Weak and strong priming cues in bumblebee contextual learning

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Summary

Bees have the flexibility to modulate their response to a visual stimulus according to the context in which the visual stimulus is seen. They readily learn that in one context a yellow target, but not a blue one, should be approached to reach sucrose and that in another context blue, but not yellow, leads to sucrose. Here we contrast the bumblebee's ability to use two types of contextual or priming cue in deciding which of two coloured targets to approach. Bumblebees could perform this task well when the pairs of colours to be discriminated were in two different places, so that the cues associated with each place indicated whether the bees should select a blue or a yellow target. In this case the priming cues were presented concurrently with the rewarded stimuli. Priming cues, which occur a little earlier than a rewarded stimulus, seem less powerful in their ability to bias a bee's choice of rewarded stimulus.

We tried with a variety of methods to train bees to use a priming colour to indicate which of two colours should be approached a few seconds later. Our only success with such sequential priming cues was when each pair of rewarded and unrewarded colours could be distinguished by additional spatial cues. Bees were trained to choose a blue–black checkerboard over a yellow–black checkerboard, after viewing a yellow priming cue, and to choose a uniform yellow target over a uniform blue one, after viewing a blue priming cue. They performed this task almost without error. To see whether bees had associated each rewarded stimulus with the relevant sequential priming cue, bees were tested with a choice between the two rewarded stimuli (the yellow target and the blue–black checkerboard). The bees' choice was biased towards the blue–black checkerboard, when the preceding priming cue was yellow, and towards the yellow target, when the priming cue was blue. We suppose that the experiment works because the presence or absence of the checkerboard provides an additional distinguishing spatial cue that can be linked to and reinforce the sequential one. Under natural conditions, as when bees follow routes, there will normally be such a synergy between spatial and sequential cues.

Key words: bumblebees, visual learning, sequential priming, contextual learning, sequence learning.

Introduction

Honeybees and bumblebees will learn to treat the same visual or olfactory target in different ways in different spatial, temporal or motivational contexts, for instance approaching a blue target and avoiding a yellow target in one context and doing the reverse in another. Such contextual influences are important in allowing insects to switch flexibly between different sensori–motor mappings, or in providing support for identifying particular local stimuli that may be of significance in one context but not in another. A honeybee will modulate its response to a local stimulus according to time of day (Kolterman, 1971; Gould, 1987; Menzel et al., 1996), the panorama around it (Collett and Kelber, 1988; Collett et al., 1997), its viewing direction (Kelber, 1989), and whether the stimulus provides access to the hive or to a feeder (Srinivasan et al., 1998a). There has been less experimental work with bumblebees, but they too will learn to approach one of a pair of stimuli on the way to their nest and the other member of the pair on their way to a feeder (Fauria et al., 2000, 2002). In all these cases, the contextual stimuli and the local rewarded and unrewarded stimuli are present concurrently.

A number of recent studies on honeybees have reported that experiencing a cue at one time can influence the insect's response to a pair of stimuli a short time later (e.g., Srinivasan et al., 1998b). Bees shown a horizontally oriented grating of black and white stripes in one compartment of a maze will learn to select a blue over a green stimulus in the subsequent compartment and do the reverse, choosing green in preference to blue when shown a vertical grating in the first compartment (Zhang et al., 1999). Honeybees will also learn to perform delayed matching to sample (Giurfa et al., 2001; Zhang et al., 2004) and delayed non-matching to sample (Giurfa et al., 2001). Evidence for sequential priming also comes from the study of more naturalistic routes (Chittka et al., 1995). Compressing the distance between several artificial landmarks

along a familiar route induces a bee to search for a known food site at a shorter distance than usual. The bee's expectation of finding food nearer the hive is not triggered just by its recognising the landmark closest to the food. Its premature landing and search is evoked more reliably when it has seen several of the landmarks in the correct sequence. Such associations across short temporal delays are of great interest for understanding behavioural sequences and their organisation.

We describe here our attempts to demonstrate that bumblebees can use a coloured priming stimulus experienced at one time to determine their choice of a coloured rewarded stimulus a few seconds later. We used situations in which there were both sequential and spatial contextual cues. Bees were trained to approach a yellow rewarded stimulus rather than a blue stimulus after experiencing one priming cue and to approach a blue rewarded stimulus rather than a yellow stimulus after experiencing another priming cue. These priming cues turned out to influence the bees' later choice of rewarded stimulus, but only when the two rewarded colours had distinguishing spatial contexts so that spatial and sequential cues could reinforce each other.

Materials and methods

Colonies of *Bombus terrestris* L. were purchased commercially and given pollen daily in their nest box. Individually numbered bees foraged for sucrose in an experimental area that they reached from the nest box through a plexiglas tunnel. The tunnel was divided into compartments by a sequence of sliding doors with which we controlled an individual bee's entry to the foraging area. Sucrose was provided in feeding compartments with entry holes that were indicated by coloured stimuli.

Rewarded stimuli

To reach the sucrose, bees chose between yellow- and blue-coloured panels, or between blue–black or yellow–black checkerboards (22 mm checks), which were presented on LCD screens. All the LCD screens used in the experiments were 35 cm wide and 29 cm high with 1 cm wide black borders on all four sides (Iiyama, model: AS 4637UT BK; Stevenage, Herts, UK). In some experiments each screen was divided into two panels, each 29 cm high and 17.5 cm wide. In other experiments, the two colours were presented on two separate screens placed side by side. Unless stated, the sides of the rewarded and unrewarded stimuli were frequently swapped between training trials.

The usual arrangement in visual discrimination learning is for bees to enter a hole in the centre of a cardboard stimulus that either does or does not lead to a sucrose reward. The use of LCD screens for stimulus presentation meant a more complex arrangement, but had the advantage of allowing the presentation of stimulus and reward to be computer controlled. Bees on seeing the rewarded panel had to fly towards the top of the panel, where a hole was cut into an overhanging ledge

that led via a tunnel to a compartment with a sucrose dispenser (Fig. 1). A door at the reward end of the tunnel was opened or shut by a servo-controlled motor so allowing or denying the bees access to sucrose, according to whether or not the associated LCD panel displayed the rewarded colour. To reduce odour differences between the entry holes above the rewarded and unrewarded panels, the tunnel doors were multiply perforated.

