

Could learning of pollen odours by honey bees (*Apis mellifera*) play a role in their foraging behaviour?

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Abstract. The role of pollen odour cues in the foraging behaviour of honey bees (*Apis mellifera* L.) is poorly understood. Using classical conditioning of the proboscis extension response, in which bees learn to associate an odour with a sucrose reward, the present study tests whether odours of bee-collected pollen from the hive environment or odours of fresh pollen on the anthers of flowers could be used in pollen foraging. Honey bees efficiently learn odours from field-bean (*Vicia faba*) bee-collected pollen and oilseed-rape (*Brassica napus*) bee-collected pollen, hand-collected pollen, anthers and whole flowers, demonstrating that honey bees can learn pollen odours associatively in biologically realistic concentrations. Honey bees learn pollen odours of oilseed rape better than field bean and, although they generalize these two odours, they easily distinguish between them in discrimination tests, suggesting that pollen odours may be used in species recognition/discrimination. There is little evidence that honey bees can recognize whole flowers based on previous experience of bee-collected pollen odour. However, they generalize the odours of oilseed-rape anthers and whole flowers, suggesting that anther pollen *in situ* may play a more prominent role than bee-collected pollen in foraging behaviour.

Key words. Discrimination, learning, odour, pollen, proboscis extension conditioning.

Introduction

Pollen is collected from flowers by foraging honey bees (*Apis mellifera* L.) to provision their colony with food. It provides a variety of essential nutrients, including starch, sterols, lipids, vitamins and minerals (Roulston & Cane, 2000). It is also the only source of protein for a colony, and is vital for brood-rearing and the glandular development of young worker bees (Winston, 1987). The survival and growth of the entire colony therefore depend on the foraging

efficiency of the workers. However, despite the importance of pollen, little is known about how honey bees use floral cues, and in particular pollen cues, to locate pollen-rewarding flowers (Dobson & Bergström, 2000; Pernal & Currie, 2002). The present study tests the odour-learning ability of honey bees, which could enable them to use pollen odour cues to maximize their foraging efficiency.

The foraging efficiency of honey bees is augmented by the foragers' communication of the location and type of forage to new recruits in the hive, and by the ability of the bees to learn and use floral cues, such as shape, colour and odour, to locate rewards on subsequent foraging occasions. Of the floral cues, odours are learnt by bees most readily, and play a prominent role in the recognition of rewarding food sources, particularly at close range (Menzel & Müller, 1996). Floral odours comprise mixtures of many volatile compounds (Knudsen

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et al., 1993). They are species-specific, enabling honey bees (and other insects) to distinguish between species (reviewed by Dobson, 1994), but they also vary intraspecifically, both qualitatively and quantitatively, with factors such as cultivar and phenology (Pham-Delègue *et al.*, 1989), damage (Dicke & van Loon, 2000) and climatic conditions (Robaker *et al.*, 1982). Therefore, once a honey bee has learnt the odour of a rewarding flower, it is unlikely to encounter another odour whose components are in exactly the same relative concentrations, even within the same floral species. To reconcile this problem in identifying future rewarding food sources with the need to remain flower-constant when foraging, honey bees both generalize nonidentical odours that they perceive to be similar, and discriminate between similar but detectably different odours (Smith, 1993; Pelz *et al.*, 1997).

Most studies investigating odour learning and recognition by honey bees have been carried out using single-compound odours; there have been very few studies performed using mixtures of volatile compounds or floral odours that can be directly related to foraging ecology (Pham-Delègue *et al.*, 1986; Laloi *et al.*, 2000; Wright *et al.*, 2002). Recognition of mixtures of volatiles/floral odours is now assumed to be based on a few key compounds present in the total mixture (Blight *et al.*, 1997; Pham-Delègue *et al.*, 1997; Laloi *et al.*, 2000). However, the origins of these compounds, and whether or not any derive from pollen, have never been investigated. In many plant species, each floral structure, including pollen, produces a distinctive blend of volatiles that contribute to the whole flower odour (Dobson *et al.*, 1990; Knudsen & Tollsten, 1991; Bergström *et al.*, 1995). Some of the behaviourally active components involved in flower recognition may derive from pollen, and may be learnt from rewarding flowers to improve future foraging efficiency of this vital resource. However, the ability of bees to learn pollen odour associatively has never been investigated to date.

Honey bees do not only come into contact with pollen when foraging. Their first experience of pollen is inside the hive from the stored pollen used as food, and from the pollen loads carried in the corbiculae of returning pollen foragers. Later, as foragers, they will encounter fresh pollen directly on the anthers of flowers. Although pollen from different species has been shown to have species-specific odours (reviewed in Dobson & Bergström, 2000), it is not known whether bees can learn these differences and subsequently use them to recognize and discriminate between species. If so, bees could learn pollen odour either within the hive from bee-collected pollen (Wenner *et al.*, 1969; Free, 1970) or in the field from pollen on the anthers of flowers, and use it to help them locate rewarding plants when foraging.

In the present study, the ability of restrained honey bees to learn pollen odours from oilseed rape (*Brassica napus* L.) and field bean (*Vicia faba* L.), two agriculturally important crops that are pollinated by bees (Free, 1993), is examined, along with their relative recognition, using the proboscis extension response bioassay (Bitterman *et al.*, 1983). The extent to which honey bees perceive odours of bee-collected pollen, fresh pollen on dehiscing anthers and whole flowers to be similar to each other is also investigated using oilseed rape as

a model plant. The experiments relate to two hypotheses regarding the potential uses of odours from bee-collected pollen and fresh pollen in flowers in the foraging behaviour of honey bees: (i) foragers use experience of bee-collected pollen from the hive in locating forage in the field and (ii) foragers use fresh pollen on the anthers of flowers in floral recognition and species discrimination and also to discriminate between pollen-rewarding and nonrewarding flowers.

Materials and methods

Honey bees

Honey bees were collected as they left the hive entrance of a colony located at Rothamsted Apiary, Harpenden, Hertfordshire, U.K. These bees were mainly foragers, but the possibility that some were guards cannot be excluded. The colony was kept outside and the bees allowed to forage normally; however, oilseed rape and field bean (the experimental species) were not flowering either at the time of the experiments or 3 weeks before the start of each, so pre-experimental conditioning was avoided. The honey bees were restrained in the laboratory in accordance with the procedure described by Bitterman *et al.* (1983). Bees were individually harnessed in small glass tubes, leaving their antennae and mouthparts free, protruding from the top. Each bee was fed with 30% sucrose solution for 5 s, then starved for 3 h. Bees were then tested for the proboscis extension reflex by touching one antenna with the sucrose solution. Only honey bees that extended their proboscis in response to this stimulation were used in the experiment; nonresponders (approximately 20%) were discarded.

