

Floral scents induce recall of navigational and visual memories in honeybees

Judith Reinhard*, Mandyam V. Srinivasan, David Guez and Shaowu W. Zhang

Research School of Biological Sciences, Visual Sciences, The Australian National University, PO Box 475, Canberra, ACT 2601, Australia

*Author for correspondence (e-mail: judith.reinhard@anu.edu.au)

Accepted 22 September 2004

Summary

During foraging flights, honeybees learn visual and chemical cues associated with a food source. We investigated whether learned olfactory cues can trigger visual and navigational memories in honeybees that assist them in navigating back to a known food source. In a series of experiments, marked bees were trained to forage at one or more sugar water feeders, placed at different outdoor locations and carrying different scents or colours. We then tested the ability of these bees to recall the locations (or colours) of these food sites and to fly to them, when the training scents were blown into the hive, and the scents and food at the feeders were removed. The results show that (1) bees, trained to a single-scented feeder at a given location, can be induced to fly to the same location by blowing the scent into the hive; (2) bees, trained to two feeders, each placed at a different location and carrying a

different scent, can be induced to fly to either location by blowing the appropriate scent into the hive; and (3) bees, trained to two feeders, each decorated with a different colour and carrying a different scent, can be induced to find a feeder of either colour by blowing the appropriate scent into the hive. Thus, familiar scents can trigger navigational and visual memories in experienced bees. Our findings suggest that the odour and taste of the nectar samples that are distributed by successful foragers on returning to the hive, may trigger recall of navigational memories associated with the food site in experienced recruits and, thus, facilitate their navigation back to the site.

Key words: associative learning, recall, memory, vision, olfaction, navigation, honeybee.

Introduction

A honeybee colony's (*Apis mellifera* L.) search for food is a well-coordinated process requiring precise orientation over distances of several kilometres (von Frisch, 1993; Gould, 1993). Foraging bees learn information about the location of a food source with respect to the hive, and on return communicate this information to their nest mates to recruit them. When a scout bee returns home after finding a good food source, she performs the so-called 'waggle dance' in the hive that informs her nest mates about the location of the food source, in terms of its distance from the hive and its direction relative to the sun (reviewed by von Frisch, 1993; Dyer, 2002). These navigational cues are learnt by the scout bee during her foraging flights. Honeybees gauge the direction of a food source using the sun and the polarized-light pattern that it creates in the sky as a celestial compass (Lindauer, 1963; Wehner, 1982; Wehner and Rossel, 1985; von Frisch, 1993). The distance to the food source is likely to be estimated through cues based on optic flow. That is, distance flown is measured by the extent to which the image of the environment moves across the visual field of the eye as the bee flies to a destination (Esch and Burns, 1995, 1996; Srinivasan et al., 1996, 1997, 2000; Esch et al., 2001; Si et al., 2003; Hrnčir et al., 2003; Tautz et al., 2004).

While it is clear that honeybee navigation relies substantially on a sun compass and an 'odometer', bees are also known to use additional visual and olfactory cues that aid the process of navigation and help guide them to their goal (Friesen 1973; Collett 1992; Tautz and Sandeman 2003). Here, we explore the use of associative learning of chemical information as one such cue. Chemical stimuli associated with a food source could be floral odours, or the taste and fragrance of the nectar collected by foraging bees and distributed in the hive on return (Winston, 1987; Kirchner and Grasser, 1998).

The neurobiology of learning and memory in honeybees and their capacity to learn environmental cues have been investigated extensively (Smith, 1991; Collett, 1992; Hammer and Menzel, 1995, 1998; Menzel and Mueller, 1996; Hammer, 1997; Joerges et al., 1997; Oleskevich et al., 1997; Galizia et al., 1998; Faber et al., 1999; Maleszka et al., 2000; Maleszka and Helliwell, 2001; Menzel and Giurfa, 2001). Honeybees are not only capable of simple associative learning by reward; they are also capable of mastering abstract relationships between stimuli (Giurfa et al., 2001). Laboratory experiments have shown that bees can match and group visual stimuli perceptually, and apply learned matching rules to new contexts (Zhang et al., 1995, 1999; Collett and Barron, 1995; Menzel et

al., 2000; Giurfa et al., 2001). They are also capable of forming associations across sensory modalities (Srinivasan et al., 1998).

Our knowledge of honeybee navigation and 'cognition' raises the question of whether previously acquired chemical-visual associations can actually facilitate honeybee navigation in the field, thus enhancing the foraging efficiency of a colony. For example, if one of the potential recruits has already foraged at the signalled location some time in the past, can the taste and smell of the nectar samples distributed by a returning scout bee trigger the recruit's memory about the site, and help the recruit relocate it readily? Some of the items that are recalled might be, for example, the flower's visual attributes, such as colour and shape, the distance of the food source from the hive, the direction in which to fly to get there, and the landmarks expected en route and at the destination.

There is evidence that bees that have previously learnt to forage at a scented feeder can be induced to visit this feeder again by blowing the same scent into the hive (Ribbands 1954; Johnson 1967; Free 1969; von Frisch, 1993; Jakobsen et al., 1995). However, in these previous studies, the feeder continued to carry the scent when the scent was blown into the hive, thus allowing the bees to find the feeder by 'homing in' on the scent that they experienced in the hive. In the present investigation, we trained the bees with scented feeders, but tested them with empty, unscented ones, similar to an early study by Johnson and Wenner (1966). Thus, when the trained bees are tested by blowing scent into the hive, they cannot simply home in on the scent; they must find the appropriate feeder by relying on previously learned navigational information that is triggered by the scent. This study is thus a true test of the existence of associative recall in honeybees, and of whether this phenomenon helps them find food under natural, outdoor conditions. A brief report on some of our results has been published elsewhere (Reinhard et al., 2004).

Materials and methods

The experiments were carried out from December 2002 to April 2003, on a field site with flower patches, bushes and trees, ensuring *ad lib* food resources and providing a variety of naturally occurring landmarks for navigation. The experimental honeybee hive (*Apis mellifera* L.) was set up in a weather-protected spot in the area. Foraging bees entered and left the hive through a Perspex tube (length 20 cm, diameter 4 cm). The tube carried an arrangement, described in detail below, to inject scent into the hive entrance when desired. Bees were trained to forage at artificial feeders, consisting of glass jars (18 cm high, 10 cm diameter) containing a 2 mol sugar solution with 3 ml scent per litre sugar solution. As scents we used Natural Flavouring Essences (Queen Fine Foods Pty Ltd., Australia): rosewater essence, lemon essence or almond essence. Depending upon the particular experiment, up to three feeders were used, each carrying a different scent. The glass jars with the scented sugar solutions were placed inverted over white saucers (diameter 15 cm), containing three layers of folded tissue paper (Fig. 1A). The tissues soaked up the sugar

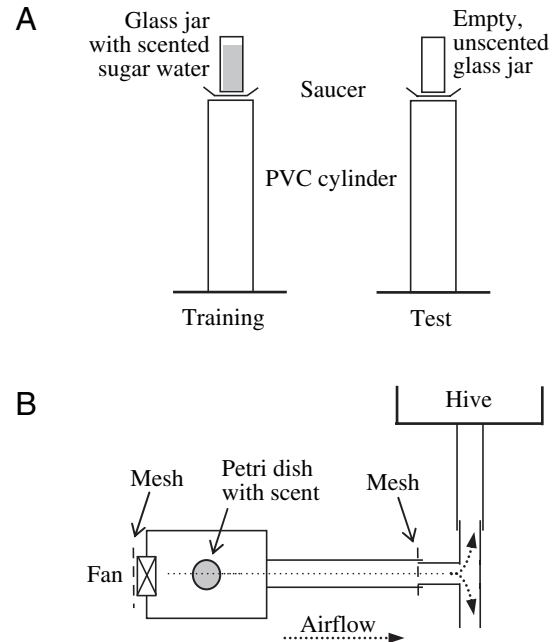


Fig. 1. (A) Design of feeders used during training and tests. (B) Setup of device used for blowing scent into the hive during the tests.

solution from the jar, thus providing a continuous flow of sugar water for the bees. Each feeder was placed on top of a white PVC cylinder (1 m high, 30 cm diameter).