It takes the bees a few seconds to move through the tunnel to the reward, but the consequent delay between seeing the stimulus and reaching the sucrose did not seem to impede the learning of colour discriminations or of spatial context. We think it unlikely that the delay would have had detrimental effects, which were specific to associations between sequential cues and rewarded colours.

Contextual and priming cues

In these experiments we used three very different priming set-ups and they are described in the Results together with the relevant experiments.

Training and testing

Groups of 6–10 bees were trained for each experiment, but by the end of the experiment the groups were usually smaller. The aim of the training process was to persuade bees to approach one coloured rewarded stimulus after viewing one priming cue and to approach the other rewarded stimulus after viewing the second priming cue. To simplify the bees' acquisition of the task, it was broken down into several steps (Fauria et al., 2002). After bees had become accustomed to foraging at the feeder, visual training began. In most experiments, training started with the bees experiencing, on different trials, the two priming cues and their associated
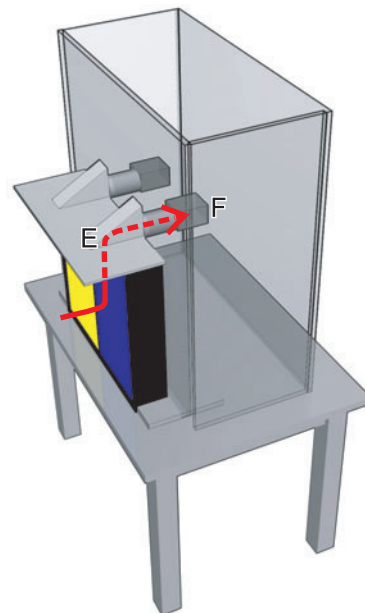

Fig. 1. Diagram of LCD screen and tunnel to feeder. E marks the entrance and F the feeder. Solid and broken lines show the bee's path.
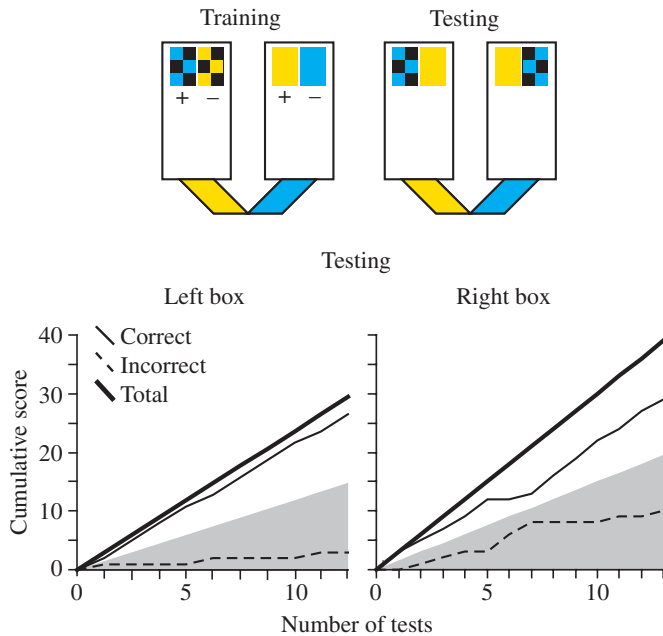
Fig. 2. Bees used spatial contextual cues to differentiate between two feeding sites. Top shows the arrangement of the apparatus with training and test stimuli. In the left flight box, the bees' task is to discriminate a rewarded (+) blue–black checkerboard from a non-rewarded (–) yellow–black checkerboard, and in the right flight box to discriminate a uniform rewarded yellow panel (+) from a blue one (–). In the absence of colour, yellow is always light grey and blue is dark grey. Bottom shows the bees' performance in tests with a choice between the reward stimuli from the two boxes. In this, and the remaining figures, choices are plotted cumulatively (ordinate) against the sequence of tests or trials for the indicated condition (abscissa), with the choices pooled over the group of bees tested (three in this case). We plot the total score, the number of correct choices and the number of wrong choices. The top edge of the grey wedge shows the null hypothesis of random choice. Bees prefer the blue–black checkerboard over the yellow panel in the left box and reverse their preference in the right box.

rewarded stimuli. Within a few trials bees directed their flight trajectories towards the rewarded stimuli, at which point the unrewarded stimuli were added. The exact way in which this last step was taken varied slightly between experiments. When the details are germane to the findings, they are described in the Results.

The only question asked in most experiments was whether bees could acquire the two discriminations. In other experiments, additional tests were given separated by training trials. Since bees tend to go where they were rewarded on the previous trial, test stimuli were arranged so that the correct choice was on the opposite side to the previously rewarded training stimulus. Experiments typically lasted two weeks with bees trained for six days a week. Chi^2 values for 2×2 contingency tables were calculated to test whether the bees' performance was independent of the two priming conditions. Each bee-trial or bee-test was considered an independent data point.

When a group of bees fail at a task it is helpful to know whether the group performance masks the success of a small minority of successful super bees. Thus, when there is enough individual data we have also analysed the performance of individual bees, again using Chi^2 values for 2×2 contingency tables to assess whether individual bees performed randomly with respect to the priming stimuli.

Odours

There is always the worry that choices may be determined by odour rather than by visual cues. Several reasons make us doubt that the results were contaminated by the effects of odours. First, the rewarded stimulus switched sides frequently and the doors were porous to odours, so that the two sides should have had very similar smells, and in tests both doors were open. Second, many experiments failed, with bees choosing randomly. If odours had been useful, the bees should have learnt to choose correctly. Third, if chemical signals deposited by bees at the tunnel entrances or further inside influenced the bees' choices, we would expect bees performing correctly to have made errors immediately after the training side was switched. There were no signs that performance was depressed in this way. Fourth, the behaviour of the bees when making decisions indicated that odours were not used. The bees flew in front of the screens and then usually flew upwards to enter one of the holes. They did not tend to fly directly between the holes.

