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Olfactory conditioning of the proboscis extension in bumble bees

D. Laloi[1], J. C. Sandoz[1], A. L. Picard-Nizou[1], A. Marchesi[1], A. Pouvreau[1], J. N. Taséi[2], G. Poppy[3] & M. H. Pham-Delègue[1,*]

[1]*Laboratoire de Neurobiologie Comparée des Invertébrés, INRA, BP 23, 91440 Bures-sur-Yvette, France;* [2]*Laboratoire de Zoologie, INRA, 86600 Lusignan, France;* [3]*Rothamsted Experimental Station, IACR, Entomology and Nematology Department, Harpenden, Hertfordshire AL5 2JQ, UK;* **Author for correspondence (Fax: 33 169 07 50 54; E-mail: pham@jouy.inra.fr)*

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Abstract

The foraging behaviour of bumble bees is well documented for nectar and/or pollen gathering, but little is known about the learning processes underlying such behaviour. We report olfactory conditioning in worker bumble bees *Bombus terrestris* L. (Hymenoptera: Apidae) obtained under laboratory conditions on restrained individuals. The protocol was adapted from the proboscis extension conditioning previously described in the honey bee *Apis mellifera* L. Bumble bees were found to be able to learn a pure odorant when it was presented in paired association with a sugar reward, but not when odour and reward were presented in an explicitly unpaired procedure. This suggests an associative basis for this olfactory learning. Bumble bees showed similar conditioning abilities when stimulated with two different floral odours. An effect of the sugar reward concentration on the learning performances was found.

Introduction

Studies on bumble bees *Bombus* sp. have mainly documented their phylogeny (Free & Butler, 1959; Estoup et al., 1996), their physiology (Röseler & van Honk, 1990), their social organization (Duchateau & Velthuis, 1988; Duchateau, 1991) and their foraging strategies (Heinrich, 1976; Fussell & Corbet, 1992; Dukas & Real, 1993a; Corbet et al., 1995). Most works on the foraging behaviour of bumble bees have been conducted in natural or semi-natural situations. In the honey bee, the success of the foraging task strongly depends on learning of landmarks and floral cues such as odours and colours (Menzel & Müller, 1996). In the bumble bee the role of visual learning in the foraging behaviour, especially in the flower constancy, was shown by Schnetter (1977) and Heinrich et al. (1977), both working with free-flying bumble bees visiting artificial feeders. More recently Laverty (1994) showed that the rate at which bumble bees learned to find nectar in various natural flowers depended on the morphological complexity of the flower. Using free-flying foragers visiting differentially rewarded flowers, Dukas & Real (1993a, b) showed that the learning performances of bumble bees were affected by nectar distribution, and that bumble bees could integrate such information from several visited flowers. Some studies focused more specifically on the learning processes such as transfer and interference (Dukas, 1995), pattern matching (Korneluk & Plowright, 1995), categorization of learnt signals (Dukas & Waser, 1994), and memory retention (Keasar et al., 1996). These works generally refered to visual learning, and specific studies on olfactory learning in bumble bees are more scarce. Pham et al. (1983) conditioned free-flying bumble bees to visit artificial feeders scented with pure odorants in a flight room. Also Jakobsen et al. (1995) studied the influence of hive scents on the orientation of workers of *Bombus terrestris* in a choice test.

In the present study we aimed to investigate the possibility of using a bioassay based on the conditioned proboscis extension response on individual bumble bees. Under natural conditions, the proboscis extension reflex is part of the foraging behaviour, and allows workers to draw up nectar from flowers. First used on Hymenoptera by Frings (1944) to study the location of olfactory organs in honey bees, the proboscis extension bioassay was initially adapted from studies on the blowfly *Cynomyiopsis cadaverina* Desvoidy (Frings, 1941). In the honey bee, this bioassay has often been used since then (Kuwabara, 1957; Takeda, 1961), and has been shown to be a pavlovian (or classical) conditioning (Bitterman et al., 1983). Ever since this bioassay has been a key-tool for studies on the neural basis of memory (Erber et al., 1980; Menzel, 1984), olfactory learning processes (Bitterman et al., 1983; Smith, 1991; Sandoz et al., 1995), genetics of learning (Brandes et al., 1988), and plant volatile discrimination abilities (Pham-Delègue et al., 1993). In the bumble bee, no attempt to use this procedure has yet been reported.