Training

Training was commenced near the hive by inducing bees to feed from a piece of tissue paper soaked with sugar solution, placed at the hive entrance, and then transporting the tissue, with the feeding bees, manually to the feeder (or feeders, depending upon the particular experiment). This procedure was repeated a few times to get the bees to learn and accept the feeders as a food source. The feeders were then moved in 10 m steps away from the hive, always ensuring that enough bees had learnt the new locations before taking the next step, until the final feeder locations were reached, 50 m from the hive (Fig. 2). During the training, the scents were offered only at the feeders: they were not blown into the hive. After the feeders had been moved to their desired final locations, the bees visiting the feeders were marked with enamel paint on the thorax and/or abdomen, until sufficient numbers of bees had been marked. During further training, unmarked bees visiting the feeders were removed whenever possible. This was done (a) to prevent the feeders from being taken over by other bees from any strong, foreign hives in the vicinity and (b) to prevent unmarked bees from being trained, thus ensuring that a fresh, naïve group of bees was used for each experiment (described below). The training to the scented feeders was carried out over 2–3 consecutive days.

Tests

At least 2 hours before starting a test, the training was

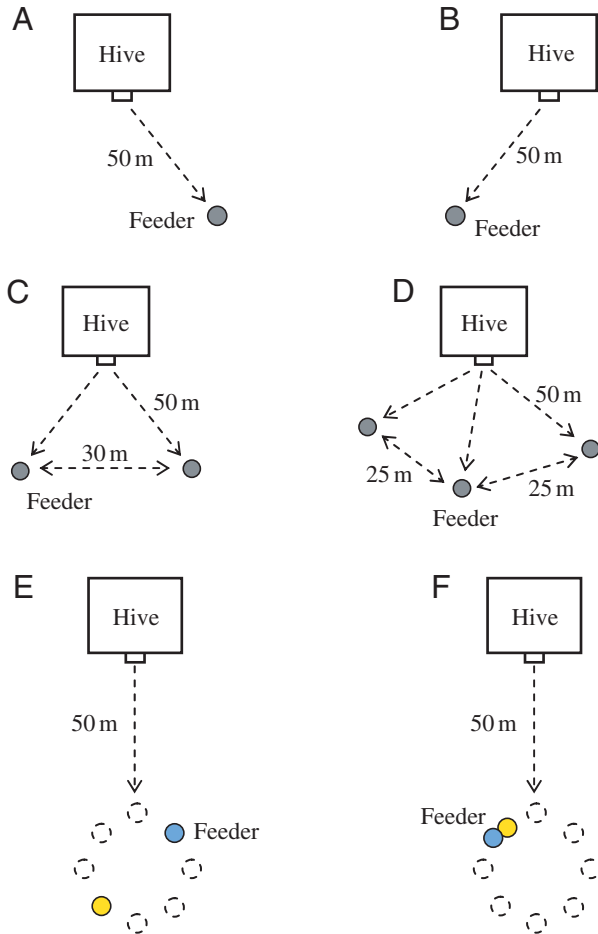


Fig. 2. Experimental configuration, showing feeder locations relative to the hive for experiment 1 (A,B), experiments 2–4 (C), experiment 5 (D), and experiment 6 (E,F).

interrupted and the feeders were removed to minimize random foraging around the feeder stations. During the tests, we offered empty, unscented feeders at the same locations. The scent was never offered at the feeders during the tests. Instead, it was blown into the hive, using a small fan (Jaycar YX-2505: 60×60 mm 12V/DC FP-108F, fan speed 4000 rpm, flow rate 0.57 m³ min⁻¹; Jaycar Electronics, Silverwater, NSW, Australia). The fan was attached to one side of a cardboard box (14.5×13.5×11 cm), which carried the scent that was blown into the hive (Fig. 1B). The scent was created by a piece of filter paper, soaked with 1.5 ml scent placed in an open Petri dish (diameter 8.5 cm) inside the box. A perspex tube exiting from the box connected to the entrance/exit tube of the hive to form a T-junction, as shown in Fig. 1B. This ensured that only some of the scent that was blown by the fan entered the hive. The rest left through the hive exit, thus reducing the risk of saturating the hive with the scent. Scent was placed in the box and the fan was run only during the tests. The bees were prevented from entering the scent box by screens of nylon mesh at the inlet end of the fan and at the T-junction. Each test lasted 8 min. The numbers and identity of bees visiting the empty,

unscented test feeders during the 8 min period of fan operation were registered. Details of scoring methods are described below. The 8 min test interval guaranteed sufficient numbers of visits while at the same time preventing bees from forming a lasting negative association, namely the lack of reward at the test feeders. Between tests, training was resumed for short periods (ca. 1 h) to maintain the level of learning and motivation.

Experiments

Six experiments were conducted, each involving training and testing. They are described below. Successive experiments were separated by one week, during which all previously marked bees were removed. This precaution, together with the procedure of removing unmarked bees visiting the feeders during training, ensured that the bees trained in each experiment had no prior experience with earlier experiments.

Experiment 1

The aim of this experiment was to investigate whether bees, trained to a scented feeder for a certain duration, can be induced to visit the feeder again when scent is blown into the hive. Honeybees were trained to a single feeder containing rose-scented sugar water, positioned 50 m to the left of the hive (Fig. 2A). The feeder was offered for 3 days, during which 300 bees visiting the feeder were marked with a dot of paint on the thorax. The feeder was then removed, and the bees were tested as follows. During the test, the training feeder was replaced by an empty, unscented feeder. We first blew air for 8 min into the hive (control), and then rose scent for 8 min. During each interval, we registered the number of marked bees visiting the feeder. Nine tests were conducted to accumulate sufficient data. The experiment was repeated with a fresh set of bees and a lemon-scented feeder, placed at a different location 50 m distant to the right of the hive (Fig. 2B). The training and testing procedures were exactly as for the rose scent, except that the feeder was scented with lemon, and lemon scent was blown into the hive during the tests.

Experiment 2

The aim of this experiment was to investigate whether bees in a colony could be induced to visit two different feeders, using two different scents. Honeybees were trained to a rose-scented and a lemon-scented sugar feeder, offered simultaneously at two different locations, each 50 m from the hive, for three consecutive days (Fig. 2C). Bees tended to remain faithful to the feeder to which they were trained and were marked accordingly: 300 bees visiting the rose-scented feeder were marked pink, and 300 bees visiting the lemon-scented feeder were marked yellow. During subsequent tests, the training feeders were replaced by empty, unscented feeders at the same locations. We first blew air for 8 min into the hive, then rose scent for 8 min, then air again for 8 min, and finally lemon scent for 8 min. During each air and scent interval, we registered all visits by pink and yellow marked bees at the two feeders. The test was carried out five times.

