of restrained bees appears to be well suited for manipulating relevant learning parameters, and this approach has been applied to the study of neurobiological and genetic bases of olfactory learning (e.g. Menzel *et al.*, 1974; Erber *et al.*, 1980; Brandes, 1988). The most commonly used protocol is the classical odour conditioning of the proboscis extension reflex based on the paired association of an odour, the conditioned stimulus (CS), and a sugar reward, the unconditioned stimulus (US), delivered to the antenna and proboscis (Frings, 1944; Kuwabara, 1957; Takeda, 1961; Vareschi, 1971). In parallel with the standard procedure, unpaired procedures (successive presentation of CS and US) have also been used (Bitterman *et al.*, 1983).

Studies on complex odour discrimination by restrained bees showed that behavioural recognition of a synthetic mixture of six floral components relied on one or two key components (Pham-Delègue *et al.*, 1993). Therefore, it appeared that such studies could be valuable in investigating complex odour recognition by the honeybee. Conclusions drawn from these experiments may then be tested with bees under more natural conditions. In the present work, we have extended the use of the coupled GC–proboscis extension assay to study mixture recognition using an extract of floral volatiles. Oilseed rape flower volatiles were chosen as the stimulus, since this crop is highly attractive to honeybees (Mesquida *et al.*, 1988). In order to investigate the effect of olfactory experience of the bees on discrimination abilities, we compared responses recorded in the coupled procedure after three different treatments had been applied: paired conditioning to the extract (conditioned), unpaired conditioning (pseudo-conditioned) or no previous experience (naive).

Materials and methods

Insects

Foraging Italian bees (*Apis mellifera ligustica*), which were loaded with pollen pellets, were collected during July, on their return to outdoor hives. The prior olfactory experience of these bees was unknown, but they could have previously have encountered flowering oilseed rape during their foraging trips or have been exposed to oilseed rape volatiles produced by stored pollen. The bees were mounted individually in glass holders, leaving the antennae and the mouthparts free. After being fed *ad libitum* with a 30% sugar solution, they were starved for 18 h at ambient temperature before use.

Oilseed rape volatiles

Volatiles were collected from flowering shoots of *Brassica napus* cv. Topas, a commercial oilseed rape variety containing low levels of both erucic acid and glucosinolates. The dynamic headspace (air entrainment) technique used for volatile collection has been described previously (Blight, 1990). In each air entrainment, 50–90 cut stems of rape, bearing buds and flowers only, were contained in water in conical flasks (50 ml) which were placed in a glass culture vessel (5 l). Volatiles were drawn from the vessel, using purified air, onto a tube of adsorbent, Porapak Q (1.5 g). At the conclusion of each air entrainment, which lasted 6–7 days, the volatiles were eluted from the Porapak with pentane and the extract was concentrated by fractional distillation of the solvent using a column of glass helices. Final concentration (to 200 µl) was done under nitrogen. Extracts from 13 air entrainments, shown by gas chromatographic (GC) analysis to be essentially the same, were combined to provide a sufficiently large, homogeneous sample of volatiles for this study. Aliquots of the combined extract were stored at –20°C, either in microvials for immediate use, or in ampoules for longer storage.

Conditioning procedures

Three procedures were used to compare discrimination of oilseed rape volatiles by three different groups of bees, i.e. conditioned, pseudo-conditioned and naive.

Conditioned bees

The conditioning procedure has been described previously (Pham-Delègue *et al.*, 1993). Bees were held initially for 15 s in an air flow of 1.6 l/min delivered through a glass tube (1 cm diameter) placed ~1 cm from the head of the bee. After this period of habituation to the mechanical stimulus, bees were subjected to five conditioning trials, with 15 min between trials. A 10 µl aliquot of the oilseed rape extract was applied to a 20 × 4 mm filter paper strip and the solvent was allowed to evaporate before the filter paper was placed in a Pasteur pipette cartridge. The vapour from the cartridge was delivered (6 s duration) into an air stream (0.6 l/min) connected to the main flow (1 l/min) passing over the head of the bee. Three seconds after the beginning of the odour stimulation (conditioning stimulus—CS), the extremity of one of the antennae was contacted with a 30% sugar solution (unconditioning stimulus—US) and the proboscis extension response (unconditioned response—UR) was rewarded by a drop of sugar solution. For each subsequent trial, the occurrence of proboscis extension during the first 3 s of the odour stimulation was recorded (conditioned response—CR). Two control trials were undertaken, comprising a 6 s presentation of hexane (10 µl) and pure air, to estimate whether the bee recognized oilseed rape volatiles specifically or whether it was responding to other associated stimuli, e.g. residual solvent and/or mechanical stimulation. Thirty-two bees were subjected to the conditioning procedure.