Results
Synopsis

We start by describing a visual discrimination task, in which bees must reverse their choice of rewarded colour according to the presence or absence of an additional spatial cue. We then show that this task is sensitive to sequential priming, but that bumblebees fail to perform a sequential priming task in which they have to choose yellow over blue, when that is preceded by yellow, but to reverse their choice when the priming stimulus is blue. In the remaining sections, we describe three attempts to enhance the strength of sequential priming. We first test whether adding discriminative positional cues to the colours to be discriminated leads to sequential priming that will persist after the positional cues are weakened. Next, we examine whether sequential priming improves if positional components are added to the priming cues. Lastly, we ask if sequential priming works if both priming cues and rewarded stimuli have positional components.

A task that is sensitive to weak contextual cues
Apparatus

This initial experiment was performed in a set up that had been designed for another purpose. A Y-shaped tunnel (40 mm by 40 mm internal cross-section, with arms 32 cm long) connected the bees' nest box to two flight boxes (Fig. 2). The left arm of the Y leading to one flight box was illuminated from above with yellow light and the right arm leading to the other

box was illuminated with blue light. Each flight box was 175 cm long, 60 cm wide and 60 cm high with white painted wooden walls and floor and a transparent ceiling. The boxes were illuminated through their ceilings by reflected, diffused light from high frequency fluorescent tubes. Patterned and black papers were fixed to the white walls of the two boxes to provide some visual texture for the bees and to differentiate the two boxes. A divided LCD screen with two feeder compartments attached was placed at the far end of each box. The bees entered one or other flight box through the Y shaped tunnel and flew to the LCD screen at the end to collect sucrose. The two flight boxes were likely to have presented the bees with slightly different contexts, provided primarily by the different patterns on the walls, and possibly light patterns in the ceiling. The differently coloured corridors at the entry to the flight boxes are unlikely to have contributed much to the bees' choices.

Findings

Bees that were trained to visit the two flight boxes were unable to learn to choose a yellow over a blue stimulus in one flight box and a blue over a yellow stimulus in the other flight box, despite many attempts in which a variety of minor changes and improvements were made to the training procedure. When an additional spatial cue was added to distinguish the two pairs of colours, bees readily learnt the task. Thus, they learnt to choose a blue–black checkerboard over a yellow–black checkerboard in one box and a uniform yellow panel over a uniform blue panel in the other box (Fig. 2). In training trials given over 8 d, bees chose correctly the blue–black checkerboard in preference to the yellow–black checkerboard on 123 out of 124 trials in the left-hand box. And they chose a yellow panel over a blue one on 161 out of 167 trials in the right-hand box.

Was the presence or absence of the checkerboard the only contextual cue that the bees had employed when making these colour discriminations? Or had they also noticed other cues that could differentiate the boxes, and had they associated these extra cues with the checkerboard in one box and with the uniform colours in the other box? We examined whether trained bees can differentiate between the boxes by giving bees a choice in each box between the positive patterns from both boxes (i.e. plain yellow *vs* the blue–black checkerboard). A tendency to choose the appropriate rewarded stimulus in each flight box would indicate that the bees had identified the box and knew which colour was rewarded there.

To ensure that the bees did not just select the stimulus or the side that they had chosen correctly last time, tests were given in the opposite flight box from the bees' most recent training experience. During the tests, both doors in the feeder tunnels were open. Bees reliably chose the rewarded stimulus associated with each box. 39 tests were given in the right-hand box and bees chose the correct yellow target in 29 tests. Bees tested in the left-hand box chose the correct blue–black checkerboard target in 27 out of 30 tests (Fig. 2). Thus, cues associated with each box biased the bees' choices in favour of the rewarded stimulus normally encountered in that box (group data: Chi^2=25.71; d.f.=1; *P*<0.001). Sufficient data were obtained from each bee to show that each individual bee passed this test.

These results led to the following conclusions. (1) The spatial cues discriminating the two boxes were too weak by themselves for bees to learn to choose blue as a rewarded stimulus in one flight box and yellow as a rewarded stimulus in the other box. (2) The combination of box-specific spatial cues with the presence or absence of a checkerboard enabled bees to choose the appropriate rewarded stimulus, when tested in each box with a yellow panel *vs* a blue–black checkerboard. (3) This task was sensitive to relatively weak contextual cues. (4) The cues specific to each box combined with either the presence or absence of the checkerboard.

Can the colour of a priming cue determine a bumblebee's later choice between two coloured reward stimuli?

Giurfa et al. (2001) showed that honeybees can perform both delayed matching to sample and delayed non-matching to sample. In one of their experiments, honeybees were shown as a priming cue a blue or a yellow coloured card at the entrance to a Y-maze. Then, according to the training regime, they learnt to choose the same or the opposite colour to reach food in the Y-maze. Can bumblebees also use the colour of a priming cue to determine their later choice of a rewarded stimulus?

Colours plus checkerboard

Apparatus and procedure

We began with bees trained to the checkerboard and plain colour tasks of the previous section. But the set up was quite different. Bees flew from their nest box into a large flight arena (3.5 m by 2.9 m and 2.2 m high) illuminated by high-frequency fluorescent lights. A tunnel (4 cm square cross-section) leading from the nest box opened into a larger transparent tunnel, 15 cm in square cross-section and 40 cm long, through which the bees flew rapidly and in which they experienced yellow or blue priming colours before entering the flight arena. The priming colours were displayed on two LCD screens. One screen faced each sidewall of the tunnel. The two screens were slightly tilted with mirrors placed below and above the tunnel.