Experiment 3

The aim of this experiment was to examine whether a given bee could learn two different locations, and associate each with a different scent. Honeybees were trained to a rose-scented and a lemon-scented sugar feeder, positioned at the same locations as in experiment 2 (Fig. 2C). But this time the feeders were offered alternately, swapping every 20–30 min, thus ensuring that the same bees visited both feeder locations. The alternate training was continued for 2 days, and 30 honeybees that regularly visited both feeders were marked individually. In subsequent tests, we offered two empty, unscented test feeders at the training locations, as well as two additional empty, unscented feeders (dummy feeders) at two randomly chosen, equidistant locations in the area. The dummy feeders were used to examine whether the bees merely searched for feeders of known visual appearance, or whether they recalled specific navigational information. We first blew rose scent for 8 min into the hive, and then lemon scent for 8 min. During each scent interval, we registered the individually marked bees visiting the four feeders, noting for each bee which feeder she visited first, the number of circlings (sightings of a bee flying around the feeder within a radius of 50 cm), the number of landings (touching down on the feeder), and the total number of visits to each feeder (sum of all circlings and landings, including first visits). Unmarked bees were not registered. The test was carried out three times.

The experiment was repeated without offering two additional dummy feeders during the test, but only two empty, unscented test feeders at the training locations. As before we blew rose scent for 8 min into the hive, then lemon scent for 8 min, and registered the individually marked bees as described above. Then we examined the possible action of air flow *per se* as a trigger by running the following controls: we first blew air for 8 min (control A), and then collected data for 8 min with the fan switched off (control B). All tests were carried out four times. The entire training/testing experiment (except for controls A and B) was then repeated using two further pairs of scents to examine if bees could be trained to any two different scents: rose and almond, in one case, and lemon and almond in another. A fresh group of bees was trained and tested for each scent pair.

Experiment 4

The aim of this experiment was to examine whether a given bee could learn to associate three different scents with three different locations. Honeybees were trained to a rose-scented, a lemon-scented, and an almond-scented sugar feeder, each placed at a different location as shown in Fig. 2D. Training was carried out in a cyclic fashion: we first offered the rose-scented feeder at location one for 20–30 min, then the lemon-scented feeder at location two for 20–30 min, then the almond feeder at location three for 20–30 min, then the rose feeder again *etc.*, thus ensuring that the same bees visited all three feeder locations. This training was continued for 2 days and the bees were marked individually, as in experiment 3. During subsequent tests, we offered three empty, unscented test

feeders placed at the training locations. We blew rose scent for 8 min into the hive, then lemon scent for 8 min, and finally almond scent for 8 min. During each scent interval, we registered the individually marked bees visiting the three feeders, noting for each bee which feeder she visited first, the number of circlings, number of landings and the total number of visits she made to each feeder. Unmarked bees were not registered. The test was carried out four times.

Experiment 5

The aim of this experiment was to investigate whether a given bee could learn to associate a particular scent with a specific target colour. In this experiment the training feeders were as in the other experiments, but in addition they were wrapped with differently coloured pieces of cardboard. Honeybees were trained alternately to a yellow, rose-scented feeder and a blue, lemon-scented feeder, swapping every 20–30 min. Bees were marked individually as described above. During training, the feeders were positioned on the perimeter of a circular area (diameter 10 m), the centre of which was located ca. 50 m from the hive (Fig. 2E). The positions of the training feeders were varied randomly on the perimeter of the circle, to ensure that the bees learnt to associate the scent of each feeder with its colour, and not its location. During subsequent tests we offered two empty, un-scented, coloured test feeders placed at random, but diametrically opposite positions on the circle perimeter (Fig. 2E). We blew rose scent for 8 min into the hive, and then lemon scent for 8 min. During each scent interval, we registered the individually marked bees visiting the two feeders, noting for each bee which feeder she visited first, the number of circlings, of landings, and the total number of visits she made to each feeder. Unmarked bees were not registered. The test was carried out four times. Four further tests were conducted in which the two test feeders were positioned immediately next to each other (Fig. 2F), to simulate a mixed flower patch.

Statistics

Statistical analysis was carried out with STATISTICA by StatSoft Inc. For experiments 1 and 2, means and s.e. of visits by marked bees during scent and air intervals were calculated and data compared using Friedman ANOVA and Wilcoxon Matched-Pairs Test. For experiments 3–5, the data collected for individual bees were added up, thus obtaining for each scent interval the overall number of first visits, circlings, landings, and total visits made to each feeder. Chi² tests and observed *vs* expected frequency Chi² tests were used to determine whether the relative preferences for the test feeders were significantly different from each other and from random-choice levels. Choice frequencies were compared separately for first visits, circlings, landings and total visits.

Results

Experiment 1

The aim of this experiment was to investigate whether bees, which have been trained to a scented feeder, can be induced to

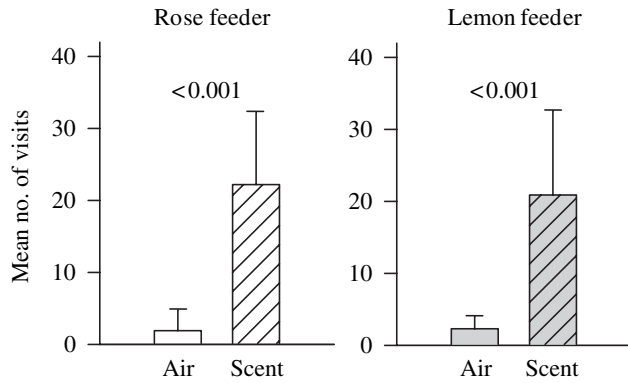


Fig. 3. Experiment 1. Scent-triggered navigation of groups of bees trained to a single feeder. Left: test results for bees trained to a rose-scented feeder (white). Right: test results for bees trained to a lemon-scented feeder (grey). The figure shows means \pm s.e. of visits by marked bees to an empty, unscented test feeder during an 8 min no-scent interval (air, unfilled bars) and an 8 min scent interval, when the respective scent is blown into the hive (hatched bars). $N=9$ tests for each scent and each no-scent interval. Numbers above bars show P -values for tests of significant difference between air and scent (Wilcoxon Matched-Pairs test).

return to that feeder, even if it is unscented and empty, merely by blowing the scent into the hive. The experiment was first carried out with a rose-scented feeder, and then repeated with a lemon-scented feeder. The results were the same for both: when air was blown into the hive (control), only a few of the trained and marked bees visited the empty unscented test feeder (Fig. 3). However, when rose scent or lemon scent, respectively, was blown into the hive, the number of visits to the test feeder was significantly higher. These results indicate that scent-trained bees can be induced to forage at a known location merely by injecting the scent into the hive, even when the destination no longer carries any food or olfactory cue.