Under restrained conditions bumble bees can extend their proboscis when antennae are contacted with a sugar solution. We hypothesized that if this reflex was elicited during an odour presentation followed by a sugar reward, thereafter we would obtain responses to the odour alone. As it has been done in honey bees (Bitterman et al., 1983) and more recently in moths (Hartlieb, 1996), the first step of the present study was to check an associative basis for this olfactory learning. We therefore compared the responses of bumble bees to explicitly paired and unpaired presentations of an odour and a sugar solution. In order to ensure that the conditioned proboscis extension could be obtained with different stimulations, we then subjected bumble bees to paired procedures with two different odours and with three different concentrations of a sugar solution.

Materials and methods

Bioassay. The classical odour conditioning of the proboscis extension reflex is based on the paired association of an odour (conditioned stimulus – CS) and a sugar solution (unconditioned stimulus – US). During conditioning, the proboscis extension reflex (unconditioned response – UR) is elicited by contacting the antennae of the bee with the US, the CS being simultaneously delivered. As the reward (R), the bee is allowed to take a drop of sugar solution. Thereafter, if the bee is properly conditioned, the delivery of the CS alone induces a conditioned proboscis extension response (conditioned response – CR).

Insects. Several colonies of bumbles bees (*Bombus terrestris* L.) were maintained separately in flight cages (each 55 × 100 × 90 cm, dome shaped) kept in a glasshouse. Pollen (pellets collected in a trap at the entrance of honey bee hives and powdered) and apiary syrup (commercial solution, 75% dry matter, composition: 15% fructose, 42% glucose, 43% maltose) were available *ad libitum* on artificial feeders placed in the flight cage. About 20 workers (of unknown age and size) were collected every day from one or several colonies. They were then harnessed individually in small glass holders, leaving their fore-legs and head free. They were starved for 6 hours, since preliminary experiments had shown this duration as giving the best hunger/mortality balance (the mortality rate was below 10% and 85% of the remaining workers showed a clear proboscis extension reflex). Only those individuals which showed the reflex after starvation were kept for the experiments. After being used in the experiments, bumble bees were marked with a colour dot on the thorax and released in the flight cage they originated from. Therefore an individual worker was never used twice in the experiments, and the strength of the colonies was not affected.

Stimulation device. Bumble bees were positioned one at a time in a constant flow of 52,5 ml/s delivered through a 1 cm glass tube, placed 1 cm in front of the bee. This flow consisted of a main airflow (50 ml/s), and a secondary one (2,5 ml/s) used for the odour stimulation. The olfactory stimulus (10 μl) was deposited onto a 40 × 3 mm piece of filter paper, inserted in a disposable Pasteur pipette. The secondary flow was delivered continuously into the main airflow, either through the pipette containing the odour source or an identical empty pipette. A solenoid low-latency valve was used to control the odour delivery. A fan was placed opposite to the stimulus delivery, so as to extract the released odours from the experimental room.

Experiment 1. Paired and unpaired training. We compared the proboscis extension responses of two groups of bumble bee workers subjected respectively to paired and unpaired presentations of CS and US. The protocol used in this experiment was adapted from procedures commonly applied in honey bee olfactory learning studies (Bitterman et al., 1983; Smith, 1991; Pham-Delègue et al., 1993; Sandoz et al., 1995).

In the paired group, bees were given 10 conditioning trials with 15-min inter-trial intervals. These conditioning trials were interspersed with blank trials in which the bees were placed in the stimulation device without delivery of any stimulus. Before each conditioning trial, the bumble bee was positioned in the air flow for 15 s, to be familiarized to the mechanical stimulation. The odour stimulus was then delivered for 12 s. During the first 6 s, the response (i.e. proboscis extension response) to the odour was recorded. Then, during the next 6 s period, the unconditioned response (UR) was elicited and the reward was provided to the bee until the end of the odour delivery. The total stimulation duration of 12 s ($6 + 6$ s) was longer than the durations commonly used in the honey bee as it had been found in preliminary experiments that shorter durations did not cause proboscis extension reflex elicitation and rewarding in bumble bees.

In the unpaired group, bees were subjected to 20 trials, i.e. 10 CS-only trials and 10 US-only trials given alternately, with 7.5 min inter-trial intervals. As above bees were familiarized to the air flow before each stimulation. During CS-only trials the odour alone was presented for 12 s, and possible proboscis extension responses during the first 6 s were recorded. During US-only trials no odour was delivered, and the US was presented for 6 s.