This priming cue was not a simple 'ganz-feld'. It presented strong luminance and chromatic contrast boundaries. The bee, on entering the tunnel, first passed a light–dark contrast boundary. It then saw briefly blue or yellow. On emerging from the tunnel, it passed a second black–white contrast boundary. Additionally, in most experiments two overlapping LCD screens, displaying the same colour as the tunnel, were placed on a brown table at short distances (35 cm and 65 cm) from the arena end of the tunnel. These screens, which we call frontal baffles, faced the bees as they flew through the tunnel and in their setting also presented a complex stimulus. While flying over the table towards the frontal baffles, the bee's ventral retina saw the dark surface of the table and its lateral and dorsal retina the white cardboard sides and fine netting that connected the end of the tunnel to the screens. The bees then
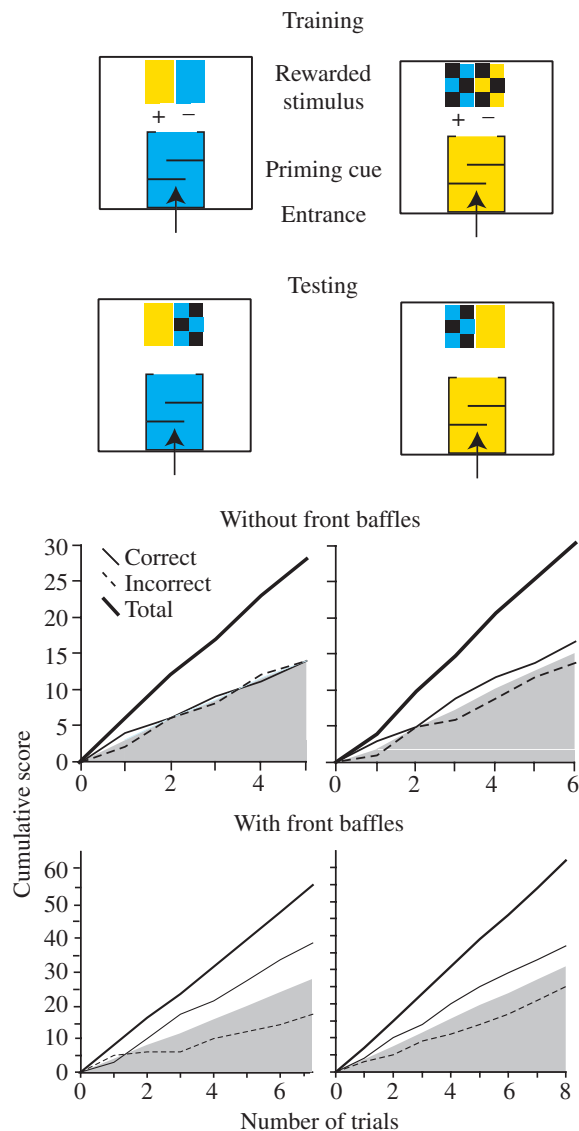
Fig. 3. Bees used a sequential priming cue to choose a rewarded stimulus that they approached after a short delay. Top shows the arrangement of training and test stimuli. Bees entered the priming set up at the arrow at the bottom of the diagram. A yellow priming stimulus in a tunnel predicted that bees should approach a rewarded (+) blue–black checkerboard rather than a non-rewarded (–) yellow–black checkerboard, and a blue priming stimulus in the same tunnel predicted that a uniform yellow panel was rewarded and that a uniform blue panel was unrewarded. The diagram shows baffles in front of the tunnel. Bottom shows the results of the tests. When bees were trained with a priming tunnel without baffles in front, their performance showed no influence of the priming cues. Six bees trained with no baffles showed no preference for the blue–black checkerboard, when the tunnel was yellow, or for the uniform yellow panel, when the tunnel was blue. Seven bees trained with frontal baffles added to the tunnel were influenced by the priming cues. They preferred the blue–black checkerboard when the priming cue was yellow and the uniform yellow panel when the priming cue was blue.

flew between the screens to reach the open arena, with the screens contrasting strongly with the arena beyond. The colour of the priming cue was switched between yellow and blue every 2–4 trials.

The bees, after flying through this priming set up, approached a table 135 cm away with a single LCD screen for presenting the unrewarded and rewarded stimuli. The screen was divided vertically into yellow and blue panels, when the tunnel was blue, and into a blue–black checkerboard and yellow–black checkerboard when the tunnel was yellow (Fig. 3). Bees were rewarded if they chose the yellow panel after flying through the blue tunnel and if they chose the blue–black checkerboard after flying through the yellow tunnel.

First, we tried the priming tunnel with no baffles. It turned out that sequential priming was only successful when the frontal baffles were present, and in all subsequent experiments with the tunnel, the frontal baffles were also in place.

Findings

Bees soon learnt to pick the blue–black checkerboard over the yellow–black checkerboard and the uniform yellow panel over the blue panel. To see whether bees could use the colour of the tunnel to select the appropriate rewarded stimulus, they were tested, as in the previous experiment, with the two rewarded stimuli – a plain yellow panel *vs* a blue–black checkerboard after passing through either the blue or the yellow tunnel. Testing with the two rewarded stimuli started after the bees' performance with the training stimuli was 95% correct (124 correct out of 130 trials) and continued for 5 d. There were eight training trials between each test. When the tunnel was blue, bees chose the yellow target on 14 tests out of 28 tests. When the tunnel was yellow, they chose the blue–black checkerboard on 17 tests out of 31 tests. Both individually and as a group, the bees' performance on these tests did not deviate significantly from chance (group data: $Chi^2=0.012$; d.f.=1; $P=0.912$). The colour of the tunnel had no detectable influence on the bees' choices, although in training the bees chose the rewarded stimulus on almost every trial.

At this stage we introduced coloured baffles as part of the priming set up and the bees' behaviour changed. The baffles ensured that the priming cue was viewed with frontal retina and it also extended the duration of the priming stimulus. The bees then tended to choose correctly when the priming cue was yellow, preferring the blue–black checkerboard over the yellow panel on 22 out of 33 tests. But their choice continued to be random on tests with the blue priming cue: they chose the yellow panel on 13 out of 28 tests. Unfortunately, the colony then stopped foraging and no more data could be obtained.