Pseudo-conditioned bees

The bees were subjected to an unpaired conditioning procedure. After 15 s of habituation to the air flow, the proboscis extension elicited by contact of one antenna with the sugar solution was rewarded (3 s duration). The odour stimulus was then presented alone for 6 s. Each bee was subjected to five trials, with 15 min between trials. The occurrence of the proboscis extension response was recorded during both the sugar and the odour stimulations. Eleven individuals underwent this procedure.

Naive bees

These individuals received the same treatment as the conditioned bees, but the odour stimulus was replaced by pure air. They were considered as naive with regard to the CS used in the experiment. The occurrence of proboscis extension responses was noted in the same way as for conditioned bees. The responses of 11 individuals were recorded.

Coupled GC–proboscis extension assay

Proboscis extension responses to the different components of the oilseed rape extract were obtained by stimulating the bees with the effluent from a GC, a system modified from Blight *et al.* (1979) for GC–electrophysiological recordings. The sample was separated using an AI-93 GC fitted with a flame ionization detector (FID) and a cold on-column injector and equipped with a 30 m × 0. 53 mm i.d. HP-1 bonded phase silica capillary column. The oven temperature was maintained at 40°C for 1 min and then programmed at 10°C/min to 250°C.The carrier gas was hydrogen. The effluent from the GC column was split approximately equally, with one part going to the FID of the GC and the remainder being directed into the air flow passing continuously over the bees. Three individuals, one from each treatment group, were aligned within the odour flow, and their respective positions were rotated between each replicate. At the end of the coupled procedure, the individuals were re-tested with the total extract.

Selection of the individuals

Of the 32 bees subjected to the paired conditioning, only 19 presented at least two conditioned responses out of the five conditioning trials and were kept for the next trials. Five bees showed a response to one or both control stimulations and were discarded. Eleven bees were chosen at random from the remaining 14, for further testing. All individuals were retained in the naive and pseudo-conditioned groups. This gave 11 clusters of three bees (conditioned, pseudo-conditioned, naive) for testing in the coupled GC procedure. In addition, two bees from the conditioned group did not respond to the total extract after the GC stimulation and were therefore considered as not being properly conditioned to the CS. They were discarded, together with the naive and pseudo-conditioned bees from the same cluster. Consequently, nine individuals in each treatment group were subjected to the whole experimental procedure and the data analysed.

Data recording and analysis

Each bee was scrutinized by an observer who closed an electrical contact connected to a 1.5 V battery. The electrical signal encoded the behavioural response with a voltage level of 1.5 V when the proboscis was extended and 0 V at rest.