The training procedures, with blank trials alternating with conditioning trials in the paired procedure, ensured that bees from the paired group and from the unpaired group received an equal number of stimulations with CS and US, and placements. Thus paired and unpaired training only differed by temporal contiguity between CS and US.

The CS was pure linalool (Sigma, 95–97%), and the US was a 75% w/w sucrose solution. This concentration was chosen in reference to that of the standard food used to rear the colonies. Four to nine individuals from each group went through the conditioning procedure every day. The final number of bumble bees subjected to the conditioning procedure was 37 in the paired group and 38 in the unpaired group.

Experiment 2. Conditioning to two different CS. In this experiment we duplicated the paired conditioning procedure of experiment 1 with pure linalool (Sigma, 95–97%), and ran the same procedure in parallel with another odorant, pure phenylacetaldehyde (Sigma, 95%). The two groups of bumble bees were given 10 conditioning trials with 15 min inter-trial intervals. The US was a 75% w/w sucrose solution. Two to eight individuals from each group were subjected to the conditioning procedure every day, for a total number of 39 and 37 individuals conditioned to linalool and phenylacetaldehyde respectively.

Experiment 3. Effect of the reward concentration. In another set of experiments, we have compared the effect of different concentrations of the sugar reward solution on the conditioning performances of bumble bees. We tested three concentrations of the apiary syrup used for the rearing of the colonies, respectively 20%, 40% and 75% w/w solutions. The first two solutions were dilutions in water of the initial 75% solution. As in the other experiments the conditioning procedure consisted of 10 paired trials, with 15 min inter-trial intervals. Preliminary experiments having shown that bumble bees could be conditioned with diluted odorants (unpublished data), to fit with more natural concentrations, the CS used in this experiment was 10% linalool (Aldrich, 97%). The solvent (hexane) was allowed to evaporate (30 s at room temperature) before insertion of the filter paper in the pipette. On each experimental day, five to seven bumble bees for each of the three sugar concentrations were subjected to the conditioning procedure, for a final number of 18 individuals in each of the three concentration groups.

Statistics. Statistical analyses were performed on the number of proboscis extension responses gained along the ten odour presentations of the experimental procedure. Each individual was ranked on the basis of this number (from 0 to 10). The ranks of the individuals were compared between two groups (experiments 1 and 2) using a Mann-Whitney test. For three groups (experiment 3) the ranks were compared with a Kruskal–Wallis test; when a significant outcome was found, standard multiple two-by-two comparisons were conducted with a significance threshold correction (Noether, 1976): the threshold level was $\alpha' = \alpha/k(k-1)$ where k was the number of tested groups.

Results

A total of 317 bumble bees were mounted in glass holders, 70% of which showed a clear proboscis extension reflex when antennae were contacted with sugar solutions.

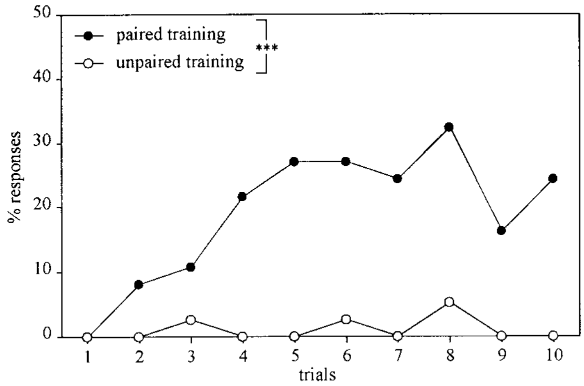

Figure 1. Percentages of *Bombus terrestris* workers showing proboscis extension responses during paired and unpaired training. A Mann–Whitney test performed on the number of responses exhibited by each individual during the procedure, showed a highly significant difference between the two groups ($z = 5.15$, $P < 0.001$)