Odour-delivery device

A device based on that developed by Pham-Delègue *et al.* (1993) was used to deliver the odour stimuli (see below) to the honey bees. A constant airflow (1.05 L min^{-1}), comprising a main airflow of 1 L min^{-1} and a secondary airflow of 5 mL min^{-1} , was delivered from a compressed air cylinder through a glass tube (1 cm in diameter). The secondary airflow was delivered continuously into the main airflow through either a disposable Pasteur pipette (glass) containing/or delivering the odour stimulus, or an identical empty 'control' pipette. New pipettes were used each time that the odour stimulus was changed. The odour stimulus was delivered over a period of 6 s, after which the secondary airflow reverted back to the default 'control' airflow. A computerized system controlled a solenoid valve used to switch between the two airflows.

Odour stimuli

Pollen. Pollen from oilseed rape and field bean was either bee-collected or hand-collected (see below). The pollen was placed in the 'odour' pipette of the secondary airflow of the

odour-delivery device. The pollen's headspace odour was thus delivered to the honey bee. A small plug of glass-wool placed at the end of the pipette prevented the pollen from falling out. The 'control' pipette of the secondary airflow in experiments using pollen odour stimuli contained a similar plug but no pollen.

Bee-collected (corbicular) pollen loads were obtained from a pollen trap fitted to the entrance of a honey bee colony placed amidst a crop of flowering field beans on Rothamsted farm. Pollen loads collected from the trap were sorted to species using colour (Kirk, 1994), and grain shape, size and exine features (Sawyer, 1981), and then stored at -20°C . Before use in an experiment, pollen samples were defrosted and reased apart using a mounted needle.

Hand-collected pollen was gathered from young oilseed-rape and field-bean flowers excised from glasshouse grown plants (cv. Aries and Alfred, respectively). With oilseed-rape flowers, the petals were held back as the pollen was gently scraped from the exposed anthers (dehiscid) using a scalpel. For field-bean flowers, the standard petal was pulled back to extrude the stamens, and the pollen was scraped off with the scalpel. The keel petals were then split apart, allowing the pollen plug within to drop into the sample.

Anthers. Anthers of glasshouse-grown oilseed rape plants (cv. Aries) that had dehiscid to expose their pollen were cut from the filaments of flowers and were placed in a pipette plugged with glass-wool as described above. The 'control' pipette used in conjunction with this stimulus in the secondary airflow was empty but similarly plugged.

Flowers. Ten oilseed-rape flowers, which were still attached to the terminal raceme of an intact glasshouse-grown oilseed rape plant (cv. Aries), were enclosed in a 100-mL glass chamber (PA Brooks Scientific Glassware, Witney, U.K.). Poly tetrafluoroethylene (PTFE) tape was wound around the stem of the raceme to prevent damage and the chamber was sealed with a small plug of Blu-Tack (Bostik Findley Ltd, U.K.). The chamber's headspace was delivered to the 'odour' dispensing pipette via PTFE tubing (1/4 inch in diameter; Supelco, Bellefonte, Pennsylvania).

Conditioning and testing procedure

The proboscis extension reflex of honey bees is elicited when the tarsi or antennae contact nectar (Minnich, 1932). The reflex can be trained in a Pavlovian (classical) conditioning procedure by presenting an odour (the 'conditioned stimulus' or CS) in temporal association with a sucrose reinforcement (the 'unconditioned stimulus' or US) (Bitterman *et al.*, 1983); the association results in the odour being able to elicit the proboscis extension response, which is known as the conditioned response.

Restrained honey bees were randomly assigned into groups. In each experiment, each group was subjected first to a conditioning phase and then to a test phase. This

procedure was continued each day until at least 30 bees had been tested to each odour. Bees were used only once in the experiments: new bees were restrained, conditioned and tested each day. The order in which the groups were used was randomized.

During the conditioning phase, each bee within a group was subjected to either three (all experiments except Experiment 2) or 16 (Experiment 2) conditioning trials. A rewarded CS-US conditioning procedure was adopted, based on the work of Bitterman *et al.* (1983), in accordance with that described by Pham-Delègue *et al.* (1993). For each conditioning trial procedure, the first bee from a test group was placed 2 cm from the end of the outlet tube of the odour-delivery device, and left for 15 s to allow it to familiarize to the airflow and experimental context. The odour stimulus was then delivered to the bee for 6 s. After the first 3 s, 30% sucrose was applied to both antennae. Proboscis extension was rewarded by allowing the bee to feed on the sucrose for the remaining 3 s of the odour presentation. The bee was then returned to her group, and the procedure was repeated until all bees in the group had received one conditioning trial in succession. During each conditioning trial, it was noted whether or not bees exhibited a proboscis extension response to the odour within the first 3 s of the odour stimulus presentation. It was also noted whether the bee licked the sucrose reward, and bees that did not take the reward in any one of the conditioning trials were excluded from all analysis (only 1% of the 654 bees conditioned in this study).

After conditioning, a test phase was conducted using the 'learners' (honey bees that had learnt the conditioning odour). 'Nonlearners' (honey bees that did not respond with at least one conditioned response during conditioning; see Laloi *et al.*, 1999), were excluded in this phase (9% of the 654 bees conditioned throughout this study). The test phase comprised a generalization or discrimination bioassay (see below) followed by a second test in which the stimulus was the clean air 'control'. The second test was not performed on honey bees that had been conditioned to the 'control' as an odour stimulus. Honey bees that showed a conditioned proboscis extension response in this latter test (5% in the study) were termed 'nonselective learners' (Laloi *et al.*, 1999) and were discarded from all analyses because it could not be certain whether they had been responding to the odour stimuli or merely to the airflow or other mechanical/physical stimulation associated with the conditioning process. Finally, all honey bees were re-tested by stimulating one antenna with sucrose solution. Those that did not respond (only three bees) were subsequently excluded from all analyses.

Experiment 1: associative learning and generalization of the odours from bee-collected pollens

This experiment was conducted to ascertain whether or not honey bees can associatively learn the odour of bee-collected pollen, and whether they perceive pollen odours from different plant species to be similar (or different).

In the conditioning phase, restrained honey bees were randomly assigned to three groups. Each group was conditioned using the procedure described above over three conditioning trials (C1–C3) to one of three odour stimuli: (i) bee-collected oilseed-rape pollen (25 mg; approximately two corbicular loads); (ii) bee-collected field-bean pollen (25 mg); or (iii) the clean airflow ('control'). The same pollen sample was used for the odour stimuli in each trial and intertrial intervals were 15 min.

In the test phase, half of the honey bees in each group conditioned to pollen odour were tested for their responses to their conditioning odour whereas the other half were tested with the alternative, novel odour stimulus. The test odour stimuli consisted of fresh samples of corbicular pollen (25 mg in both cases) that were presented to the bee as in conditioning trials, but unrewarded. A positive (a conditioned proboscis extension response) or negative (no proboscis extension) response was recorded over the duration (6 s) of the odour presentation for each bee.