Experiment 2

The aim of this experiment was to examine whether different scents can selectively trigger different groups of bees within a hive to fly to different locations. Groups of honeybees had been trained simultaneously to a rose-scented feeder (pink marked group) and a lemon-scented feeder (yellow marked group), each offered at a different location (Fig. 2C). In subsequent tests when air was blown into the hive (control), the mean numbers of marked bees visiting the empty, unscented test feeders at the former rose and lemon feeder locations were low and about equal (Fig. 4). The few visits of bees at the rose feeder location were only from pink bees; and at the lemon feeder location, they were from yellow bees. When rose scent was blown into the hive, the number of visits to the rose feeder location increased significantly, with all visits being from pink bees (Fig. 4, upper panel). By contrast, the mean number of visits to the lemon feeder location during the rose scent interval did not differ from the control, and only yellow bees continued to visit there. When blowing lemon scent, the results were

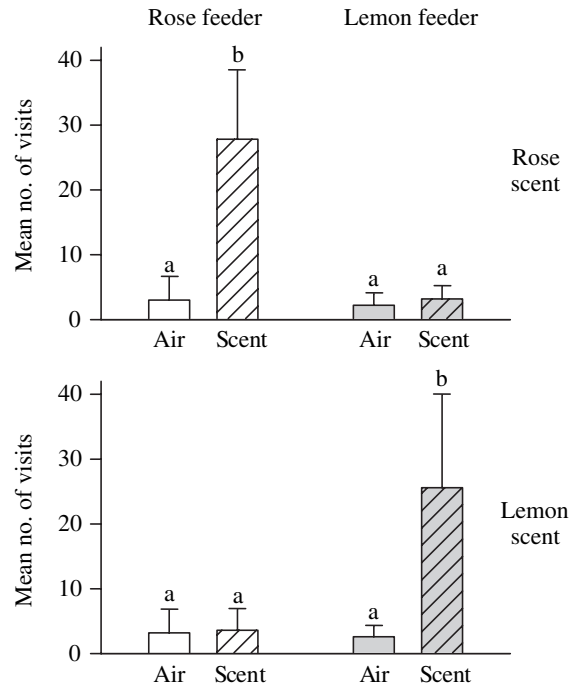


Fig. 4. Experiment 2. Scent-triggered navigation of groups of bees trained simultaneously to a rose-scented and a lemon-scented feeder placed at two different locations. The figure shows means \pm s.e. of visits by marked bees to empty, unscented test feeders (white, rose feeder; grey, lemon feeder) during an 8 min no-scent interval (air, unfilled bars) and an 8 min scent interval (hatched bars), when either rose scent (above) or lemon scent (below) was blown into the hive. $N=5$ tests for each scent and each no-scent interval. Different letters above bars denote significant difference at a level of at least $P<0.05$ (Friedman ANOVA and Wilcoxon Matched-Pairs test).

reversed: the number of visits to the lemon feeder location increased significantly, with visits being from yellow bees (Fig. 4, lower panel). Conversely, the visits to the rose feeder location did not differ from the control (Fig. 4, lower panel). These results indicate that different scents can selectively trigger different groups of bees within a hive to fly to different locations.

Experiment 3

The aim of this experiment was to investigate whether a given bee can simultaneously learn two different locations, and associate each location with a different scent. In the first round we used rose scent and lemon scent during training. During the tests we offered not only empty, unscented test feeders at each of the former training feeder locations but, in addition, two randomly placed 'dummy' feeders. When rose scent was blown into the hive, the majority of individually marked bees visited the rose feeder location. About a third of this number (or fewer) also visited the lemon feeder, and fewer than 5% visited the dummy feeders (Fig. 5). This was true regardless of how the bees' choices were measured: first visits, circlings, landings or total visits. When blowing lemon scent, it was the lemon feeder location that received the majority of visits, while

the rose feeder location received significantly fewer visits and the dummy feeders again fewer than 5%. This experiment demonstrates that bees can indeed learn two different food locations simultaneously, and associate each location with a different scent. It also shows that scent blown into the hive does not simply elicit a random search for a feeder in a certain area. Rather, it triggers bees to fly to the specific location associated with the scent.

When repeating the experiment without dummy feeders during the test interval, the results were the same; not only for rose and lemon, but also for the other scent pairs tested: rose and almond, and lemon and almond (Fig. 6). During each test,

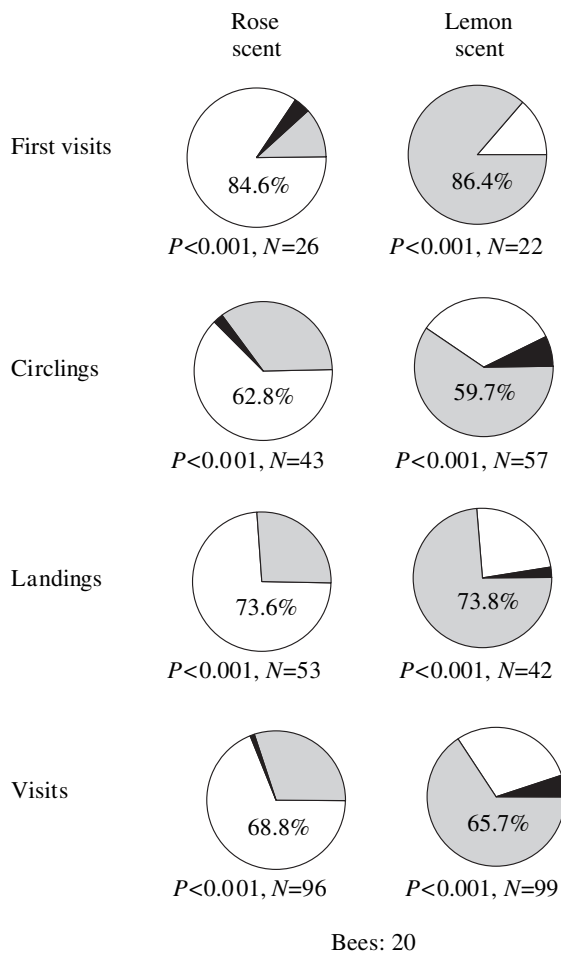


Fig. 5. Experiment 3. Scent-triggered navigation in individual bees, trained alternately to a rose-scented and a lemon-scented feeder placed at two different locations. Pie charts show distribution of visits to four empty, unscented test feeders during an 8 min rose scent interval, and an 8 min lemon scent interval (white, former rose feeder; grey, former lemon feeder; black, two dummy feeders placed randomly in the area). Data are accumulated from three tests for each scent, and are shown separately for first visits, circlings, landings and total visits. The number of individually marked bees that visited both test feeders, the number of choices (N) and P -values (observed *vs* expected frequency χ^2 Test) are shown below the pie charts. The expected frequency, based on random choice among the four test feeders, was 25%.

the majority of bees first visited the feeder location associated with the scent blown into the hive. Furthermore, the number of circlings, landings and total visits was significantly biased towards the associated feeder location. These results demonstrate that the ability of bees to associate two different feeding locations with two different scents is rather general, and not restricted to two specific scents.

During the control tests (control A, blowing air; control B, keeping the fan switched off), far fewer trained bees emerged from the hive in response to either of these conditions. Typically, the number of visits by marked bees to either test feeder was then only a fraction of that observed in the tests with scents (55 and 60 total visits, respectively, from four tests per control). More importantly, the emerging bees showed no preference for either location (total visits, control A, location 1, 52.7%, location 2, 46.3%, $P=0.517$, $N=55$, 9 bees; total visits, control B, location 1, 50%, location 2, 50%, $P=1.000$, $N=60$, 10 bees). Therefore, the airflow created by the fan elicited very little foraging on its own. It was the scent blown into the hive that triggered the bees to forage at specific locations.