To extend these initial findings, a second colony was trained on the same task, with the frontal baffles in place from the start. As before, bees performed almost without error on training trials. Bees chose correctly in 1240 trials out of a total of 1281 training trials, with most of the errors at the start of training. Bees also tended to choose correctly in tests, but at a lower
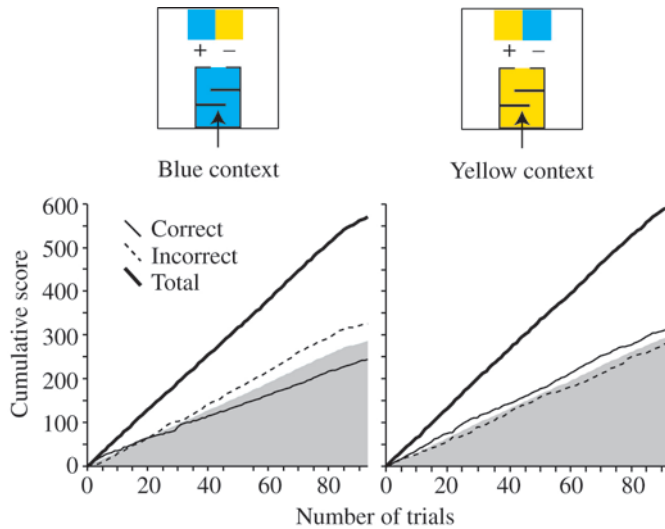
Fig. 4. Bees failed to use a sequential priming cue to switch their choice of colour. Top shows the arrangement of training stimuli. A yellow priming stimulus in a tunnel with frontal baffles indicated that bees should approach a yellow rather than a blue panel for a reward (+). A blue priming stimulus indicated that the blue panel was rewarded (+) and the yellow panel unrewarded (−). Bottom: seven bees failed to perform the task when trained for 180 trials. The group developed a slight preference for the yellow panel.

level of accuracy than in training. After a blue priming cue, bees chose the yellow panel over the blue–black checkerboard on 38 out of 55 tests. After the yellow priming stimulus, they chose the blue–black checkerboard over the yellow panel on 37 out of 62 tests (Fig. 3). The priming colour had a significant effect on the bees' choice of rewarded stimulus (group data: Chi^2=8.58; d.f.=1; P<0.005).

Both doors to the feeder were open during the first three days of testing and it seemed that during this period the bees' performance in tests, but not in training, started to deteriorate. We wondered whether the bees might have been learning that on trials with two rewarded stimuli, it did not matter which stimulus they chose. To counter this possibility, we changed the training and testing regimes. The number of training trials between tests was reduced to three and the test trials also became training trials. A wrong choice of yellow panel or blue–black checkerboard was now met by a closed door that blocked access to the sucrose.

This new regime did not influence the bees' performance during training trials when they were presented with plain yellow *vs* blue, or with the blue–black *vs* yellow–black checkerboards. Bees chose correctly on 392 trials out of 397 trials. It also did not improve the bees' performance during tests. Over four days of tests, the bees primed with yellow chose the blue–black checkerboard over the yellow target on 48 tests out of 70 tests and when primed with blue they chose the yellow target over the blue–black checkerboard over the yellow target on 37 tests out of 70 tests (Chi^2=5.74; d.f.=1; P<0.02). It seems that bees do make some use of the priming

colours in their choice of a rewarded stimulus, provided that the frontal baffles are present.

Can sequential priming cues reverse a bumblebee's choice of colour?

Can bumblebees given the choice between two plain colours learn to approach one colour after viewing one sequential priming cue and to approach the other colour after viewing a different priming cue. This task, which honeybees can perform successfully (Giurfa et al., 2001), makes greater demands of the sequential cue. Bees must switch between approaching and avoiding a colour without the help of any distinguishing spatial cues. An attempt was made to train bumblebees to choose a uniform blue panel after viewing a blue priming stimulus and to choose a uniform yellow panel after viewing a yellow priming stimulus. Bees again flew through a yellow or a blue tunnel and then passed between the frontal baffles displaying the same colour. The LCD screen carrying the rewarded and unrewarded stimuli was divided into uniform yellow and blue panels. Bees were rewarded for selecting the yellow panel after passing through the yellow tunnel and baffles, and they were rewarded for selecting the blue panel after passing through the blue tunnel and baffles (Fig. 4). Bees trained for 180 consecutive trials failed to associate their subsequent choice with what they had seen previously in the tunnel and on the baffles. They performed close to chance throughout the experiment. The blue priming cue was followed by the choice of the blue rewarded stimulus on 231 out of 571 trials and the yellow rewarded stimulus was chosen after the yellow priming cue on 311 out of 590 trials (Chi^2=2.076; d.f.=1; P=0.15). None of the seven individual bees that were trained showed any sign of learning.

It seems that bumblebees can use sequential cues to prime their later choice of a colour, if the sequential cues can be combined with spatial cues that distinguish the rewarded colours, as in Fig. 3, but not if such distinguishing spatial cues are absent (Fig. 4).

Attempts to enhance sequential priming
Does success at any easy task help in the later acquisition of a similar but more difficult task?

Honeybees, after successfully learning an easy visual discrimination problem, can be trained to perform, or will generalise to, a harder but related discrimination problem, which they would have found impossible without prior training on the easy task (e.g. Wehner, 1971). Can a similar method be used to enhance the effect of sequential priming cues? We asked whether the repeated experience of a sequential priming stimulus followed by the correct rewarded stimulus might allow bees to link the priming and rewarded stimuli.