Experiment 2: discrimination between the odours of bee-collected pollens

This experiment was conducted to ascertain whether the odours from bee-collected oilseed-rape and field-bean pollens contain any differences that can be learnt by honey bees and used to differentiate between them (i.e. can bees discriminate pollen odours from different plant species?). To determine whether a bee can discriminate one odour from another may require differential (discriminative) conditioning, involving presentations of a rewarded conditioning odour stimulus (CS⁺) and an un-rewarded or punished conditioning odour stimulus (CS⁻) (Bitterman *et al.*, 1983; Getz & Smith, 1987). The use of salt solution as a negative reinforcement of the CS⁻ (Getz & Smith, 1987; Bhagavan & Smith, 1997), which forces animals to show their best discriminative ability, was adopted for this experiment.

In the conditioning phase, restrained honey bees were randomly assigned to two groups. Each group was conditioned using the procedure described above except that for one group (OSR⁺/FB⁻), the odour of oilseed-rape (OSR) pollen (bee-collected; 25 mg) was reinforced with a positive reward of 30% sucrose (CS⁺); and presentations of field-bean (FB) pollen odour (bee-collected; 25 mg) were negatively reinforced by application of salt solution (3 M sodium chloride; CS⁻) to the antennae and proboscis if it was extended (although bees never actually fed on this solution). The reverse conditioning procedure was carried out for the other group (FB⁺/OSR⁻).

Each pollen odour was presented eight times, such that 16 conditioning trials were carried out in total, honey bees receiving either positively or negatively rewarded odours in individual trials with intertrial intervals of 8 min. The same pollen sample was used for each of the odour stimuli in each trial and conditioning odours in the trials were presented in a pseudo-randomized order (Smith & Getz, 1994):

OSR⁺/FB⁻ group: 2 × (FB⁻ OSR⁺ OSR⁺ FB⁻ OSR⁺ FB⁻ FB⁻ OSR⁺)

FB⁺/OSR⁻ group: 2 × (OSR⁻ FB⁺ FB⁺ OSR⁻ FB⁺ OSR⁻ OSR⁻ FB⁺)

In the testing phase, half the conditioned bees in each group were tested to a fresh sample of the CS⁺ odour whereas the other half were tested to a fresh sample of the CS⁻ pollen odour. The tests were unrewarded and the number of positive (conditioned proboscis extension) and negative (no proboscis extension) responses during test odour presentations were recorded.

Experiment 3: associative learning and generalization of the odours from hand-collected pollens

This experiment was conducted to ascertain whether bees could associatively learn the odour of hand-collected pollen and whether they perceive odours of hand-collected pollen from different plant species to be similar or different. This second point enabled a comparison with bee-collected pollen, to determine if generalization of bee-collected pollen odours, as seen in Experiment 2, is due to 'bee-derived' volatile components present in both pollens (a result of the collection process by the honey bee), rather than due to there being similarities between pollen odours. The experiment was conducted exactly as Experiment 1 in all aspects, except that hand-collected pollen (25 mg for both species) was used in place of bee-collected pollen.

Experiment 4: associative learning and generalization of the odours from bee-collected pollen, anthers and flowers

The response of honey bees to the odours of bee-collected pollen, pollen on freshly dehiscing anthers and the whole flower were examined to determine how similar bees perceive these odours to be.

In the conditioning phase, restrained bees were randomly assigned to three groups. Each group was conditioned using the procedure described above over three conditioning trials (C1–C3) with 10-min intertrial intervals. The conditioning odour stimulus for each group was either: bee-collected oilseed-rape pollen (approximately 60 mg; 4–5 pollen loads; oilseed-rape anthers (60 dehiscing anthers weighing approximately 0.06 g from 10 flowers); or oilseed-rape flowers (10 flowers attached to the raceme of an intact potted plant). Thus, similar quantities of pollen were present in each sample. The same samples were used for each of the odour stimuli in each conditioning trial.

In the test phase, a generalization test was conducted in which all honey bees were tested to all three odour stimuli, presented unrewarded, one after the other in a randomized order. A positive or negative response was recorded as above.

Data analysis

Odour acquisition. For Experiments 1, 3 and 4, the proportion of honey bees exhibiting a proboscis extension response during the first 3 s of the conditioning odour presentation at each conditioning trial (C1–C3) was plotted to produce odour acquisition curves for the three odour stimuli tested in each of these experiments. Differences in learning efficiency at C3 were tested in each experiment using a log-linear model (McCullagh & Nelder 1989) with terms for the three conditioning groups and the responses. If a difference was found, pairwise comparisons of the means were conducted using Fisher's exact test. All analyses carried out in this study were conducted using GenStat software (GenStat, 4.2, VSN International Ltd, U.K.). For Experiment 2, the proportions of proboscis extension responses exhibited on each of the eight conditioning trials for both the positive (CS⁺) and negative (CS⁻) conditioning odour were plotted to produce acquisition curves for the two groups of bees (OSR⁺/FB⁻ and FB⁺/OSR⁻). To compare responses with the CS⁺ and the CS⁻ in each group, a Wilcoxon's Matched Pairs test (signed ranks; Siegel & Castellan, 1988) was used, counting how many times each bee responded to each stimulus (in theory, between 0 and 8 times).

Generalization test. The generalization test measures the similarity between two odours by testing whether responses conditioned to one stimulus can also be elicited by other odours (Kalish, 1969; Smith & Getz, 1994). It compares the proportion of bees exhibiting a conditioned proboscis extension response to the conditioning odour with the proportion of responses to a novel odour at the test. A difference in the proportions indicates that bees discriminate between these two odours; no difference

indicates generalization (but does not mean that the bees cannot discriminate). For Experiments 1 and 3, the proportions of positive and negative responses to the two pollens were compared for both groups (oilseed rape-conditioned and field bean-conditioned in Experiment 1, and OSR⁺/FB⁻ and FB⁺/OSR⁻ in Experiment 3) in two Fisher's exact tests. In Experiment 4, differences in the responses of bees to the three test odour stimuli were analysed within each of the three groups using Cochran's Q-test (Siegel & Castellan, 1988); a planned contrast was then carried out between the two 'fresh' flower odours untouched by bees (anthers and whole flowers) with the probability of a type 1 error controlled using $\alpha/2$, where α is the level of significance (Marasculio & McSweeney, 1967).

Discrimination test. For Experiment 2, the proportion of positive and negative conditioned proboscis extension responses when tested to field-bean or oilseed-rape pollen odour was compared for each group by Fisher's exact test.