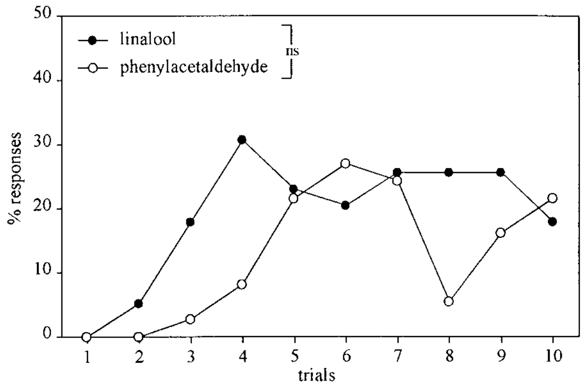

Figure 2. Percentages of *Bombus terrestris* workers showing proboscis extension responses during conditioning to linalool and phenylacetaldehyde. A Mann–Whitney test performed on the number of responses exhibited by each individual during the procedure, showed no significant difference ($z = 1.13$, $P = 0.26$)

Experiment 1. Paired and unpaired training. The percentages of proboscis extension responses obtained during both paired and unpaired procedures are presented in Figure 1. In the paired group, the proportion of bumble bees responding to the CS increased with successive conditioning trials, reaching a level of 32% at the eighth trial. Twenty-four individuals out of 37 (64.8%) responded to the odour, each exhibiting from 1 to 8 responses during the 10 odour presentations. By contrast the unpaired training produced negligible levels of response: one bumble bee responded twice, and two others once each out of 38 individuals. The comparison of the numbers of responses in the two groups showed a highly significant difference (Mann–Whitney test, $z = 5.15$, $P < 0.001$).

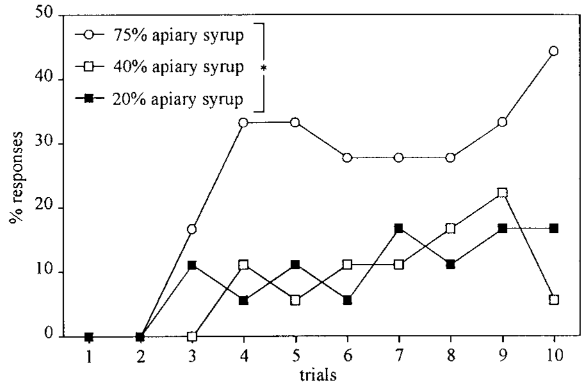

Figure 3. Percentages of *Bombus terrestris* workers showing proboscis extension responses during conditioning with 75%, 40% and 20% w/w sugar reward solutions. A Kruskal–Wallis test performed on the number of responses exhibited by each individual during the procedure, showed a significant difference between the three groups ($H = 5.95$, $P = 0.05$)

Experiment 2. Conditioning to two different CS. Figure 2 shows the percentages of proboscis extension responses obtained for bumble bee workers conditioned either to linalool or to phenylacetaldehyde. The acquisition curve for linalool was similar to that of experiment 1, reaching 30% of CR by trial four. Twenty three individuals out of 39 (59.0%) responded to linalool, each producing from 1 to 8 responses during the 10 conditioning trials. Bees conditioned to phenylacetaldehyde also showed an increase of the conditioned response, reaching a level of 27% at the sixth trial. Nineteen individuals out of 37 (51.3%) exhibited between 1 and 5 responses to phenylacetaldehyde during the conditioning procedure. The comparison of the numbers of responses in the two groups yielded a non-significant outcome (Mann–Whitney test, $z = 1.13$, $P = 0.26$).

Experiment 3. Effect of the reward concentration. The percentages of CR obtained during the 10 conditioning trials for the three apiary syrup concentrations are plotted in Figure 3. In the group rewarded with 75% apiary syrup, the proportion of bees responding to the CS increased with successive trials, reaching 33% at the fourth trial, and up to 44% at the end of the procedure. Twelve individuals out of 18 (66.7%) responded to the CS, each producing from 1 to 8 responses during the 10 conditioning trials. Lower levels of responses were obtained with 20% and 40% solutions of apiary syrup, both groups reaching 16.7% and 22.2% of CR respectively. With 20% apiary syrup, only seven individuals out of 18 (38.9%)

responded to the CS, each producing between 1 and 4 responses. With 40% apiary syrup, nine bumble bees out of 18 (50.0%) showed between 1 and 3 responses. A Kruskal–Wallis test performed on the number of responses of bees from the three groups yielded a significant difference ($H = 5.95$, $P = 0.05$). The two-by-two comparison of 20% and 40% apiary syrup groups gave non significant results. The two other comparisons (20% vs 75%, and 40% vs 75% apiary syrup groups) showed only near significant differences ($P < 0.05$ in both cases, with corrected threshold $\alpha' = 0.0083$). Based on these data we considered the overall significance as relying mainly on a higher level of responses in the group of bees rewarded with 75% apiary syrup.