Bees were first trained to find food in two different places in the arena. In one place, they were rewarded for choosing a blue over a yellow panel and in the other location for choosing a yellow over a blue panel (Fig. 5). The yellow location was rewarded when bees had passed through the blue tunnel and baffles, and the blue location was rewarded when bees passed
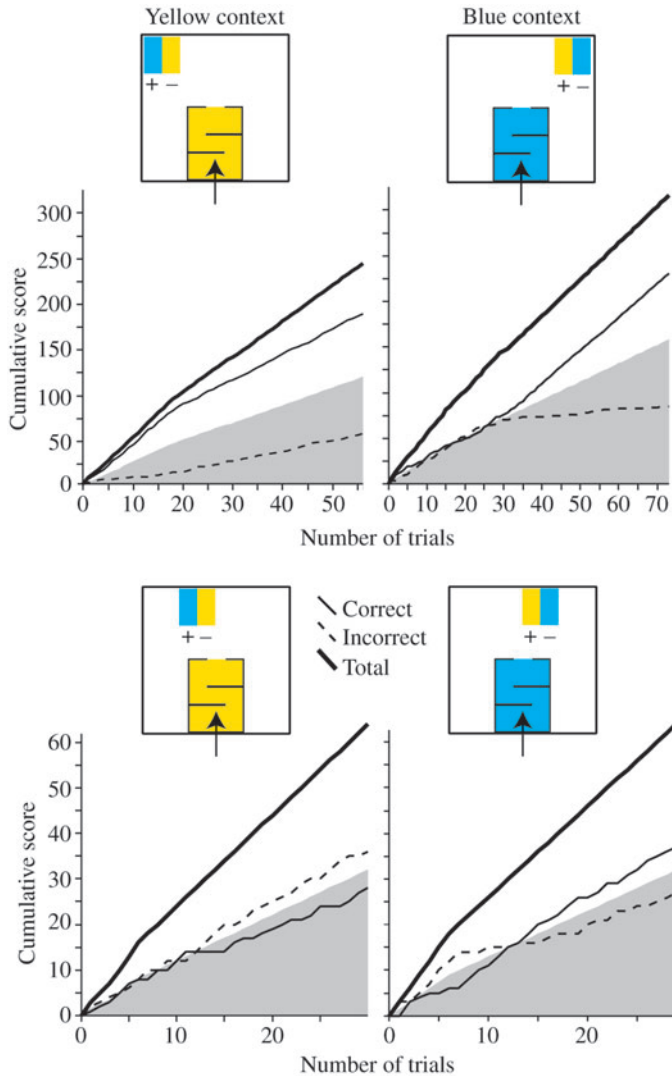
Fig. 5. Bees trained in a two-step experiment failed to use a sequential priming cue to switch their choice of colour. Top: in the first step of the experiment, the two screens were placed in opposite corners of the arena with their centres 210 cm apart, providing a different positional cue for each screen. A group of four bees then learnt to approach the rewarded blue panel in the left corner and the rewarded yellow panel in the right corner significantly more often than the unrewarded panel. Bottom: the two screens were moved close together with their centres 60 cm apart. One bee had ceased foraging during the first stage of the experiment. The remaining three bees failed to choose correctly and their performance did not recover over a further 60 trials. They exhibited a slight preference for the blue panel, whether the priming cue was blue or yellow.

through the yellow tunnel and baffles. Bees, at this stage of the experiment, had spatial and sequential priming cues to help them choose the appropriate rewarded colour. After the bees had successfully acquired this initial task, the two screens were moved closer together, so reducing the spatial priming cues. Did the first stage of training forge a strong enough link between sequential priming cues and rewarded stimuli, so that

the sequential priming cues on their own could now continue to guide the bees' correct choices?

At the beginning of training bees had to choose between the rewarded and unrewarded stimuli in the blue rewarded location, but they just saw yellow in the yellow rewarded location. The colours of the tunnel and the rewarded location were changed every 2–4 trials. To reduce confusion, the screen displaying the rewarded stimulus in the irrelevant location was blanked out. When the unrewarded stimulus was first introduced in the yellow rewarded location, bees began by choosing blue and yellow equally often, while preferring blue over yellow in the blue rewarded location (Fig. 5). After about 45 training trials on the two sides, the bees' behaviour switched. They now almost always chose blue in the blue location and their preference for yellow in the yellow location became weaker (Fig. 5). Overall, when the priming cue was yellow, bumblebees chose the blue panel in the left-hand corner of the room on 188 out of 244 trials, and, when the priming cue was blue, they chose the yellow panel in the right hand corner on 231 out of 316 trials (group data: $Chi^2=136.6$; d.f.=1; $P<0.001$). All four trained bees performed the task successfully.

Did this repeated experience of a priming cue followed by a rewarded stimulus help bees choose the correct rewarded stimulus when the spatial separation between the pairs of rewarded and unrewarded stimuli was reduced? The two screens and associated feeders were moved closer together and the same training schedule continued. The bees' choices dropped close to 50% correct and remained that way throughout the subsequent training period. Now, when the priming cue was blue, they chose the blue panel on 37 trials out of 64 trials, and, when the priming cue was yellow, they chose the yellow panel on 28 out of 64 trials (group data: $Chi^2=0$; d.f.=1; $P=1$). None of the trained bees transferred what they had learnt on the first task to the second more difficult one.

There are several possible reasons why pre-training with a spatial component to the rewarded cues was of no help in performing the task when the spatial component was missing. (1) No links were formed between the sequential priming cue and the rewarded stimulus during the first task. (2) Links were formed between each priming cue and the associated rewarded colour, but the links were specific to a combination of rewarded colour and location. (3) Links were formed between priming colour and rewarded colour, but they were too weak to support sequential priming.

Does the addition of a spatial component to the priming cue enhance sequential priming?

In the experiments described so far, the bees were not required to behave differently in the two priming contexts. The bees flew through the tunnel and nothing changed there except the colour. Might bees be more attentive to a priming cue, if they had to choose actively where they exited a priming compartment? To answer this question, we added a positional component to the coloured priming cue.
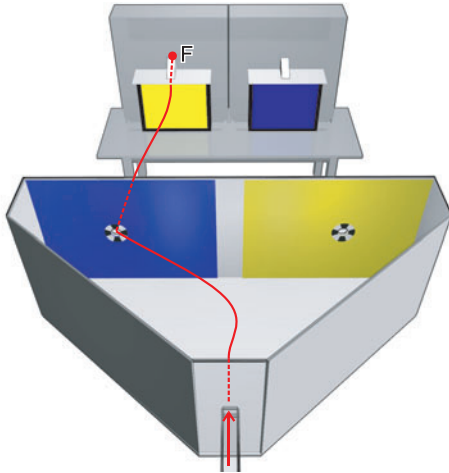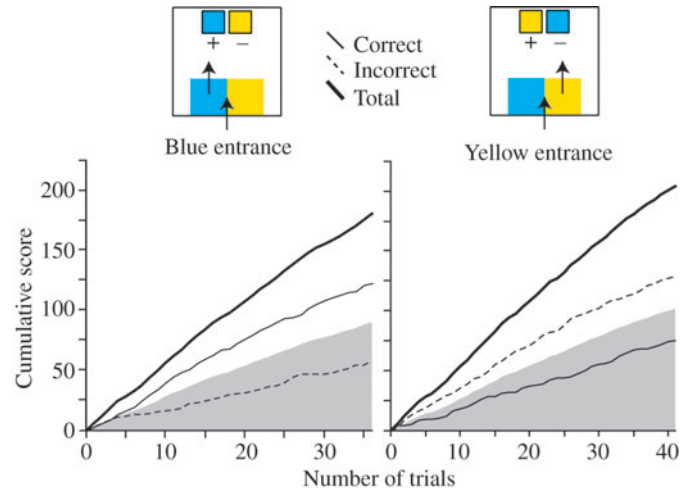
Fig. 6. Diagram of the priming compartment.