Results

Experiment 1: associative learning and generalization of the odours from bee-collected pollens

The number of proboscis extension responses to pollen odour increased from 13 to 20% spontaneous responses (see Menzel *et al.*, 1993) at the first conditioning trial (C1) to 90–100% at C3, indicating that learning occurred quickly (Fig. 1A). Conversely, the proportion of honey bees responding to the blank air 'control', when this was presented as a conditioning stimulus, did not alter between conditioning trials, remaining below 10% (Fig. 1A). There

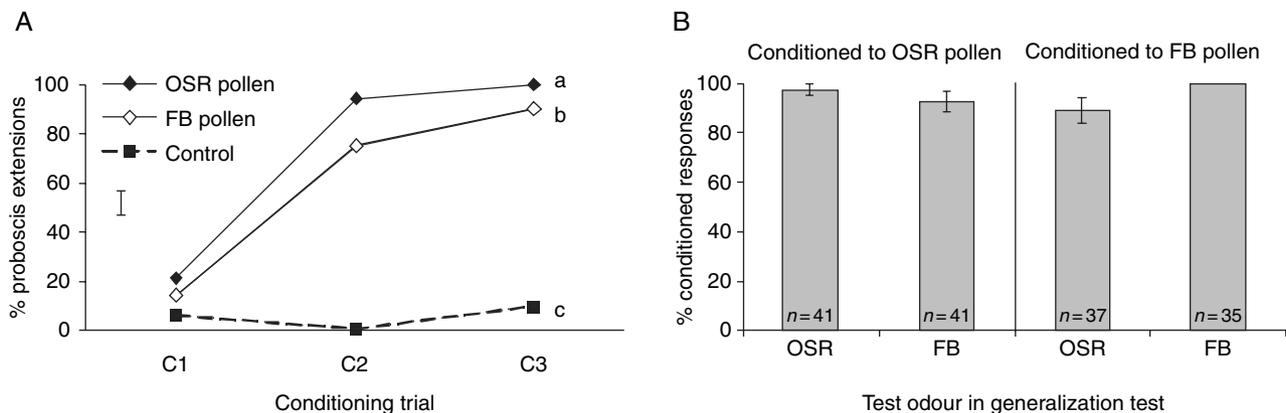


Fig. 1. Learning and generalization of bee-collected pollen by restrained honey bees. (A) Odour acquisition curves of oilseed-rape (OSR; $n = 82$) and field-bean (FB; $n = 80$) bee-collected pollen odours and clean air (control; $n = 35$). Data points represent the percentage of bees showing a proboscis extension response during the first 3 s of odour presentation in three conditioning trials (C1–C3); at C3, 'a' differs significantly from 'b' ($P < 0.01$) and both differ from 'c' ($P < 0.001$). The error bar indicates the maximum \pm SE, based on the binomial distribution. (B) Percentage of bees (\pm SE) conditioned in (A) to either oilseed-rape or field-bean bee-collected pollen odours showing a conditioned proboscis extension response when presented with oilseed-rape or field-bean odours in an unrewarded test. Neither test is significantly different ($P > 0.05$).

was a difference in the learning efficiency of the three groups at C3 (log-linear model $\chi^2 = 126.3$, d.f. = 2, $P < 0.001$) with both pollen odours being different from the control (both Fisher's exact $P < 0.001$), and the number responding to oilseed-rape pollen was higher than to field-bean pollen (Fisher's exact $P = 0.006$) (Fig. 1A).

Honey bees generalized between oilseed-rape and field-bean bee-collected pollen odours (Fig. 1B) with 90–100% bees responding to both odours, regardless of conditioning. Honey bees conditioned to oilseed-rape pollen did not differ significantly in the proportion of conditioned proboscis extension responses exhibited to either the odour of oilseed-rape or field-bean pollen (Fisher's exact $P = 0.616$). Similarly, bees conditioned to field-bean pollen odour did not differ significantly in their responses when tested to these odours (Fisher's exact $P = 0.115$). The results might indicate that the honey bees were unable to differentiate between the two bee-collected pollen odours. This possibility was further examined in Experiment 2.

Experiment 2: discrimination between the odours of bee-collected pollens

After some initial generalization, honey bees quickly learnt to discriminate the odours, learning to respond to the CS⁺ odour associated with the sucrose reward and not to the CS⁻ odour associated with salt. For both groups, responses to CS⁺ started at a rate of 28–40% at C1 and reached 96–100% at C8, whereas responses to CS⁻ started between 21 and 30%, increased to approximately 80% at C2 then sharply decreased to < 4–12% by C8 (Fig. 2A, i and ii). Differences in responses to the CS⁺ and CS⁻ were significant for both groups: Wilcoxon test statistic = 0.000, $n = 63$, $P < 0.001$ for the OSR⁺/FB⁻ group, and for the FB⁺/OSR⁻

group, Wilcoxon test statistic = 0.000, $n = 56$, $P < 0.001$ (Fig. 2A, i and ii).

Significantly more honey bees in both groups responded to the odour associated with the positive reward than the negatively rewarded odour (OSR⁺/FB⁻ group: Fisher's exact $P < 0.001$; FB⁺/OSR⁻ group: Fisher's exact $P < 0.001$; Fig. 2B). These results clearly show that although honey bees may generalize between OSR and FB pollen odours, they are able to differentiate between them.

Experiment 3: associative learning and generalization of the odours from hand-collected pollens

Honey bees learnt the three odours with significantly different degrees of efficiency (log-linear model $\chi^2 = 40.95$, d.f. = 2, $P < 0.001$; Fig. 3A). Responses ranged from < 5% for all three odours at C1 to 82% to oilseed-rape pollen odour, 57% to field-bean pollen odour and 16% for the control at C3. Both pollen odours produced more responses than the control (both Fisher's exact $P < 0.001$) and oilseed-rape pollen was learnt better than field-bean pollen (Fisher's exact $P = 0.010$). Bees therefore learnt to associate the odours of both oilseed-rape and field-bean hand-collected pollens with the sucrose reward, but not the clean air control.

Honey bees from both groups generalized between the odours. Bees conditioned to the odour of hand-collected field-bean pollen responded equally well (94%) to oilseed-rape and field-bean odours in the generalization bioassay (Fisher's exact $P = 0.332$; Fig. 3B). When conditioned to the odour of hand-collected oilseed-rape pollen, 96% bees responded to the presentation of oilseed-rape pollen odour but fewer (76%) responded to the field-bean pollen (Fisher's exact $P = 0.056$; Fig. 3B). These results indicate that hand-collected field-bean pollen was less rapidly learnt

