

# Circadian timed episodic-like memory – a bee knows what to do when, and also where

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## Summary

This study investigates how the colour, shape and location of patterns could be memorized within a time frame. Bees were trained to visit two Y-mazes, one of which presented yellow vertical (rewarded) *versus* horizontal (non-rewarded) gratings at one site in the morning, while another presented blue horizontal (rewarded) *versus* vertical (non-rewarded) gratings at another site in the afternoon. The bees could perform well in the learning tests and various transfer tests, in which (i) all contextual cues from the learning test were present; (ii) the colour cues of the visual patterns were removed, but the location cue, the orientation of the visual patterns and the temporal cue still existed; (iii) the location cue was removed, but other contextual cues, i.e. the colour and orientation of the visual patterns and the temporal cue still existed; (iv) the location cue and the orientation cue of the visual patterns were removed, but the colour cue and temporal cue still existed;

(v) the location cue, and the colour cue of the visual patterns were removed, but the orientation cue and the temporal cue still existed. The results reveal that the honeybee can recall the memory of the correct visual patterns by using spatial and/or temporal information. The relative importance of different contextual cues is compared and discussed. The bees' ability to integrate elements of circadian time, place and visual stimuli is akin to episodic-like memory; we have therefore named this kind of memory circadian timed episodic-like memory.

Supplementary material available online at  
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## Introduction

Studies of the natural foraging behaviour of bees suggest that individuals have the capacity to learn and remember not only the colour and shape of flowers that are bountiful in pollen and nectar, but also how to get to them (Wehner, 1981; Lehrer et al., 1995; Chittka et al., 1993; Vorobyev and Menzel, 1999; Collett et al., 2003). The species of flowers that are in bloom, say this week, are likely to be replaced by a different species at a different location next week, and different flower species have different peak times of nectar secretion during the day (Kakutani et al., 1989). So the bee needs not only spatial information, such as the features and location of the flowers, but also temporal information, and has indeed evolved an impressive ability to learn colours, odours, shapes and routes, within a time frame, quickly and accurately.

Bees can learn the time of day when flowers start secreting nectar. In an early study, when bees were trained to visit a feeder at a particular hour of the day, almost all of the trained bees visited the feeder during the hour-long reward period (Behling, 1929). This 'Zeitgedächtnis' or time-sense persists for 6–8 days, and thus can outlast short periods of bad weather (Wahl, 1932).

It was also shown that bees can recall 9 different times per day, with an accuracy of 20 min (Koltermann, 1971). In Koltermann's experiments, the bees could associate scents with an artificial feeder at a particular time.

Honeybee foragers possess a circadian rhythm, with an activity period during the day and a sleep-like state at night (Lindauer, 1975; von Frisch, 1993; Bloch and Robinson, 2001; Bloch et al., 2001; Moore, 2001). A special feature of the honeybees' circadian rhythm is its flexibility. In typical circadian rhythms, a particular behaviour is fixed to a special phase of the cycle. The honeybee 'Zeitgedächtnis' enables the bee to continuously adjust its behaviour according to its memory and the time of day (Chalifman, 1950; Lindauer, 1954; Wittekindt, 1955).

Honeybees have the ability to flexibly change their preference for a visual pattern according to the context in which a discrimination task is carried out. Context cues help to carve up the world into distinct regions, and so can aid animals to cope with possible confusions (Colborn et al., 1999; Fauria et al., 2002; Cheng, 2005; Dale et al., 2005). Honeybees can learn to treat the same stimulus in different ways, depending on the

context in which the stimulus is presented (Gould, 1987; Menzel et al., 1996; Srinivasan et al., 1998; Colborn et al., 1999; Zhang et al., 2006). Menzel et al. investigated whether and how contextual parameters, such as time of day and features characterizing the location, can be utilized to determine choice behaviour (Menzel et al., 1996). They claimed that time of day cannot by itself elicit a conditioned response, but can control different behaviours, such as image-matching, navigation and timing of motivation to forage, and thus act as an occasion setter for a sensory-motor routine (Menzel et al., 2006).