Fig. 7. Bees failed to use the colour and position of the exit hole in a priming compartment to select the colour of a rewarded stimulus. Top shows the training arrangement. The bees' task is to approach the blue panel after passing through the blue exit hole and the yellow panel after passing through the yellow exit hole. Blue- and yellow-rewarded stimuli frequently switched sides. Bottom shows the performance of a group of six bees when passing through the blue exit hole on the left (with which training began) or the yellow exit hole on the right. Over a training period of 79 trials, bees failed to learn to pick the rewarded yellow screen after passing through the yellow exit hole. Bees developed a blue preference that was independent of the exit hole through which they left the priming compartment.

Apparatus and procedure

The 40 cm long tunnel and baffles were removed. The 4 cm wide tunnel from the nest box opened into a wedge-shaped, priming compartment (Fig. 6) that was 75 cm deep and 50 cm high. It was 20 cm wide where the bees entered and 145 cm wide at its end wall, where the bees exited. Half of the end wall was constructed of yellow paper and half of blue paper with a light strip separating the two coloured sections. Bees reached the arena from the priming compartment through one of two 4 cm diameter holes in its end wall. The holes were set 85 cm apart, with one in the blue and the other in the yellow half, and they were made conspicuous by surrounding 2.5 cm wide black and white rings. Only one exit hole was open on each trial and its colour and position determined whether the bee should approach a yellow or a blue LCD screen to reach the feeder (Fig. 6). The colour of the LCD screens frequently switched sides following a schedule that avoided any correlation between the side of the exit hole and the position of the rewarded stimulus.

Findings

Bees failed to use any of the available cues (the colour surrounding the open exit hole, or the position of the exit hole in the priming compartment, or their own position when entering the flight arena) to determine their subsequent choice of rewarded stimulus. The bees developed a preference for blue, which they expressed whether they left the priming compartment through the blue or the yellow exit hole (Fig. 7). When the priming hole was blue, bees chose the blue panel on 120 out of 175 trials, and when the priming hole was yellow they chose the blue panel on 130 out of 205 trials (group data: $Chi^2=0.898$; d.f.=1; $P=0.344$). No individual bee showed signs of learning the relation between exit hole and rewarded stimulus. Bees typically flew from side to side in front of the two possible exits until they detected and flew through the open hole. It is arguable, but to us unlikely, that this initial inspection of the two holes and the coloured panels confused the bee about which coloured hole it exited.

Sequential cues and route learning

Finally, in a variant of this method, we asked whether bees might be able to link together the sequential components of each of two stable routes. Both the priming cues and the rewarded stimuli were in fixed locations so that bees either flew one route, from the priming compartment to one feeding site in the arena, or they flew a second route to the other feeding site. Exit through the yellow hole indicated that approaching a yellow screen on the left would be rewarded and exit through the blue hole signalled that the blue screen on the right would be rewarded (Fig. 8).

To reduce the chances that the bees were simply learning two manoeuvres, one associated with each exit hole, the bees were not allowed direct access to the flight arena from the priming compartment. Instead, they flew from the priming compartment into an adjoining compartment that was 30 cm deep. From here they could enter the flight arena through a centrally placed exit tunnel that was 20 cm wide and 50 cm high (Fig. 8). The length of the tunnel was either 24 cm or 42 cm. It turned out that this small difference in the length of the tunnel caused the bees to behave very differently. Practised bees flew very quickly through this last compartment into the arena and towards one of the LCD screens displaying the rewarded or unrewarded stimulus. Thus, in this task, bees had to learn two routes with an overlapping segment in the middle.

We began with a 24 cm long exit tunnel and six trained bees. When the priming hole was blue, bees chose the blue panel on
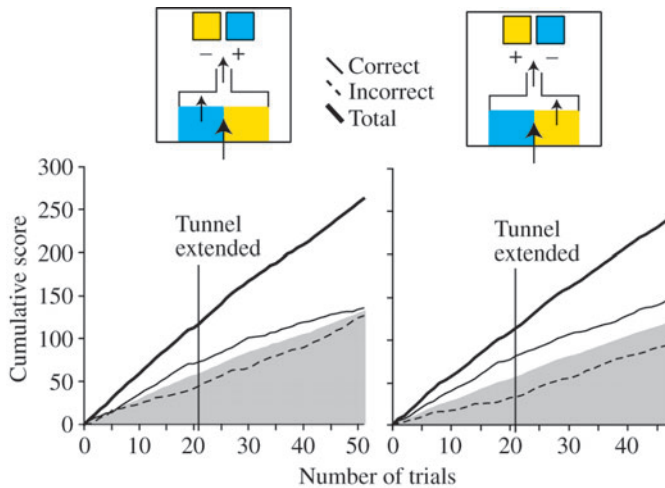
Fig. 8. Bees failed to use a coloured exit hole to select a rewarded stimulus that was in a fixed location. Top shows the training arrangement. The blue and yellow exit holes from the priming compartment led to a further compartment from which the bees entered the flight arena via a centrally placed exit tunnel. The bees' task was to approach the rewarded blue screen when the blue exit hole was open and to approach the yellow screen when the yellow exit hole was open. Bottom shows the performance of a group of six bees during training. At the start of training, the exit tunnel was 24 cm long. When, after 41 trials, it was clear that the bees had acquired the task, the exit tunnel was extended to 42 cm. Performance then broke down and did not recover over a further 55 trials, as shown by the correct and incorrect choice lines which run parallel to or converge upon the random choice line. The vertical lines through the graphs mark when the tunnel was extended.

the right on 71 trials out of 113 trials, and when the priming hole was yellow they chose the yellow panel on the left on 87 trials out of 125 trials (group data: Chi^2=23.86; d.f.=1; P<0.001). It could be that the short exit tunnel allowed the bees to fly in a single manoeuvre from the priming compartment to the rewarded stimulus and that the bees had learnt no more than a motor pattern, in which they veered to the left when leaving one exit hole and to the right when leaving the other.