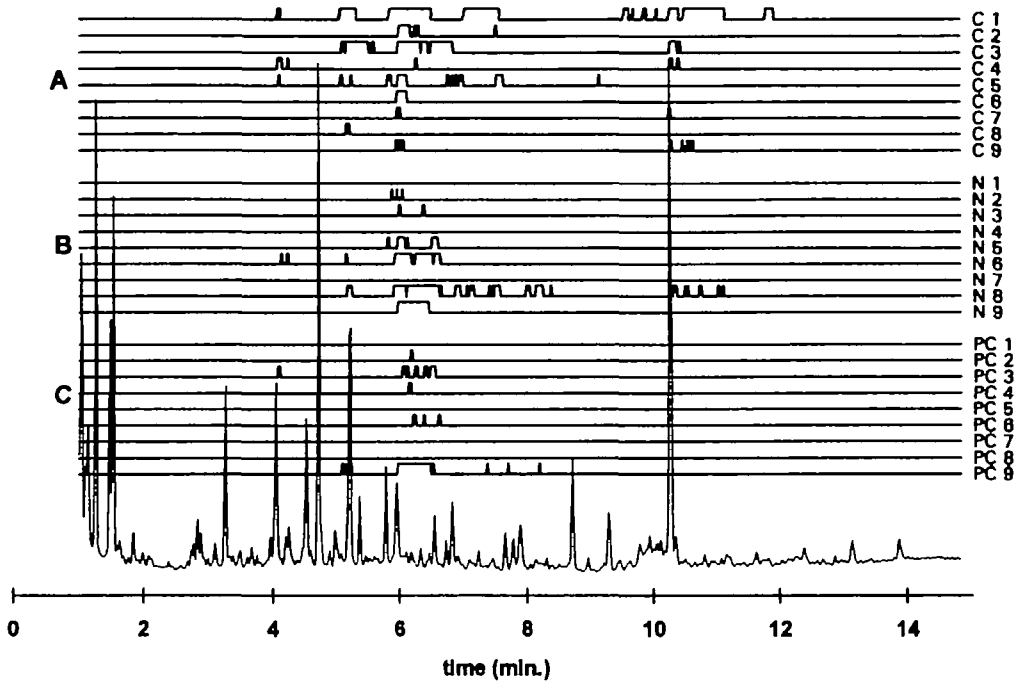

Figure 1 Individual proboscis extension responses of the three groups of nine bees, during stimulation with an oilseed rape extract separated by GC Prior experimental procedure **(A)** conditioned bees, **(B)** naive bees, **(C)** pseudo-conditioned bees

These electrical signals (one bee per channel), together with the recorder output from the FID, were stored on tape (Racal store) and simultaneously sampled on a micro-computer with a data acquisition card (Data Translation DT2821, USA; 12 bits precision; 200 Hz per channel). They were stored as binary files using custom software developed initially for spike analysis (Marion-Poll and Tobin, 1992). These data were later undersampled down to 10 Hz and exported as a series of voltage values to Excel using a Windows data analysis program (Awave, Marion-Poll, 1995). Each experiment lasted up to 15 min, and generated four columns with 900 values. These raw data were then adjusted in three ways. First, the arrays were temporally aligned with a reference chromatogram. This was performed by offsetting the cells until major peaks of the chromatograms were exactly superimposed (the elution times were constant). Second, the bee responses were converted to values of 0 (no response) and 1 (proboscis extension). Lastly, the response of all bees subjected to the same treatment were summed over 1 s periods, in order to build an overall response profile. The amplitude of a profile at a particular time thus represented the number of bees which responded positively to the components eluting at that time.

Statistics

Non-parametric statistics were used to analyse the data. Two-by-two comparisons of the response durations in the different treatment groups were performed using Mann–Whitney tests. χ^2 tests were used for multiple comparisons of frequencies of response to the active areas of the extract or among treatment groups. When significant, these tests were followed by two-by-two comparisons using χ^2 tests (1 df). When conditions for the application of the χ^2 test were not fulfilled according to Cochran's (1954) rule, Fisher's exact method was used (Scherrer, 1984). The significance threshold was 5% divided by n, with n being the number of comparisons in which each data set was used.

Results

Responses of individual bees from the three groups are shown in Figure 1. In the conditioned group (A), all nine bees exhibited at least one response during the elution of the components, but only six in the naive (B), and five in the pseudo-conditioned (C) groups responded. The total durations of response of the conditioned (median, 12 s) and naive bees (median, 4 s) did not differ significantly, and neither did those of the pseudo-conditioned (median of 1 s)