Experiment 4

Here we investigated whether individual honeybees can learn to associate three different scents (rose, lemon and almond) with three different locations (Fig. 2D). During the tests, each of the three scents was blown into the hive, in turn. In contrast to the results of the tests in experiment 3, the honeybees did not show any preference towards the feeder location associated with the scent, irrespective of the scent that was blown (Fig. 7). The locations of first visits, circlings, landings and total visits appeared to be arbitrarily distributed among the three feeders. Some preferences were registered, however they were not correlated with the scents blown into the hive. When rose scent was blown the bees showed a preference in landings for the lemon feeder, and when lemon scent was blown they showed a preference in landings for the rose feeder. Also, in the test in which almond scent was blown, the bees showed a preference for the rose feeder location, rather than the almond feeder location. These results suggest that bees have difficulty in learning to associate three different scents with three different locations.

Experiment 5

The aim of this experiment was to investigate whether a given bee can learn to associate a particular scent with a specific target colour. For training, a yellow rose-scented feeder and a blue lemon-scented feeder were used with positions randomly varying on the perimeter of a circular area, as described in 'Materials and methods'. In the first type of test, when the differently coloured, but empty and unscented test feeders were placed 10 m apart on the perimeter of the training circle (Fig. 2E), the majority of trained bees visited the feeder that carried the colour associated with the scent that was blown into the hive. That is, the bees showed a strong and statistically significant preference for the yellow feeder when

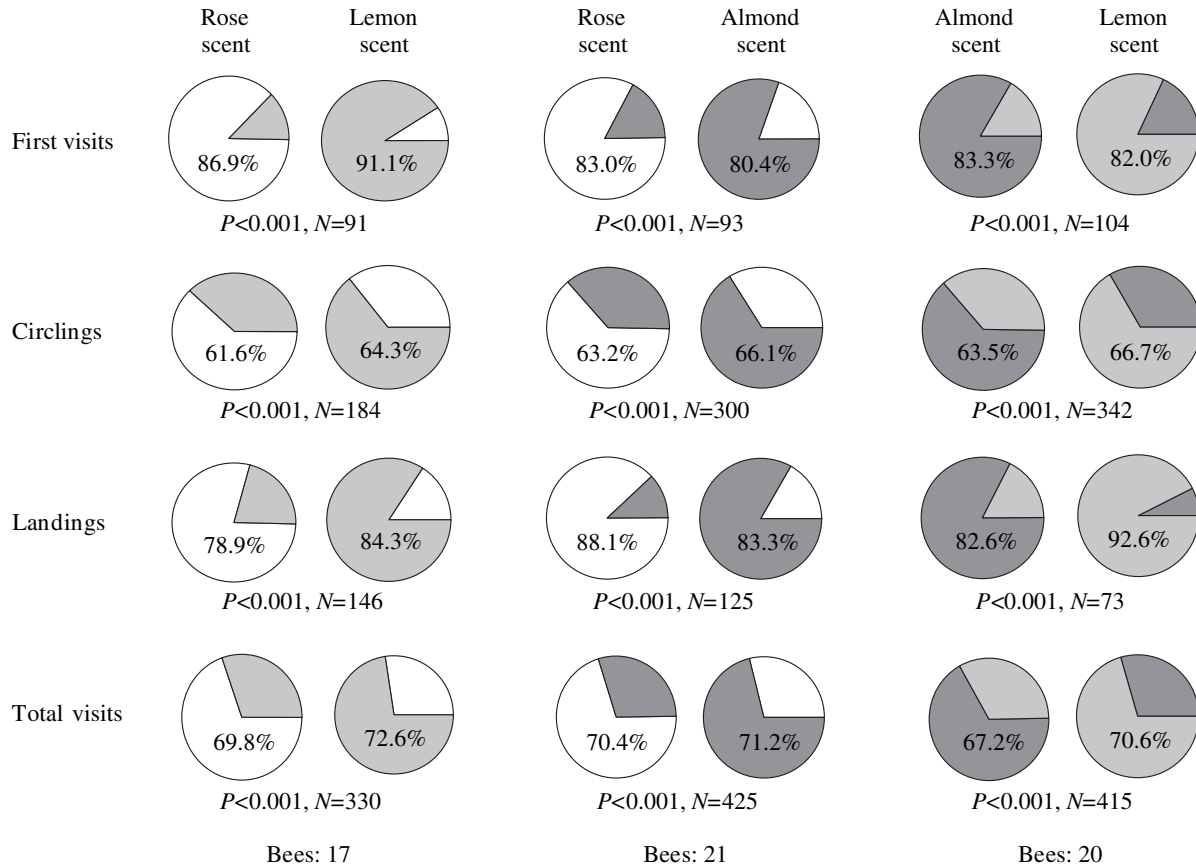


Fig. 6. Experiment 3. Scent-triggered navigation in individual bees, trained alternately to two feeders, each placed in a different location and carrying a different scent. The figure shows the results of experiments using the following scent combinations: rose and lemon, rose and almond, and almond and lemon. Pie charts show distribution of visits to the two test feeders in each experiment, when the respective scents were blown into the hive (white, former rose feeder; light grey, former lemon feeder; dark grey, former almond feeder). In each experiment data are accumulated from four tests for each scent, and are shown separately for first visits, circlings, landings and total visits. The number of individually marked bees that visited both test feeders, the number of choices (N) and P -values for tests of significant difference between the two feeders when the respective scents were blown (χ^2 test) are shown below the pie charts.

rose scent was blown, and for the blue feeder when lemon scent was blown (Fig. 8A). In the other type of test, the test feeders were placed immediately next to each other, as shown in Fig. 2F, simulating a mixed flower patch. In these tests, the bees visited both feeders equally often (Fig. 8B). Only landings were registered in this type of test, because first visits and circlings were difficult to attribute to an individual feeder when the two feeders were placed next to one another. The landings are displayed as ‘total visits’ in Fig. 8B. The results of this experiment indicate that bees are able to associate two different scents with two differently coloured targets, provided that the targets are not positioned very close together.

Discussion

The results of this study reveal that honeybees employ their capacity for associative learning and cross-modal recall to aid navigation. In correspondence to an earlier study (Johnson and Wenner, 1966), our results show, first of all, that a group of bees trained to forage at a scented feeder can be triggered to

return to the feeder’s former location merely by injecting the scent into the hive, even when the destination no longer bears the food or the scent (experiment 1). Second, different scents can be used to train and deploy different groups of bees to different locations (experiment 2). Thus, after training bees in a hive to simultaneously visit two feeders, each positioned at a different location and carrying a different scent, injection of one of the scents into the hive will selectively trigger one group of bees to visit one location, while injecting the other scent will trigger a different group of bees to visit the other location.