Accordingly, we continued to train the same bees with the exit tunnel extended to 42 cm. After the exit tunnel was lengthened, the bees failed to perform correctly, though they still flew smoothly through the tunnel. Over a further 60 training trials, the five remaining bees showed no sign of acquiring the correct behaviour (Fig. 8). When the priming hole was blue, bees chose the blue panel on the right on 65 trials out of 150 trials, and when the priming hole was yellow they chose the yellow panel on the left on 61 trials out of 117 trials (Fig. 8, group data: Chi^2=0.377; d.f.=1; P=0.539). The individual scores of three bees were significantly better than chance, when trained with the shorter exit tunnel. When the tunnel was extended one of these bees preferred blue whatever the priming cue, one preferred yellow and the other just chose incorrectly. The priming cue no longer determined which rewarded stimulus the bees approached. The frequently

experienced correlation in the first part of the experiment between each priming cue and the rewarded stimulus did not provide a useful scaffold to help the bees perform correctly after the tunnel was extended.

One interpretation of these results is that the bees' success with the shorter tunnel was caused by their associating a different manoeuvre with each coloured exit hole that could continue uninterrupted through the tunnel to the rewarded stimulus. The strategy may have broken down with the extended tunnel, because bees had to begin a new manoeuvre on leaving it, and, at this time, the priming signal was too weak to determine whether the bee should fly to the right or left. A second interpretation is that bees, when trained with the shorter tunnel, associated each rewarded colour in its fixed location with the appropriate hole in the priming compartment, but that the extended tunnel introduced an extra route segment across which the sequential priming signal failed to propagate.

Discussion

It is easy to show that spatial contextual cues have a powerful effect. Bees readily learn to approach a blue target rather than a yellow one to reach a feeder in one location and to approach a yellow target and not a blue target to reach a feeder in a second location (Fig. 5). No special effort was needed to make the two locations memorable, bees just picked up the distinguishing cues that were present. We suppose that the particular cues associated with each place become linked to the rewarded colour there. Subsequently, recognition of these positional cues enhances the bee's response to the appropriate colour. A similar easily forged link between spatial and chromatic cues may account for the readiness with which bees can learn to choose simultaneously in a single location a blue–black checkerboard over a yellow–black checkerboard and a plain yellow target over a blue one.

By contrast, it was difficult to demonstrate sequential priming in bumblebees. A blue or yellow priming cue did not influence a bumblebee's choice of rewarded stimulus a few seconds later when bees had to choose a yellow target after seeing one priming colour and a blue target after experiencing the other priming colour (Fig. 4). Conversely, sequential priming did work when bees were trained in a single place to make two independent discriminations, choosing a blue–black checkerboard over a yellow–black checkerboard and a plain yellow target over a plain blue target. In training, the check patterns were seen shortly after experiencing a yellow priming cue and the plain colours appeared after bees experienced the blue priming cue. The effect of sequential priming was evident in later tests. Bees tended to choose the blue–black checkerboard over the yellow target after experiencing the yellow priming stimulus and to choose the yellow target over blue–black checkerboard after the blue priming stimulus. Thus, a persisting signal that is induced by briefly viewing yellow or blue is enough to tip the bees' later choice in favour of the associated rewarded stimulus.

Why was sequential priming harder to demonstrate in

bumblebees than in honeybees (Srinivasan et al., 1998b; Zhang et al., 1999; Giurfa et al., 2001)? One possibility is that the priming cues we used were not appropriate. Not every stimulus works. Thus, flying through a short brightly lit blue or yellow tunnel did not on its own bias the bees' behaviour. Addition of the frontal baffles was necessary for any priming to occur (Fig. 3). Perhaps robust sequential priming requires a particular arrangement of priming cues that we have not been able to establish.

A second possible reason for weak sequential priming is that priming signals decay rapidly or are subject to interference from irrelevant stimuli. In the classical experiments of Grice (1948), rats found it difficult to learn stimulus–response contingencies across short delays of a few seconds, and were unable to learn at all when the delay was prolonged to 10 s. Recent experiments in mice show that fear conditioning to a stimulus predicting an electric shock is easily disrupted by distracting stimuli, if there is a substantial time interval (18 s) between the end of the conditioning stimulus and the application of the shock, and further, that the anterior cingulate cortex, which is thought to be part of an attentional system, is essential for such trace conditioning (Han et al., 2003).

A third possibility, which we favour, is that the sequential priming cues were adequate, but that spatial cues interacted and interfered with the sequential cues. To test for sequential priming, two stimuli were presented in essentially the same location in a large flight arena, and the correct choice between the two stimuli depended upon what colour the bumblebee had seen a few seconds earlier. Each of the potentially rewarded stimuli was therefore linked to the same set of spatial cues, but to different sequential cues. The common spatial cues could thus have masked the weaker, but distinguishable, sequential cues. Once an animal has arrived at a place that can be reached by several routes, it may be hard to attach importance to the particular route that was taken.

The experiments in honeybees were conducted in small Y mazes, rather than in an open arena. Switching the positions of stimuli between the arms of a Y maze has a very large effect on their global location within the maze. Consequently, colours presented in the Y maze may be learnt without a fixed spatial context so that there are no spatial cues to weaken the effect of sequential priming. This is not the case for the bumblebee experiments. Reversal of the positions of the colours on an LCD screen in a large arena hardly alters their position within the room. The two rewarded colours thus have the same spatial context associated with them, which may prevent the weaker sequential cues from being effective.

If we accept this argument, two conclusions can be drawn from our data. First, signals associated with spatial context are more effective than, and can mask signals associated with, sequential context. Second, sequential cues can combine with correlated spatial cues to determine a bee's behaviour. Under natural conditions, sequential priming is likely to be of particular help in binding together the learnt components of routes. Spatial and sequential cues will then usually reinforce each other, rather than compete. In the one test situation that did work (Fig. 3), spatial cues (presence or absence of a checkerboard) and sequential cues could act synergistically and together overcome the effect of a common location.

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