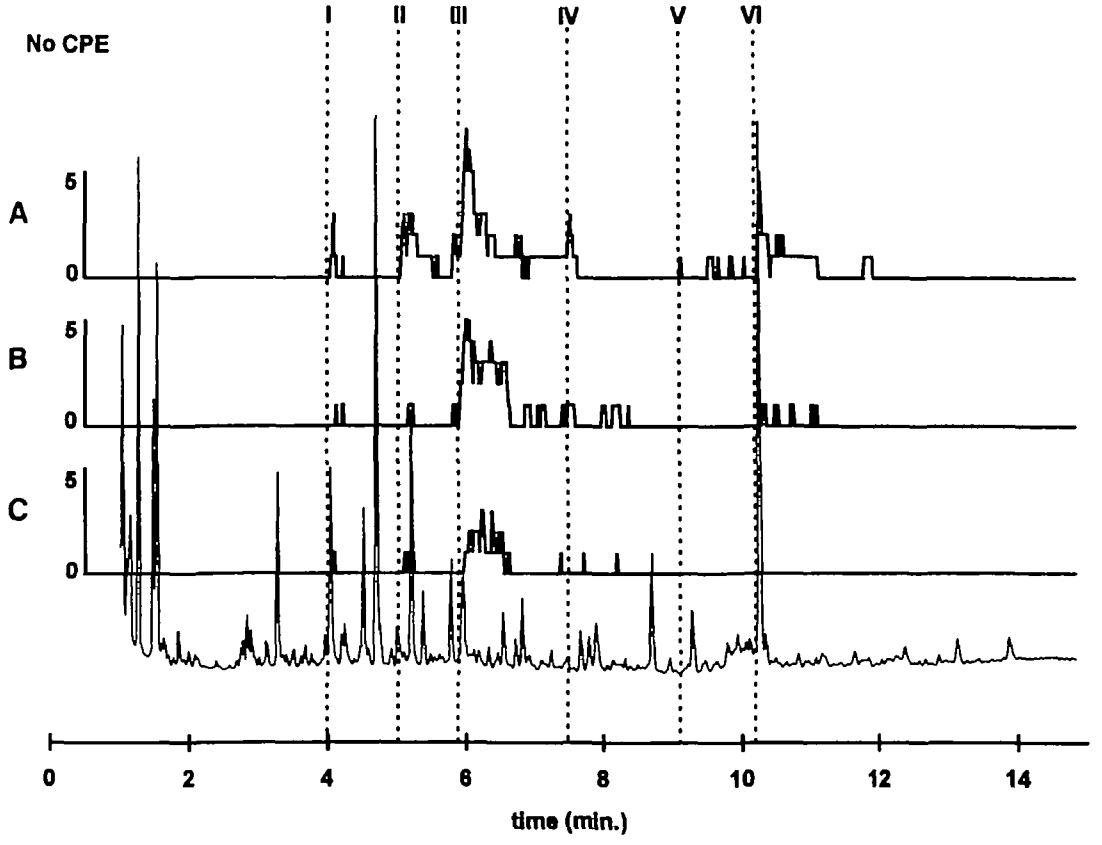

Figure 2 Cumulated proboscis extension responses of the three groups of nine bees **(A)** conditioned bees, **(B)** naive bees, **(C)** pseudo-conditioned bees during stimulation with an oilseed rape extract separated by GC Responses were converted into bins cumulated over 1 s to build response profiles I–VI areas inducing behavioural activity

and naive bees. The durations of response of conditioned bees were significantly longer than those of the pseudo-conditioned bees (Mann–Whitney test, $P < 0.05$).

To facilitate the identification of areas of the chromatogram eliciting the main behavioural activity, the individual responses were cumulated to express the frequency of the responses by each treatment group (Figure 2). During the coupled GC–proboscis extension assay of the oilseed rape extract, regions of activity were identified. This led to the definition of six active areas on the chromatogram of the extract, but only four corresponded to a clear start of activity, and only these, i.e. areas I, II, III and VI, were considered in this comparative study. When considering the maximum frequencies of response obtained in each group and in each activity area, it appeared that the frequency of response was always higher in area III than in the other areas, with seven, five and three individuals out of nine responding simultaneously in area III for groups A, B and C respectively (Figure 2). Except for the activity elicited in area VI by the most abundant component in the extract, subsequently identified as (*E*,*E*)-α-farnesene (Pham-Delègue *et al.*, 1992; Blight *et al.*, 1997), the other responses were associated with minor components.

Differences also appeared between the three groups in the number of individuals responding to each active area (Figures 1 and 2). Some discrepancy may arise in the number of individuals responding at a given time, when comparing either individual recordings (Figure 1) or the cumulated responses (Figure 2). This is caused by the smoothing effect of pooling all activities during 1 s bins. In particular, two individuals may respond to the same peak but with a slight delay and without overlapping. When they are recorded in the same bin, they are actually expressed as two cumulated responses; when they are recorded in two separate and successive bins, they appear in the cumulated representation as one long lasting response.

Conditioned bees (C1–9)

Three bees (C1, C4, C5) exhibited the CPE response to area I. Two of them (C1, C5) and two additional bees (C3, C8)

responded to area II. The highest CPE activity was elicited by area III, to which seven individuals responded. An additional bee (C4) also responded, but with a slight delay. Three bees (C1, C2, C5) responded to area IV, and only one (C5) to area V. The major component, (*E*,*E*)-α-farnesene, in area VI, elicited proboscis extension in five bees (C1, C3, C4, C7, C9). Only one bee (C1) responded to five of the six areas.