In contrast to earlier studies where the feeders or food sites always carried odour (Ribbands, 1954; Johnson 1967; Free, 1969; von Frisch, 1993; Jakobsen et al., 1995), we used unscented test feeders similar to Tautz and Sandeman (2003). Therefore, the bees could not find the food site by tracking its smell. In contrast to the study by Wells and Rathore (1995) our test feeders did not carry sugar water. Since the test feeders were empty, the bees were not rewarded, and consequently would not have danced upon return to the hive to recruit further bees to the location. Therefore, when scent was blown during

the test, the significant increase in the number of bees arriving at the feeders can only have been induced by the scent blown into the hive. But scent does not unleash foraging activity in an unspecific way, triggering all foragers to leave the hive in anticipation of food. Rather, scent induces marked bees to fly to the feeder, i.e. those that had acquired a prior memory of the location of the feeder. This observation indicates that a familiar scent indeed leads to a recall of a specific navigational memory. Unmarked bees were observed visiting the test feeders in experiments 1 as well as 2, but their number was comparatively low and remained constant regardless of whether scent or air was blown into the hive. They were most likely untrained foragers inspecting the feeders by chance. This supports the above notion, that injection of scent does not increase the general foraging activity of the hive. Rather, it only triggers a reaction in bees that have formed the scent-associated memory.

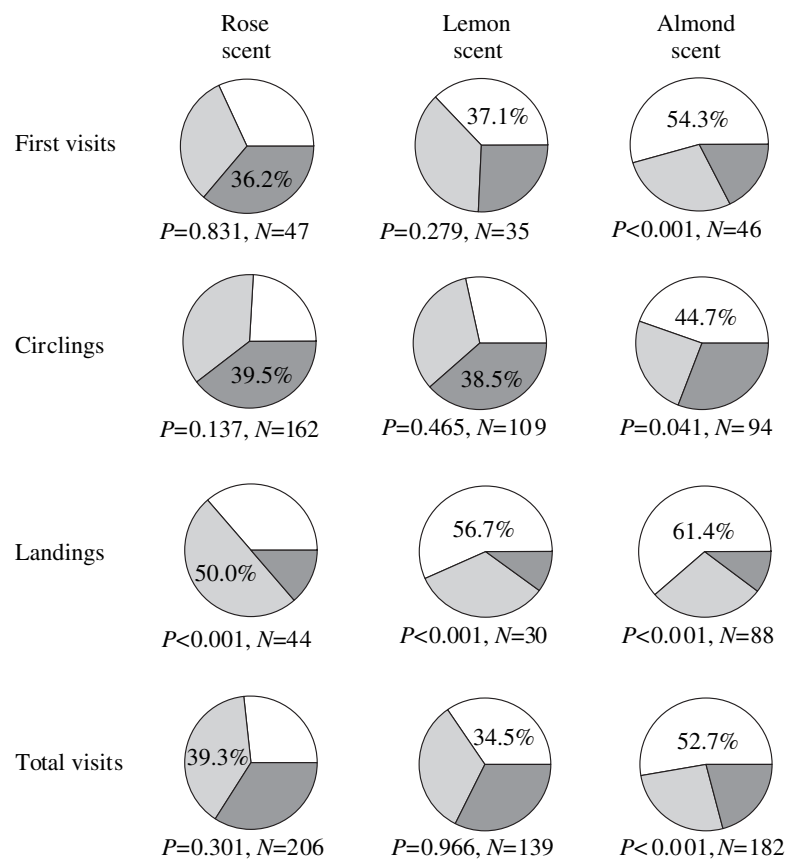


Fig. 7. Experiment 4. Scent-triggered navigation in individual bees, trained cyclically to a rose-scented, a lemon-scented, and an almond-scented feeder, each placed at a different location. Pie charts show distribution of visits to the three test feeders when the respective scents were blown into the hive (white, former rose feeder; light grey, former lemon feeder; dark grey, former almond feeder). In each experiment data are accumulated from four tests for each scent, and are shown separately for first visits, circlings, landings and total visits. The number of individually marked bees that visited all three test feeders, the number of choices (N) and P -values (observed vs expected frequency Chi² Test) are shown below the pie charts. The expected frequency, based on random choice among the three test feeders, was 33%.

When a group of bees is trained to visit to two feeders that are simultaneously presented, one subgroup tends to lock on to one feeder, and another subgroup to the other feeder. Apparently, the bees in each subgroup tend to remain faithful to the feeder that they first visited, and cross-visitation is rare (Johnson and Wenner, 1966). The percentage of marked bees in experiment 2 that visited both feeders during training was rather low: between 3 and 4% (unpublished observations). Consequently, the number of bees that could have arrived at a particular test feeder after having first visited the other feeder was likely to have been negligible. The majority of scent-triggered foragers flew directly to the appropriate test feeder, making it very likely that they were using scent-triggered navigational memory.

The above conjecture was tested more rigorously in experiment 3, where we ensured that each individually marked bee was trained alternately to two different locations, each associated with a different scent. In these experiments we only analysed data from individual bees that visited the feeders during both scent intervals in the test, because only of those could we be certain that they had learnt both associations. In each experiment, this was the majority of the 30 trained and individually marked bees. These experiments demonstrate clearly that bees can learn to associate specific scents with specific locations. Furthermore, an individual bee can remember at least two locations, and associate each one with a different scent. The experiment with the dummy feeders reveals that injection of scent does not simply trigger the bees to search for a feeder with the correct visual appearance. Rather, it causes the bees to search in the location that was associated with the scent during training.

Experiment 5 demonstrates that bees can also learn to associate different scents with targets of different colours. When the positions of the feeders are randomised during training, as they were in this experiment, but the association between scents and colours is preserved, trained bees can be induced to visit a feeder of a specific colour, regardless of its location, by blowing the associated scent into the hive. In nature, a good food source is not likely to change its position or colour, at least in the short term. Therefore, it is likely that, in natural foraging, bees learn to associate a floral scent with a location as well as a colour. Our results suggest that bees are better at learning to associate scents with locations, rather than colours: the frequency of correct choices is slightly higher in the location recall experiment (Fig. 6) than in the colour recall experiment (Fig. 8). Nevertheless, when the two coloured test feeders were offered 10 m apart, the honeybees clearly distinguished between the colours associated with the scents injected into the hive, making the correct choice in at least two thirds of the cases.