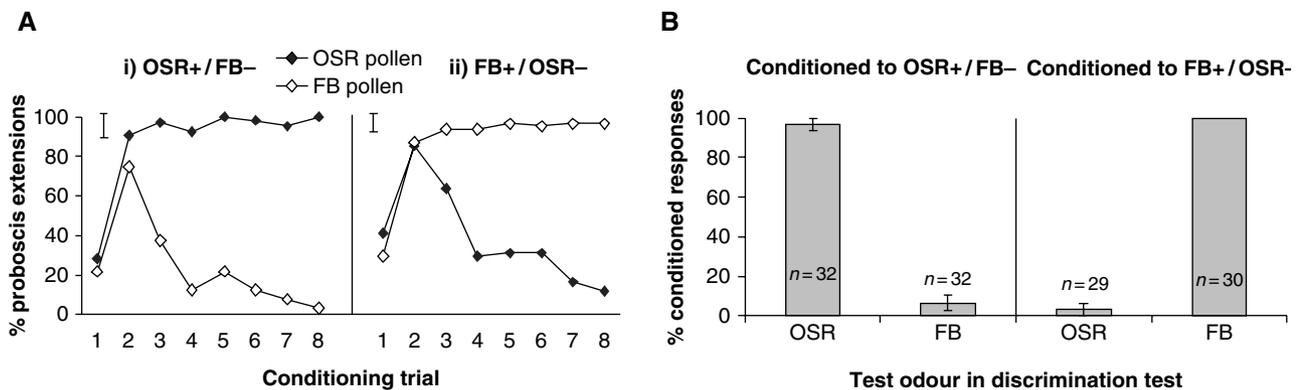


Fig. 2. Discrimination of bee-collected pollen odours by restrained honey bees. (A) The percentage of proboscis extension responses exhibited during eight successive paired presentations of a positively reinforced (sucrose solution) and negatively reinforced (salt solution) odour [either oilseed-rape (OSR) or field-bean (FB) bee-collected pollen] during discriminative conditioning: (i) bees positively conditioned to oilseed rape, negatively conditioned to field bean (OSR⁺/FB⁻) ($n = 64$), where the curves differ significantly ($P < 0.001$); (ii) bees positively conditioned to field bean, negatively conditioned to oilseed rape (FB⁺/OSR⁻) ($n = 61$), where the curves differ significantly ($P < 0.001$). The error bar represents the maximum \pm SE, based on the binomial distribution. (B) Percentage of bees (\pm SE) from (A) showing a conditioned proboscis extension response when presented with the odour of oilseed-rape or field-bean bee-collected pollen odours in an unrewarded test after conditioning to either OSR⁺/FB⁻ or FB⁺/OSR⁻. Both tests differ significantly ($P < 0.001$)

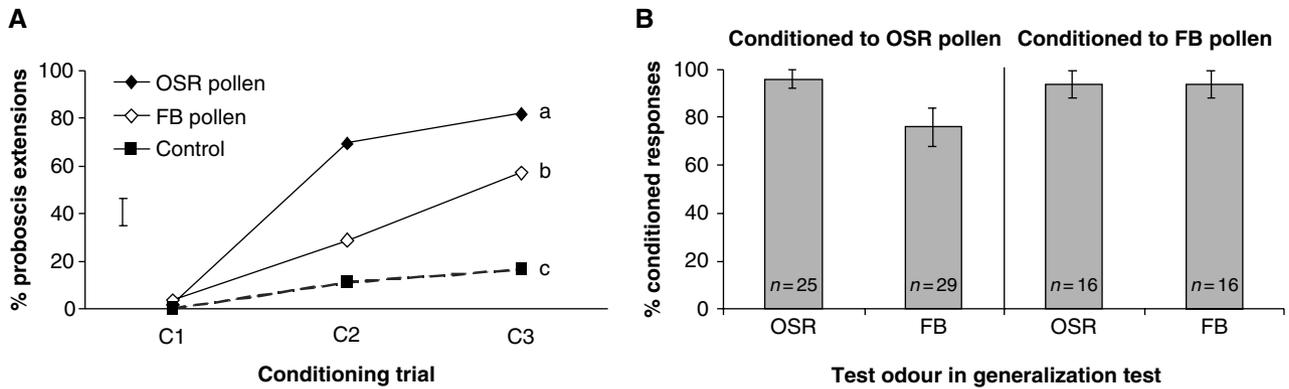


Fig. 3. Learning and generalization of hand-collected pollen by restrained honey bees. (A) Odour acquisition curves of oilseed-rape (OSR; $n = 66$) and field-bean (FB; $n = 56$) hand-collected pollen odours, and of clean air (control, $n = 36$). Data points represent the percentage of bees showing a proboscis extension response during the first 3 s of odour presentation during three conditioning trials (C1–C3); at C3, 'a' and 'b' differ significantly ($P < 0.01$) and both differ from 'c' ($P < 0.001$). The error bar represents the maximum \pm SE, based on the binomial distribution. (B) Percentage of bees (\pm SE) from (A) conditioned to either oilseed-rape or field-bean hand-collected pollen odours showing a conditioned proboscis extension response when presented with oilseed-rape or field-bean hand-collected pollen odours in an unrewarded test. Neither test is significantly different ($P > 0.05$).

than oilseed-rape pollen, and that generalization was slightly, but not significantly lower from hand-collected oilseed rape to field bean than from field bean to oilseed rape.

Experiment 4: associative learning and generalization of the odours from bee-collected pollen, anthers and flowers

All three odours were learnt quickly; responses rising from 2 to 16% at C1 to 81–95% at C3, with no differences in response between the three odours (log-linear model $\chi^2 = 4.331$, d.f. = 2, $P = 0.115$; Fig. 4A).

In the generalization test, a high proportion (95–100%) of honey bees in each group responded to their conditioning odour (Fig. 4B), indicating efficient learning of all three odours. There were significant differences between bees' responses to the three test odour stimuli for each of the groups and, in each case, this was due to the difference in responses between bee-collected pollen odours and the 'fresh' floral odours because there were no significant differences in the contrasts between anthers and whole flowers (bees conditioned to bee-collected pollen: $Q = 32.08$, $P < 0.001$, contrast = 2.83, $P = 0.058$; bees conditioned to anthers: $Q = 9.33$, $P < 0.01$; contrast = 0.33, $P = 0.585$;