There has, however, been little experimental work investigating bees' abilities to modulate their behaviour in response to multiple contextual cues in the spatial and/or temporal domain. In a previous study, we showed that honeybees are able to reverse their pattern preference according to the task at hand and the time of day (Zhang et al., 2006). In these experiments, the bees learned to make opposite decisions when foraging and when homing (task), and also in morning and afternoon (time). These contextual cues help the bees to memorize the rules for navigating an experimental maze, and to recall the correct memory in the associated context. In the present study, we further investigated how the colour, shape and location of patterns could be memorized within a time frame, and examined the importance of different contextual cues.

## Materials and methods

### General

The experiments were conducted at the Australian National University, Canberra, and the set-up was located in a small

greenhouse covered by an opaque PVC sheet. We added additional styrofoam sheets beneath the PVC sheet above mazes A, B and C so that the mazes were always in the shadow, while ensuring a homogenous illumination of the mazes and some heat protection for the observers. The greenhouse was separated by two blinds into three compartments, so that the bees could not see maze A or maze B from the hive entrance. Nor could they see maze A from maze B and *vice versa*. The flight distance was approximately 4 m from the hive to maze A and maze B, and 2 m to maze C (see Fig. 1). The hive had two entrances at opposite sides, and was mounted on the wall, so that the bees were able to forage both inside and outside. At the beginning of each experiment, about 20 foraging bees (*Apis mellifera* L.) were individually marked and trained to visit a feeder with a 0.5 mol l<sup>-1</sup> sugar solution in the Y-mazes. Bees entering the Y-mazes were trained to choose one of two patterns, which indicated the position of the feeder reward.

### Maze set-up

Three compound Y-mazes were used in the experiments. Each was made of four cylinders of 25 cm height and 25 cm diameter, and covered by a Perspex<sup>TM</sup> lid. The four cylinders were connected by holes, 4 cm in diameter, through which the bees could fly from one cylinder to the next. The holes were positioned in the middle of the cylinder wall, halfway up from the base (12.5 cm from both ends). The first cylinder carried two holes on opposite sides. The bee would enter through the entrance hole, and fly through the next hole into the second

cylinder. The second cylinder had three holes, one serving as entrance, and two others, 90° apart, as exits leading to the next two cylinders. Each of the two holes carried a visual stimulus, between which the bees had to choose (Fig. 1). One of the two patterns indicated the position of the feeder reward. If the bee made a positive decision by flying through the correct pattern (termed positive), it would enter the third cylinder, and find a feeder with sugar solution as a reward. If the bee chose the wrong (termed negative) pattern, it found an empty cylinder, and was released to try again. A bee choosing between visual patterns could not see whether the next cylinder contained the feeder or not, because the feeder was placed on the floor of the maze, and a cardboard baffle was placed behind the entrance holes of the reward cylinders. This prevented the bees from seeing into the reward cylinder from the decision cylinder. The entrance of the decision cylinder also had a baffle to slow the bees down, which made observation easier, and gave the bees more time to look at the visual stimuli. This maze set-up is well established in honeybee behavioural research (Srinivasan and Lehrer, 1988; Zhang et al., 1992; Zhang and Srinivasan, 1994; Zhang et al., 1995; Zhang et al., 1996; Zhang et al., 1999).

During training, the positions of the positive and negative patterns at the mazes were regularly swapped every 30 min, so that the bees could not use position as a cue to find the feeder. Similarly, the positions of the