Naive bees (N1–9)

One bee (N6) responded briefly to area I and two bees (N6, N8) to area II. Six bees responded to area III. One bee, N8, was particularly responsive, and was the only one that responded to area VI, the (*E*,*E*)-α-farnesene peak.

Pseudo-conditioned bees (PC1–9)

Only one individual (PC3) showed a response to area I, and another one (PC9) to area II. Five bees responded to area III and none to area VI.

The sum of response frequencies over the four activity areas was 18, 8 and 5 for A, B and C treatment group respectively (out of the 36 potential response frequency that could have been obtained if all nine bees of each group had given a response in each area). These values differed significantly between the treatment groups ($\chi^2 = 12.5$, 2 df, $P < 0.01$). Further two-by-two comparisons between groups showed that a significantly higher level of activity was induced in the conditioned group compared with the naive group ($\chi^2 = 6.0$, 1 df, $P < 0.025$) and to the pseudo-conditioned group ($\chi^2 = 10.8$, 1 df, $P < 0.005$).

When the extract was tested at the end of the experiment, all nine individuals from the conditioned group responded which can be explained by the fact that they were selected according to this criterion. However, it should be noted that two of the 11 conditioned bees tested did not respond to the extract at the end of the testing protocol and were discarded from the data set. Only one bee from the naive group (N3) and none from the pseudo-conditioned group exhibited proboscis extension to the extract, although bees from these groups responded consistently to the eluted components of the extract.

Discussion

Discrimination of oilseed rape volatiles by honeybee foragers has been studied using a coupled GC–behavioural technique with bees subjected to different conditioning protocols. Six areas of behavioural activity were elicited during the elution of the sample of oilseed rape flower volatiles, one of which (area VI) corresponded to a major component in the sample, (*E*,*E*)-α-farnesene. All three groups of bees, irrespective of the conditioning protocols used, showed some proboscis extension responses to the components eluting from the GC column. However, as expected, paired conditioning significantly increased the responsiveness of individuals in terms of the total frequency of response to the areas of activity, compared with naive and pseudo-conditioned bees. Conditioned bees also exhibit responses of significantly longer duration than pseudo-conditioned bees. Nevertheless, in all three groups, responses were still obtained to some components, with area III eliciting the highest level of activity. In contrast, the response to (*E*,*E*)-α-farnesene, with the exception of one bee in the naive group, was only elicited after paired conditioning to the oilseed rape extract. This suggests that components eluting in area III elicit activity irrespective of the prior experimental procedure used. However, this interpretation must be treated with some caution, since the experiments were conducted using foragers of unknown olfactory experience. Further experiments with bees with known olfactory experience are in progress in order to address this problem.

Unpaired conditioning tended to decrease the responsiveness of the bees, although no significant differences were found in the profiles or durations of the responses of the naive and pseudo-conditioned bees. This is consistent with the data of Bitterman *et al.* (1983), who showed that unpaired training was not associatively neutral, but gave resistance to later learning.

Despite the fact that high activity was obtained from naive and pseudo-conditioned bees to components eluting from the GC column, only one bee in these two groups responded to the total extract when it was presented at the end of the GC stimulation. This suggests the presence of an inhibition process in naive and pseudo-conditioned bees, with components being behaviourally active when presented individually, but not when presented in a mixture. This inhibition appears to be removed by classical conditioning, which induced responses to the total extract and to individual components, in addition specifically to cueing the response to a particular compound, (*E*,*E*)-α-farnesene. The mechanism of this inhibition is unknown, but it could be mediated by one or several compounds in the sample.

Alternatively, whilst individual compounds might give rise to the proboscis extension response, their simultaneous presentation without classical conditioning may inhibit activity. Such synergistic/inhibitory effects have been shown previously with binary mixtures (Smith and Cobey, 1994). These authors found that a CPE response to an alcohol after training in an aldehyde background was significantly lower than when the bee was trained to the same alcohol in the background of another odorant.

This study, using a novel coupled GC–behavioural technique, has confirmed that key compounds are involved in the discrimination of floral volatiles by foraging honeybees, as previously shown by CPE responses to synthetic mixtures (Pham-Delègue *et al.*, 1993) and by the responses of free-flying bees to floral blends (Pham-Delègue *et al.*, 1986). The identification of 16 behaviourally active compounds in extracts of oilseed rape flower volatiles has now been achieved and is reported elsewhere (Blight *et al.*, 1997). Further work on the behavioural activity of these compounds is in progress.

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Received May 25, 1996; accepted January 21, 1997