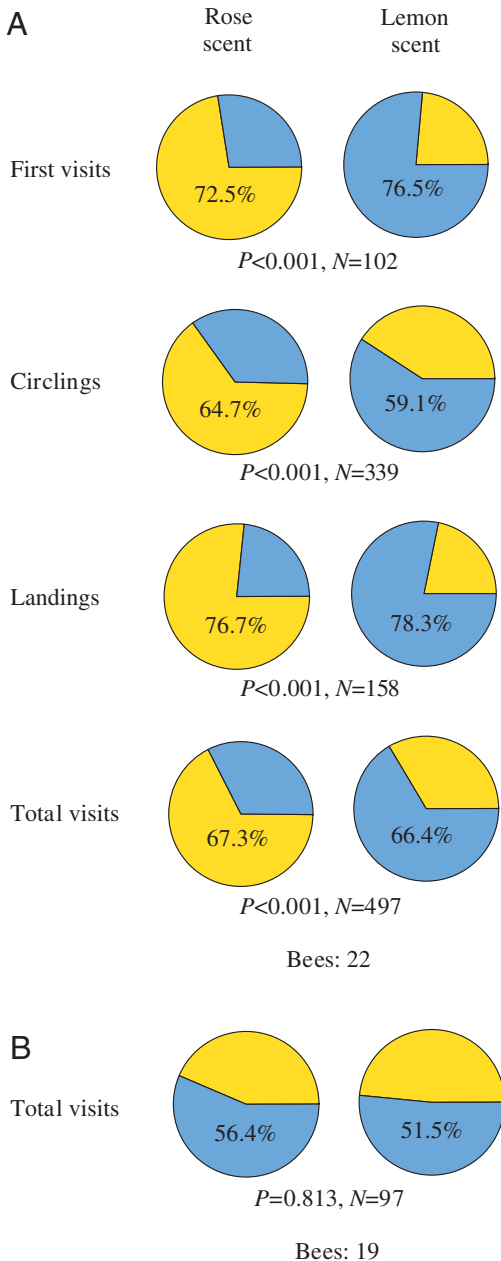


Fig. 8. Experiment 5. Scent-triggered navigation in individual bees, trained alternately to a yellow rose-scented and a blue lemon-scented feeder. The positions of the feeders were randomly varied on the perimeter of a circular area (diameter 10 m), to train bees to the colours of the feeders rather than their positions. Pie charts show distribution of visits to two empty, unscented, but coloured test feeders when the respective scents were blown into the hive (yellow, yellow test feeder; blue, blue test feeder). (A) Test feeders were placed 10 m apart, at diametrically opposite positions on the circle perimeter. (B) Test feeders were placed next to each other. In each case, data are accumulated from four tests for each scent. For A results are shown separately for first visits, circlings, landings and total visits, while for B only total visits could be registered. The number of individually marked bees that visited both test feeders, the number of choices (N), and P-values for tests of significant difference between the two feeders when the respective scents were blown (Chi² Test) are shown below the pie charts.

When the coloured test feeders were presented in close proximity to each other, the bees seemed to make no distinction between them. It is possible that this apparent disappearance of colour discrimination is simply a consequence of our testing procedure. With the two test feeders positioned next to each other, a bee approaching the correct colour would inevitably also see the other colour, and possibly treat the two feeders as a single object because of their close proximity (see also Huber et al., 1994) and because both were associated with a reward during training. This does not imply that bees are unable to distinguish between different colours when they are presented next to each other. We carried out a control experiment in which bees were specifically trained to discriminate between the same two coloured stimuli placed next to each other, by associating only one of them with a sugar reward and frequently swapping the positions of the two stimuli to prevent positional learning. When the trained bees were tested, they did not treat the two stimuli as a single object. They showed an excellent ability to discriminate between the two colours: they chose the correct stimulus with a frequency of 97.5% (158 visits, $P < 0.001$, Chi²-Test). Thus, the apparent failure of the bees to distinguish between the differently coloured feeders when they were juxtaposed in experiment 5 is not due to lack of colour discrimination *per se*.

Experiment 4 reveals that, when honeybees are trained to visit three differently scented feeders, each at a different location, injecting a scent into the hive does not cause them to return preferentially to the specific location that was associated with the scent during the training. Instead, the bees visit all three locations either randomly or if showing a preference it is not correlated with the scent blown into the hive. This lack of discrimination cannot be due to the inability of the bees to discriminate the three scents: the results of experiment 3 demonstrate clearly that the bees can discriminate all of the scents: rose, lemon and almond, when they are trained to distinguish between them in pair-wise fashion. Possibly, honeybees are not able to learn three different scent-location associations simultaneously. That is, when more than two scents and locations are involved, they associate every scent with every location. This finding is analogous to Menzel's early study (1969) showing that bees have difficulty in learning more than two colour-reward associations at the same time. It may well be that the honeybee's capacity for associative recall is limited to two separate items at any one time, if the recall involves multiple and multimodal cues, as it does in our experiments. If the bees are required to learn and recall more than two such complex scent-associated locations, they might prefer switching to the strategy of learning a simple general rule, instead of learning numerous specific cues and associations. In the present context, the bees in experiment 4 might have simply learnt the rule 'scent in the hive equals food in a known area'. Scent would then trigger vector memories of the general area where food was offered during training, and the bees would inspect all feeders in the area, leading to a random distribution of

visits. The reason for the weak feeder preferences that were nevertheless registered in our experiment remains uncertain, especially as the preferences were not correlated with the scents blown into the hive. Clearly, the experiment with three scents requires further investigation, to examine whether the apparent inability to learn three different scent-location associations persists even when the distances between the feeders, as well as the differences between their angular bearings as seen from the hive, are increased.

Von Frisch's classic studies of the honeybee's dance language (1993) have shown how scout bees recruit their nest mates to visit an attractive food source by conveying information on the direction and distance of the destination through the dance. Wenner and co-workers have always maintained the notion that honeybee recruitment is achieved primarily through olfactory cues (Johnson and Wenner, 1966; Johnson, 1967). Our findings do not diminish the importance of the dance, but they do point to a way in which olfactory cues could be used, through associative recall, as additional mechanisms that might facilitate recruitment. We find that scent injected into the hive triggers recall of the location of a formerly visited food source, as well as of some of its visual properties, such as its colour. A given bee can learn at least two locations, or colours, and associate each with a specific scent. Further investigation is required to explore whether scent also triggers recall of other features of a food site, such as the shapes of the flowers, or the properties and layout of the surrounding landmarks. In nature, the taste and scent of nectar samples distributed by successful foragers returning to the hive could trigger recall of a variety of visual and navigational memories associated with the food site in experienced recruits, and thus expedite their journey to the site.

We thank S. Moritz and H. Zhu for help with the experiments, and J. Srinivasan for the use of her property for conducting the experiments. J.R. was supported by a Feodor-Lynen Research Fellowship from the Alexander von Humboldt Foundation (FLF-1093207), by the Centre for Visual Sciences, ANU, and by a grant from the Australian Research Council to S. W. Zhang and M. V. Srinivasan (DP0450535). D.G. was supported by a grant from the Australian Research Council to M. V. Srinivasan and S. W. Zhang (DP0208683).