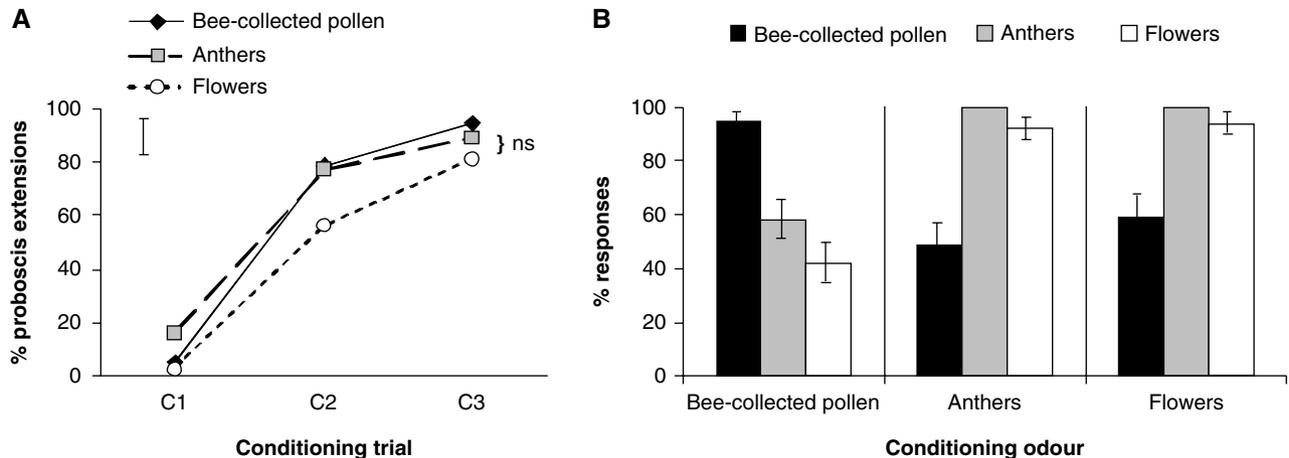


Fig. 4. Learning and generalization of odours from oilseed-rape bee-collected pollen, anthers and whole flowers by restrained honey bees. (A) Odour acquisition curves: bee-collected pollen ($n = 43$), anthers ($n = 44$), whole flowers ($n = 43$). Data points represent the percentage of bees showing a proboscis extension response during the first 3 s of odour presentation during three conditioning trials (C1–C3); at C3, not significant ($P > 0.05$). The error bar represents the maximum \pm SE of the proportion, based on the binomial distribution. (B) Percentage of bees (\pm SE) conditioned in (A) to the odour of either bee-collected pollen ($n = 43$), anthers ($n = 39$) or flowers ($n = 34$), showing a conditioned proboscis extension response when presented with each of these odours in unrewarded tests. In each group, responses to the three odours differ significantly ($P < 0.001$) but responses between anthers and whole flowers are not significantly different ($P > 0.05$).

bees conditioned to whole flowers: $Q = 30.47$, $P < 0.001$, contrast = 0.35, $P = 0.563$; Fig. 4B). The odour of bee-collected oilseed-rape pollen is therefore perceived by bees as different from the odours of pollen-bearing anthers and whole oilseed-rape flowers, which are perceived to be similar.

Discussion

The present study shows that honey bees can learn to associate the odour of bee-collected pollen with a sucrose reward, which they generalize between oilseed-rape and field-bean bee-collected pollen odours, but they can discriminate these two pollen odours. Honey bees can also learn the odours of pollen from dehiscing anthers (hand-collected in this study) and generalize between hand-collected pollen types. Honey bees generalize between the odours of anthers and whole flowers, but less so between each of these odours and bee-collected pollen. These experiments demonstrate that honey bees can associatively learn pollen odours. Moreover, pollen odours are learnt quickly and efficiently at biologically realistic concentrations. The roles that odours of bee-collected pollen and fresh pollen *in situ* could therefore play in the foraging behaviour of honey bees is discussed below.

The role of bee-collected pollen odours in foraging

Because honey bees, at all stages of their lives, are in contact with bee-collected pollen odours in the hive, they could learn such cues and use them at a later stage when foraging. There are two possible ways by which this might happen: (i) via passive exposure or (ii) by learning, during development and/or as adults. It is possible that bees could learn pollen odours during development when they are fed by nurse bees and thereafter use these odour cues to help them locate forage as adults. The present study does not test the ability of honey bee immatures to learn pollen odours. However, previous studies indicate that it is unlikely that any such olfactory conditioning influences foraging behaviour. Boelter & Wilson (1984) found that honey bees reared on pollen from a range of species did not exhibit foraging preferences for the pollen upon which they had been fed as they developed. Furthermore, Sandoz *et al.* (2000) found that the odourous environment during pupal development of honey bees had little influence on their later behaviour. Moreover, the pollen from the same flowers upon which bees were reared upon may no longer be available by the time the bee finally begins to forage if the flowering period is short.

The possibility that honey bees could use bee-collected pollen odours in foraging, through learning as adults the odours from the corbicular loads of returning foragers (Von Frisch, 1967; Wenner *et al.*, 1969; see also Pernal & Currie, 2002), appears to be of more adaptive value because it could assist them to locate flowers from currently rewarding species. The odour learning could occur passively (through simple exposure), or possibly through associative

conditioning if the odours from the pollen loads are reinforced with a reward given through trophallaxis (Calderone & Johnson, 2002). Odour exposure at the early adult stage in honey bees can influence later orientation behaviour; bees subjected to either passive olfactory exposure or to a Pavlovian associative learning procedure afterwards orientated in a choice test towards the conditioning odour whereas naïve bees avoided it (Sandoz *et al.*, 2000). In addition, the introduction into the hive of an odour previously learnt when foraging has been shown to stimulate foragers to return to the site where the odour was presented with reward (Reinhard *et al.*, 2004). Given that odours in the hive may influence later behaviour, bees must be able to recognize fresh pollen on dehiscing anthers or the whole flower, based on experience of bee-collected pollen odours, for there to be a possible role of bee-collected pollen in foraging. It may also be beneficial if bees could discriminate between different plant species on the basis of pollen odour alone because bees could use this information as an additional cue in finding rewarding forage upon recruitment.

The evidence presented here suggests that bees can indeed discriminate between species on the basis of pollen odours. When honey bees are conditioned to associate the odour of bee-collected oilseed-rape pollen with a sucrose reward, they generalize their response to the novel odour of field-bean pollen and vice versa (Experiment 1). This indicates that these odours have important similarities. These could be due to the intrinsic similarities in field-bean and oilseed-rape pollen volatiles (pollen-derived components) or to odours added by the bees during the pollen-collecting process (bee-derived components). The generalization tests using hand-collected pollen (Experiment 3) show high generalization between field-bean and oilseed-rape pollen odours, and it is likely that pollen-derived components are at least partially responsible for the generalization observed in Experiment 1. Despite such similarities between bee-collected oilseed-rape and field-bean pollen odours, honey bees are able to discriminate between them (Experiment 2), which further indicates that bee-collected pollens contain enough species-specific material to allow discrimination. This supports chemical evidence indicating that pollen odours are species specific (Dobson & Bergström, 2000), and suggests that honey bees can detect these differences and could learn to use them in species discrimination. Indeed, previous studies have suggested that bees can discriminate between different plant species on the basis of their pollens (Levin & Bohart, 1955; Schmidt & Johnson, 1984; Cook *et al.*, 2003). However, the present study demonstrates that bees can discriminate between plant species based on pollen odour alone.

The precondition for use of bee-collected pollen odour cues in foraging is that honey bees can recognize whole flowers on the basis of bee-collected pollen odour. The present experiments reveal that bees generalize only to a limited extent between bee-collected oilseed-rape pollen odours and those from either oilseed-rape anthers or whole oilseed-rape flowers (Experiment 4). This indicates that bee-collected pollen odours are somewhat different

from those of the pollen present on anthers *in situ* (and this is supported by chemical analysis; Cook, 2000) and of whole flowers (note also that generalization is lower between bee-collected pollen and anthers/flowers in Experiment 4 than it is between the two sorts of bee-collected pollen in Experiment 1). Therefore, the data suggest that the collection process induces a thorough modification of the pollen odour, and that probably only a few components learnt by bees in bee-collected pollen odour are present in the aroma of either pollen on dehiscing anthers or whole flowers. Further tests on a wider range of pollens than those used in the current study are needed to confirm this. However, based on the available data, it is unlikely that honey bees use the odour learnt from bee-collected pollen to forage selectively in the field. However, this does not preclude that odour from fresh pollen (see below) on the bodies of returning foragers could influence the behaviour of recruits (Von Frisch, 1967), nor that recognition of bee-collected pollen plays a role in foraging (e.g. as an stimulus for workers to collect pollen; Calderone & Johnson, 2002).