Discussion

Adapting the proboscis extension conditioning procedure commonly used for honey bees, we succeeded for the first time in conditioning the proboscis extension response in workers of *Bombus terrestris*. The comparison between paired and unpaired training showed that a temporal relation between CS and US is necessary to obtain conditioned responses to the CS. In the paired group, 64.8% of the tested bees were actually conditioned to the odour, whilst only 7.9% of individuals exhibited responses to the same odour when CS and US were presented in an unpaired procedure. This finding suggests a probable associative basis for the conditioning of the proboscis extension in the bumble bee, as it was concluded after similar studies in the honey bee *Apis mellifera* (Bitterman et al., 1983) and moths *Heliothis virescens* and *Helicoverpa armigera* (Hartlieb, 1996). Additional experiments will be carried out to confirm the associative nature of the observed behavioural changes. In particular, to study possible non-associative components in these changes, such as sensitization or habituation (Alloway, 1972; Rescorla, 1988), CS-only and US-only treatments will be conducted.

Considering the level of conditioned responses following the paired training procedure, the asymptote value of about 30% might appear weak when compared to honey bee responses reaching 70–100% after a single conditioning trial (Bitterman et al., 1983; Sandoz et al., 1995). But similar levels of conditioned responses were obtained with moths (Hartlieb, 1996). This may actually account for the relative importance of olfactory learning in finding plants for these different species. It can also be hypothesized that the experimental procedure did not optimize the recording of olfactory learning performance. However, as we did not observe any decrease in the motivational state of the workers during the conditioning procedure (as they still took up the reward during trials), we could exclude the effect of satiation. The relatively low level of responses seemed to be rather related to high individual variation in the learning performances, with some individuals displaying many conditioned responses whilst others did not respond. Further experiments will be undergone to analyse possible effects of age, size, or previous experience on the individual ability to perform a learning task.

Using different stimulations we showed that the conditioning of the proboscis extension in the bumble bee can be obtained with different CS and with different US. We observed similar responses with two pure floral odorants, linalool and phenylacetaldehyde. Many works on free-flying bumble bees have shown that these insects can use odours, as well as colours and shapes, as orientation cues in their foraging behaviour (Heinrich et al., 1977; Pham et al., 1983; Jakobsen et al., 1995). Nevertheless little is known about the way by which bumble bees use pure or complex odorants. In the honey bee, studies based on the proboscis extension assay have shown that learning and discrimination performances vary according to the odour stimuli (e.g. Smith & Menzel, 1989; Getz & Smith, 1991; Smith, 1991; Pham-Delègue et al., 1993). As it allows a good control of the stimulations, the proboscis extension procedure applied to bumble bees will help to screen out quality/quantity parameters of odour recognition in these insects.

As for the effect of the sugar reward, we showed an effect of the concentration on the individual learning performances, workers conditioned with the 75% apiary syrup showing higher level of responses than those conditioned with 20% or 40% concentrations of the same mixture. Besides, the 75% apiary syrup induced conditioning performances similar to that obtained with the 75% sucrose solution used in the two other experiments. In the honey bee very few data are currently available on the effect of the concentration of reward on proboscis extension conditioning. The only account to our knowledge is an experiment by Bitterman et al. (1983) who showed a slower acquisition of the conditioned response with a 7% sucrose solution compared to 20% or 40% solutions, all the three groups reaching, however, the same asymptotic level within eight trials. Consistently with our data, these authors found that higher concentrations induced higher performances. The 10-trials procedures used in our experiment may not be adequate to ensure that the responses of bumble bees conditioned to the three concentrations have reached an asymptotic level. Further experiments conducted with longer procedures and/or other sugar concentrations will be needed to address this question.

The proboscis extension conditioning procedure has been used in numerous studies in the honey bee. In this insect strong similarities have been observed between the results with restrained and free-flying individuals (Pham-Delègue et al., 1993; Mauelshagen & Greggers, 1993). The development of the proboscis extension bioassay in the bumble bee may become a helpful tool for the understanding of olfactory learning mechanisms involved in the orientation to floral sources.

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