References

- Collett, T. S. (1992). Landmark learning guidance in insects. *Philos. Trans. R. Soc. Lond. B* **337**, 295-303.
- Collett, T. S. and Barron, J. (1995). Learnt sensory-motor mappings in honeybees: interpolation and its possible relevance to navigation. *J. Comp. Physiol. A* **177**, 287-298.
- Dyer, F. C. (2002). The biology of the dance language. *Annu. Rev. Entomol.* **47**, 917-949.
- Esch, H. E. and Burns J. E. (1995). Honeybees use optic flow to measure the distance of a food source. *Naturwissenschaften* **82**, 38-40.
- Esch, H. E. and Burns J. E. (1996). Distance estimation by foraging honeybees. *J. Exp. Biol.* **199**, 155-162.
- Esch, H. E., Zhang, S., Srinivasan, M. V. and Tautz, J. (2001). Honeybee dances communicate distances measured by optic flow. *Nature* **411**, 581-583.
- Faber, T., Joerges, J. and Menzel, R. (1999). Associative learning modifies neural representations of odours in the insect brain. *Nat. Neurosci.* **2**, 74-78.
- Free, J. B. (1969). Influence of the odour of a honeybee colony's food stores on the behaviour of its foragers. *Nature* **222**, 778.
- Friesen, L. J. (1973). The search dynamics of recruited honey bees, *Apis mellifera ligustica* Spinola. *Biol. Bull.* **144**, 107-131.
- Galizia, C. G., Naegler, K., Hoelldobler, B. and Menzel, R. (1998). Odour coding is bilaterally symmetrical in the antennal lobes of honeybees (*Apis mellifera*). *Eur. J. Neurosci.* **10**, 2964-2974.
- Giurfa, M., Zhang, S., Jenett, A., Menzel, R. and Srinivasan, M. V. (2001). The concepts of 'sameness' and 'difference' in an insect. *Nature* **410**, 930-933.
- Gould, J. L. (1993). Ethological and comparative perspectives on honey bee learning. In *Insect Learning* (ed. D. R. Papaj and A. C. Lewis), pp. 18-50. New York: Chapman and Hall.
- Hammer, M. (1997). The neural basis of associative reward learning in honeybees. *Trends Neurosci.* **20**, 245-252.
- Hammer, M. and Menzel, R. (1995). Learning and memory in the honeybee. *J. Neurosci.* **15**, 1617-1630.
- Hammer, M. and Menzel, R. (1998). Multiple sites of associative odor learning as revealed by local brain microinjections of octopamin in honeybees. *Learn. Mem.* **5**, 146-156.
- Hrcncir, M., Jarau, S., Zucchi, R. and Barth, F. G. (2003). A stingless bee (*Melipona seminigra*) uses optic flow to estimate flight distances. *J. Comp. Physiol. A* **189**, 761-768.
- Huber, B., Couvillon, P. A. and Bitterman, M. E. (1994). Place and position learning in honeybees (*Apis mellifera*). *J. Comp. Psych.* **108**, 213-219.
- Jakobsen H. B., Kristjansson K., Rohde B., Terkildsen M. and Olsen, C. E. (1995). Can social bees be influenced to choose a specific feeding station by adding the scent of the station to the hive air? *J. Chem. Ecology* **21**, 1635-1648.
- Joerges, J., Kuettner, A., Galizia, G. and Menzel, R. (1997). Representations of odours and odour mixtures visualized in the honeybee brain. *Nature* **387**, 285-288.
- Johnson, D. L. (1967). Communication among honey bees with field experience. *Anim. Behav.* **15**, 487-492.
- Johnson, D. L. and Wenner, A. M. (1966). A relationship between conditioning and communication in honey bees. *Anim. Behav.* **14**, 261-265.
- Kirchner, W. H. and Grasser, A. (1998). The significance of odor cues and dance language information for the food search behavior of honeybees (Hymenoptera: Apidae). *J. Insect Behav.* **11**, 169-178.
- Lindauer, M. (1963). Kompassorientierung. *Ergebn. Biol.* **26**, 158-181.
- Maleszka, R. and Helliwell, P. (2001). Effect of juvenile hormone on short-term olfactory memory in young honeybees (*Apis mellifera*). *Horm. Behav.* **40**, 403-408.
- Maleszka, R., Helliwell, P. and Kucharski, R. (2000). Pharmacological interference with glutamate re-uptake impairs long-term memory in the honeybee, *Apis mellifera*. *Behav. Brain Res.* **115**, 49-53.
- Menzel, R. (1969). Das Gedächtnis der Honigbiene fuer Spektralfarben. II. Umlernen und Mehrfachlernen. *Z. vergl. Physiologie* **63**, 290-309.
- Menzel, R. and Giurfa, M. (2001). Cognitive architecture of a mini-brain: the honeybee. *Trends Cog. Sci.* **5**, 62-71.
- Menzel, R. and Mueller, U. (1996). Learning and memory in honeybees: from behavior to neural substrates. *Annu. Rev. Neurosci.* **19**, 379-404.
- Menzel, R., Brandt, R., Gumbert, A., Komischke, B. and Kunze, J. (2000). Two spatial memories for honeybee navigation. *Proc. R. Soc. Lond. B* **267**, 961-968.
- Oleskevich, S., Clements, J. D. and Srinivasan, M. V. (1997). Long-term synaptic plasticity in the honeybee. *J. Neurophysiol.* **78**, 528-532.
- Reinhard, J., Srinivasan, M. V. and Zhang, S. W. (2004). Scent-triggered navigation in honeybees. *Nature* **427**, 411.
- Ribbands, C. R. (1954). Communication between honeybees. I: The response of crop-attached bees to the scent of their crop. *Proc. R. Entomol. Soc. London A* **29**, 141-144.
- Si, A., Srinivasan, M. V. and Zhang, S. W. (2003). Honeybee navigation: properties of the visually driven 'odometer'. *J. Exp. Biol.* **206**, 1265-1273.
- Smith, B. (1991). The olfactory memory in the honeybee *Apis mellifera*. I. Odorant modulation of short- and intermediate-term memory after single-trial conditioning. *J. Exp. Biol.* **161**, 367-382.
- Srinivasan, M. V., Zhang, S. W., Lehrer, M. and Collett, T. S. (1996).

- Honeybee navigation en route to the goal: visual flight control and odometry. *J. Exp. Biol.* **199**, 237-244.
- Srinivasan, M. V., Zhang, S. W. and Bidwell, N.** (1997). Visually mediated odometry in honeybees. *J. Exp. Biol.* **200**, 2513-2522.
- Srinivasan, M. V., Zhang, S. W. and Zhu, H.** (1998). Honeybees link sights to smells. *Nature* **396**, 637-638.
- Srinivasan, M. V., Zhang, S. W., Altwein, M. and Tautz, J.** (2000). Honeybee navigation: nature and calibration of the 'odometer'. *Science* **287**, 851-853.
- Tautz, J. and Sandemann, D. C.** (2003). Recruitment of honeybees to non-scented food sources. *J. Comp. Physiol. A.* **189**, 293-300.
- Tautz, J., Zhang, S. W., Spaethe, J., Brockmann, A., Aung Si and Srinivasan, M.** (2004). Honeybee odometry: performance in varying natural terrain. *PLoS Biology* **2**, 915-923.
- von Frisch, K.** (1993). *The Dance Language and Orientation of Bees*. London: Harvard University Press.
- Wehner, R.** (1982). Himmelsnavigation bei Insekten. Neurophysiologie und Verhalten. *Neujahrsbl. Naturforsch. Ges. Zuerich* **184**, 1-132.
- Wehner, R. and Rosell, S.** (1985). The bee's celestial compass – a case study in behavioral neurobiology. *Fortschr. Zool.* **31**, 11-53.
- Wells, H. and Rathore, R. R. S.** (1995). Discriminant conditioning of foragers in the Asian honey bees *Apis cerana* and *A. dorsata*. *Ecol. Entomol.* **20**, 374-379.
- Winston, M.** (1987). *The Biology of the Honey Bee*. Cambridge: Harvard University Press.
- Zhang, S. W., Srinivasan, M. V. and Collett, T.** (1995). Convergent processing in honeybee vision: Multiple channels for the recognition of shape. *Proc. Natl. Acad. Sci. USA* **92**, 3029-3031.
- Zhang, S. W., Lehrer, M. and Srinivasan, M. V.** (1999). Honeybee memory: Navigation by associative grouping and recall of visual stimuli. *Neurobiol. Learn. Memory* **72**, 180-201.