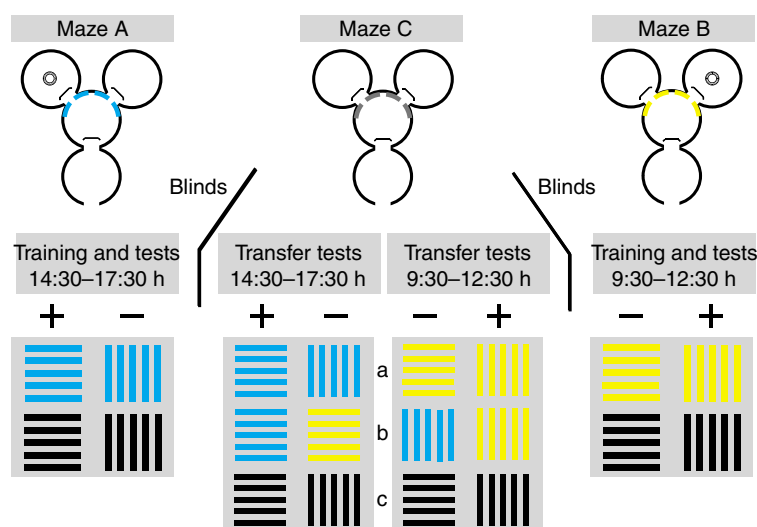


Fig. 1. The experimental set-up and visual patterns. Yellow patterns were used in the training and learning test in the morning at Maze B; blue patterns were used in the training and learning test in the afternoon at Maze A; black patterns were used in experiment 1, the transfer tests at Maze A and B. Various transfer tests were conducted at Maze C: (a) experiment 2: yellow training patterns in the morning and blue training patterns in the afternoon; (b) experiment 3: blue and yellow vertical patterns in the morning and blue and yellow horizontal patterns in the afternoon; (c) experiment 4: black horizontal and vertical patterns in the morning and in the afternoon. The rewarded patterns were denoted by (+) and the unrewarded patterns were denoted by (-). See text for further details.

positive and negative patterns were interchanged every 10 min in the middle of the learning tests and every 5 min in the middle of the transfer tests.

#### *Visual stimuli*

The stimuli were presented as 18 cm×18 cm squares (grating patterns) or 18 cm diameter circles (sector and ring patterns, details in Fig. S1 in supplementary material) on the exits of the second cylinder. They were printed on normal copy paper using a Fuji Xerox Document Centre C360 PS colour printer. The training stimuli in maze A were always blue/white, and the training stimuli in maze B were always yellow/white. The stimuli for the transfer tests at the mazes A and B were black/white. The stimuli for the transfer tests at maze C were blue/white, yellow/white or black/white. Horizontal *versus* vertical gratings (Figs 1, 3, 4) and sector *versus* ring patterns (Fig. S1a,b in supplementary material) were used as visual stimuli with different groups of bees. The rewarded pattern, which provided access to a feeder, was termed 'positive', the unrewarded pattern was termed 'negative'.

#### *Training and testing procedure*

The bees were trained for 3 days before testing began, and thus experienced the circadian reward pattern three times, *circa* an average of 20 rewards on each training pattern per bee. During training and testing, the number of bees in the apparatus was carefully controlled: if two or more bees were seen in the decision cylinder, all were released without a reward, and allowed to attempt the task again. Each bee that reached the reward cylinder, and collected the sugar solution in the feeder, was released by lifting the Perspex<sup>TM</sup> lid. Thus, it did not have to trace its way back through the maze. Training was carried out daily over two sessions. In the morning session (09:30–12:30 h), the bees were trained to forage at maze B. In the afternoon session (14:30–17:30 h), the bees were trained to forage at maze A. During the break from 12:30 h to 14:30 h, and during the night, a feeder was placed in a neutral position to keep the bees motivated to fly inside the greenhouse. During the morning training at maze B, maze A did not contain a feeder, and the Perspex<sup>TM</sup> lids were open. Similarly, throughout the afternoon training at maze A, maze B did not contain a feeder, and the Perspex<sup>TM</sup> lids were open. During all learning and transfer tests, there was no difference between the mazes. Both mazes contained a feeder, and the Perspex<sup>TM</sup> lids were closed. The mazes were accessible to the bees at all times, except during the transfer tests at maze C.

During training, the yellow vertical grating provided access to the feeder at maze B in the morning, and the blue horizontal grating indicated the feeder position at maze A in the afternoon (Fig. 1). When the sector and ring patterns were used, the yellow sector pattern was positive at maze B in the morning, and the blue ring pattern was positive at maze A in the afternoon (Fig. S1a in supplementary material). Using the two colours blue and yellow at the two training mazes made learning easier for the bees, probably because cues stay longer in memory when offered in combination with other, simultaneously offered cues (Lindauer, 1970; Colborn et al., 1999; Fauria et al., 2002; Cheng, 2005).

#### *Data collection*

During the learning tests and the transfer tests at Maze A or Maze B, both mazes were observed, and every positive and negative decision in the mazes was recorded. Only the first choice of each bee during one foraging flight was included in the data. The reward continued to be offered during all tests, to prevent bees from losing their motivation to visit the apparatus (Zhang et al., 1999). The learning tests lasted for 20 min, and the transfer tests lasted for 10 min. In the middle of each testing period (after 10 min in the learning tests, and after 5 min in the transfer tests), the positions of the patterns were swapped in order to cancel out any effect of a possible side bias. All transfer tests were followed by at least 30 min of normal training at maze A or B. This shorter testing period, and subsequent training under normal conditions, ensured that the bees did not learn during the transfer test conditions. Each bee was allowed to make a maximum of three rewarded visits in each of the transfer tests, which is not enough to learn a pattern discrimination task. Additionally, there was a break of at least 24 h before a transfer test condition was repeated. When testing the bees at maze C, mazes A and B were disassembled, in order to make the bees visit maze C.