The role of anther pollen odour in foraging

A totally different question is whether pollen volatiles are part of the floral aroma learnt by honey bees when foraging. Floral odours are recognized by bees on the basis of a few key components of the mixture (Blight *et al.*, 1997; Pham-Delègue *et al.*, 1997; Laloi *et al.*, 2000). Some of the key components learnt and used in flower recognition could derive from pollen. Furthermore, these compounds may be used to determine pollen-rewarding from nonrewarding flowers, thereby increasing foraging efficiency. For these hypotheses to be plausible, honey bees must be able to detect and learn pollen odours presented in realistic concentrations. Honey bees do associatively learn the odours from oilseed-rape and field-bean hand-collected pollens, but less well than they learn the odour of bee-collected pollens (cf. Experiments 1 and 3). This may be due to differences in their concentrations. Odours at high concentration support stronger associations (Pelz *et al.*, 1997) and are learnt faster than odours of lower concentrations (Wright & Smith, 2004a). However, the odour of anthers from only 10 oilseed-rape flowers was learnt well, and these results suggest that, at least for oilseed rape and field bean whose flowers occur in groups on racemes, pollen odours exist in nature in concentrations that can be detected and learnt by bees, alluding to a role in foraging behaviour.

This study further suggests that pollen odours are a significant part of the whole flower aroma. In Experiment 4, bees generalize the odours from oilseed-rape whole flowers and their anthers, indicating that these odours are perceived to be similar; at least some of the components learnt in the recognition of one odour are present and detectable in the other. Bees conditioned to the odour of whole flowers also respond to the odour of anthers and this indicates that some of the key components learnt in whole flower recognition derive from anthers. Because bees can detect the odour of anthers

amongst the whole floral odours and can detect species-specific differences in pollen odours, they could be used as an additional cue in species discrimination during foraging.

Both Von Aufsess (1960) and Von Frisch (1967) indicate that honey bees can differentiate between pollen odours and other floral parts, suggesting that odours are distinct within the whole flower context. However, analyses conducted to date on the floral volatiles from different species that reveal the chemical basis of these differences (reviewed in Dobson & Bergström, 2000) have not yet been extended to oilseed rape. To test whether or not there are detectable differences between the aroma of whole oilseed-rape flowers and the odour of the anthers, a discrimination experiment involving these two odours should be conducted. Such studies would shed light on whether or not honey bees could use pollen odours to discriminate pollen-rewarding from nonrewarding flowers, as has been suggested for bumble bees (Dobson *et al.*, 1999).

The importance of pollen odours in foraging behaviour may vary between plant species. There is some evidence from the present study that oilseed-rape pollen is learnt more efficiently than field-bean pollen. As with differences between bee- and hand-collected pollens, this could be attributed to differences in concentration, and may explain why, in Experiment 3 (the generalization bioassay involving hand-collected oilseed-rape and field-bean pollens), bees conditioned to field bean generalize to oilseed rape, but bees conditioned to oilseed-rape pollen generalize less, but not significantly less, to field bean. At low concentrations, generalization by honey bees between similar odours is higher than at high concentrations (Bhagavan & Smith, 1997; Wright & Smith, 2004a, b).

The present study uses restrained honey bees and classical conditioning methods to investigate the learning and recognition abilities of honey bees with respect to pollen and floral odours. These experiments show that honey bees possess the odour-processing abilities to learn these odours, and to generalize or discriminate between them. Thus, although it appears unlikely that honey bees learn the odour of bee-collected pollen within the hive and use this knowledge when foraging, pollen odours from dehiscing anthers could play an important role in honey bee foraging behaviour through their use in floral recognition, species discrimination and, possibly, in assessing reward availability. Previous experiments have demonstrated that the results of the proboscis extension response bioassay closely match those investigating instrumental learning (based on operant behaviour) in bees using free-flying bees in a choice situation (Mauelshagen & Greggers, 1993; Pham-Delègue *et al.*, 1993; Laloi *et al.*, 2000). However, tests must also be carried out using free-flying bees in more natural situations, where they can learn as a result of their choices, to show whether or not pollen odours do play a role in honey bee foraging behaviour.

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References

- Bergström, G., Dobson, H.E.M. & Groth, I. (1995) Spatial fragrance patterns within the flowers of *Ranunculus acris* (*Ranunculaceae*). *Plant Systematics and Evolution*, **195**, 221–242.
- Bhagavan, S. & Smith, B.H. (1997) Olfactory conditioning in the honey bee, *Apis mellifera*: effects of odor intensity. *Physiology and Behavior*, **61**, 107–117.
- Bitterman, M.E., Menzel, R., Fietz, A. & Schäfer, S. (1983) Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). *Journal of Comparative Psychology*, **97**, 107–119.
- Blight, M.M., Le Métayer, M., Pham-Delègue, M.-H. *et al.* (1997) Identification of floral volatiles involved in recognition of oilseed rape flowers, *Brassica napus* by honeybees, *Apis mellifera*. *Journal of Chemical Ecology*, **23**, 1715–1727.
- Boelter, A.M. & Wilson, W.T. (1984) Attempts to condition the pollen preference of honey bees. *American Bee Journal*, **124**, 609–610.
- Calderone, N.W. & Johnson, B.R. (2002) The within-nest behaviour of honeybee pollen foragers in colonies with a high or low need for pollen. *Animal Behaviour*, **63**, 749–758.
- Cook, S. (2000) The use of pollen cues in resource location by a pollinator and a pest. PhD Dissertation, Nottingham University, U.K.
- Cook, S.M., Awmack, C.S., Murray, D.A. & Williams, I.H. (2003) Are honey bees' foraging preferences affected by pollen amino acid composition? *Ecological Entomology*, **28**, 622–627.
- Dicke, M. & van Loon, J.J.A. (2000) Multitrophic effects of herbivore-induced plant volatiles in an evolutionary context. *Entomologia Experimentalis et Applicata*, **97**, 237–249.
- Dobson, H.E.M. (1994) Floral volatiles in insect biology. *Insect-Plant Interactions* (ed. by E. A. Bernays), Vol. V, pp. 47–81. CRC Press, Boca Raton, Florida.
- Dobson, H.E.M. & Bergström, G. (2000) The ecology and evolution of pollen odors. *Plant Systematics and Evolution*, **222**, 63–87.
- Dobson, H.E.M., Bergström, G. & Groth, I. (1990) Differences in fragrance chemistry between flower parts of *Rosa rugosa* Thunb. (*Rosaceae*). *Israel Journal of Botany*, **39**, 143–156.
- Dobson, H.E.M., Danielson, E.M. & Van Wesep, I.D. (1999) Pollen odor chemicals as modulators of bumble bee foraging on *Rosa rugosa* Thunb. (*Rosaceae*). *Plant Species Biology*, **14**, 153–166.
- Free, J.B. (1970) The flower constancy of bumble bees. *Journal of Animal Ecology*, **39**, 395–401.
- Free, J.B. (1993) *Insect Pollination of Crops*, 2nd edn. Academic Press, U.K.
- Getz, W.M. & Smith, K.B. (1987) Olfactory sensitivity and discrimination of mixtures in the honeybee *Apis mellifera*. *Journal of Comparative Physiology A*, **160**, 239–245.
- Kalish, H.I. (1969) Stimulus generalization. *Learning: Processes* (ed. by M. H. Marx), pp. 207–297. Macmillan, New York, New York.
- Kirk, W.D.J. (1994) *A Colour Guide to Pollen Loads of the Honey Bee*. International Bee Research Association, U.K.
- Knudsen, J.T. & Tollsten, L. (1991) Floral scent and intrafloral scent differentiation in *Moneses* and *Pyrola* (*Pyrolaceae*). *Plant Systematics and Evolution*, **177**, 81–91.
- Knudsen, J.T., Tollsten, L. & Bergström, L.G. (1993) Floral scents – a checklist of volatile compounds isolated by head-space techniques. *Phytochemistry*, **33**, 253–280.
- Laloi, D., Roger, B., Blight, M.M. *et al.* (1999) Individual learning ability and complex odor recognition in the honey bee, *Apis mellifera* L. *Journal of Insect Behavior*, **12**, 585–597.
- Laloi, D., Bailez, O., Roger, B. *et al.* (2000) Recognition of complex odors by restrained and free-flying honeybees, *Apis mellifera*. *Journal of Chemical Ecology*, **26**, 2307–2319.
- Levin, M.D. & Bohart, G.E. (1955) Selection of pollens by honey bees. *American Bee Journal*, **95**, 392–393 & 402.
- Marasculio, L.A. & McSweeney, M. (1967) Nonparametric post hoc comparisons for trend. *Psychological Bulletin*, **67**, 401–412.
- Mauelshagen, J. & Greggers, U. (1993) Experimental access to associative learning in honeybees. *Apidologie*, **24**, 249–266.
- McCullagh, P. & Nelder, J.A. (1989) *Generalized Linear Models*, 2nd edn. Chapman & Hall, U.K.
- Menzel, R. & Müller, U. (1996) Learning and memory in honeybees: from behavior to neural substrates. *Annual Review of Neuroscience*, **19**, 379–404.
- Menzel, R., Greggers, U. & Hammer, M. (1993) Functional organization of appetitive learning and memory in a generalist pollinator, the honey bee. *Insect Learning: Ecological and Evolutionary Perspectives* (ed. by D. Papaj and A. C. Lewis), pp. 79–125. Chapman & Hall, U.K.
- Minnich, D.E. (1932) The contact chemoreceptors of the honey bee *Apis mellifera* Linn. *Journal of Experimental Zoology*, **61**, 375–393.
- Pelz, C., Gerber, B. & Menzel, R. (1997) Odorant intensity as a determinant for olfactory conditioning in honeybees: roles in discrimination, overshadowing and memory consolidation. *Journal of Experimental Biology*, **200**, 837–847.
- Pernal, S.F. & Currie, R.W. (2002) Discrimination and preferences for pollen-based cues by foraging honeybees, *Apis mellifera* L. *Animal Behaviour*, **63**, 369–390.
- Pham-Delègue, M.-H., Bailez, O., Blight, M.M. *et al.* (1993) Behavioural discrimination of oilseed rape volatiles by the honeybee *Apis mellifera* L. *Chemical Senses*, **18**, 483–494.
- Pham-Delègue, M.-H., Masson, C., Etievant, P. & Azar, M. (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., Etievant, P., Guichard, E. & Masson, C. (1989) Sunflower volatiles involved in honeybee discrimination among genotypes and flowering stages. *Journal of Chemical Ecology*, **15**, 329–343.
- Pham-Delègue, M.-H., Blight, M.M., Kerguelen, V. *et al.* (1997) Discrimination of oilseed rape volatiles by the honeybee: combined chemical and biological approaches. *Entomologia Experimentalis et Applicata*, **83**, 87–92.
- Reinhard, J., Srinivasan, M.V. & Zhang, S.W. (2004) Scent-triggered navigation in honeybees. *Nature*, **427**, 411–411.

- Robaker, D.C., Flottum, P.K., Sammataro, D. & Erickson, F.H. (1982) Effects of climatic factors on soybean flowers. *Field Crops Research*, **122**, 267–278.
- Roulston, T.H. & Cane, J.H. (2000) Pollen nutritional content and digestibility for animals. *Plant Systematics and Evolution*, **222**, 187–209.
- Sandoz, J.C., Laloi, D., Odoux, J.F. & Pham-Delegue, M.H. (2000) Olfactory information transfer in the honeybee: compared efficiency of classical conditioning and early exposure. *Animal Behaviour*, **59**, 1025–1034.
- Sawyer, R. (1981) *Pollen Identification for Beekeepers*. University College Cardiff Press, U.K.
- Schmidt, J.O. & Johnson, B.E. (1984) Pollen feeding preference of *Apis mellifera*, a polylectic bee. *Southwestern Entomologist*, **9**, 41–47.
- Siegel, S. & Castellan, N.J. (1988) *Nonparametric Statistics for the Behavioral Sciences*, 2nd edn. McGraw-Hill, New York, New York.
- Smith, B.H. (1993) Merging mechanism and adaption: an ethological approach to learning and generalization. *Insect Learning: Ecological and Evolutionary Perspectives* (ed. by D. Papaj and A. C. Lewis), pp. 126–157. Chapman & Hall, U.K.
- Smith, B.H. & Getz, W.M. (1994) Nonpheromonal olfactory processing in insects. *Annual Review of Entomology*, **39**, 351–375.
- Von Aufsess, A. (1960) Geruchliche nahorientierung der biene bei entomophilen und ornithophilen blüten. *Zeitschrift für Vergleichende Physiologie*, **43**, 469–498.
- Von Frisch, K. (1967) *The Dance Language and Orientation of Bees*. Harvard University Press, Cambridge, Massachusetts.
- Wenner, A.M., Wells, P.H. & Johnson, D.L. (1969) Honey bee recruitment to food sources: Olfaction or language? *Science*, **164**, 84–86.
- Winston, M.L. (1987) *The Biology of the Honey Bee*. Harvard University Press, Cambridge, Massachusetts.
- Wright, G.A. & Smith, B.H. (2004a) Different thresholds for detection and discrimination of odors in the honey bee (*Apis mellifera*). *Chemical Senses*, **29**, 127–135.
- Wright, G.A. & Smith, B.H. (2004b) Variation in complex olfactory stimuli and its influence on odour recognition. *Proceedings of the Royal Society of London Series B – Biological Sciences*, **271**, 147–152.
- Wright, G.A., Skinner, B.D. & Smith, B.H. (2002) Ability of honeybee, *Apis mellifera*, to detect and discriminate odors of varieties of canola (*Brassica rapa* and *Brassica napus*) and snapdragon flowers (*Antirrhinum majus*). *Journal of Chemical Ecology*, **28**, 721–740.

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