#### *Tests at mazes A and B*

The performance of individual bees was recorded in the learning tests. During training, the bees learned for each of the mazes A and B where and when to go, and what to do there. The constant control of the learning level ensured that the bees were well trained throughout the transfer tests. These tests were repeated with a different group of bees, using the sector and ring patterns (Fig. S1a in supplementary material).

In experiment 1, we investigated whether honeybees can distinguish the patterns at two locations without the colour cue, using black patterns in mazes A and B (Fig. 1). If the bees were still able to choose the positive pattern, we could be certain that they had used the maze location cue (where), the shape cue (what), and the time cue (when), independently of the colour cue. These tests were repeated with a different group of bees, using sector and ring patterns (Fig. S1b in supplementary material).

#### *Transfer tests at maze C*

In experiment 2, we examined whether honeybees can choose the learned training patterns at a novel location, namely in maze C (Fig. 1a). The bees had never visited maze C before, and thus the maze location cue was excluded. They had to base their decision on the pattern colour and shape (what) and the time of day (when), transferring their knowledge of what to do in a certain timeframe to a new 'where'.

In experiment 3, we examined whether honeybees can choose the trained colour independently of the location and shape cue. Yellow and blue vertical gratings were used in the morning, and yellow and blue horizontal gratings were presented in the afternoon (Fig. 1b). Thus, the bees could not use the pattern shape and the location cue for decision making. Since both patterns had the same (positive) shape, the bees had to decide between yellow and blue according to the time of day at the new location.

In experiment 4, we examined whether honeybees can choose the trained shape independently of the location and colour cue.

We excluded the colour and location cues by presenting black gratings at maze C (Fig. 1c). The bees had to choose a pattern shape according to the time of day, without relying on pattern colour or maze location as cues.

#### Analysis of performance

We first performed analyses of variance (ANOVA) across all repeated tests for individual bees and for each type of experimental condition, using the statistical software SYSTAT 11 to check the homogeneity of the data. Once the data were found to be homogeneous, the performance of each bee was evaluated separately by pooling its correct choices and visits over all repeated tests, and calculating the ratio of the number of correct choices to the number of visits. Then, the average performance for a particular experimental condition was obtained by averaging choice frequencies across bees. The sample size ( $N$ ) was the number of bees, rather than the number of individual choices, ensuring that the samples were truly statistically independent. Mean values of choice frequency, standard deviation (s.d.) and standard error of the mean (s.e.m.) were calculated. In the text and in the figures, performance is indicated by the mean choice frequency ( $\pm$  s.e.m.). In the analysis, we included only bees that visited both of the mazes regularly. A visit at the correct maze was counted when the bee entered the maze and made a decision. A visit at the wrong maze was counted when the bee entered the maze, and also when it hovered in front of the maze.

The performances in the morning and the afternoon tests were compared by GraphPad Prism statistical software, using two-way repeated measurement ANOVA (Time: morning *versus* afternoon; Repeated measurements) to determine whether performance changed significantly during the time and between the repeated measurements. *Post-hoc* comparisons were done by means of the Bonferroni  $t$ -tests, which compared each repeated measurement in the morning and in the afternoon (for example, percentage of choices for the vertical pattern in the afternoon, compared with the percentage of choices for the vertical pattern in the morning). Control experiments were carried out at the end of experiments to test for a possible side bias. We conducted a simple dual choice test at Maze A and Maze B, for which  $2 \times 2$  McNemar tests were used for statistical analysis.

### Results

#### *The honeybees learned to forage at maze B in the morning and at maze A in the afternoon*

For a comparison of the bees' location preference in the morning and the afternoon, the total visits to Maze A and Maze B were recorded at the same time in each learning test and transfer test. The ratio of the number of visits at Maze B (or A) to the number of total visits was calculated for each test in the morning (or afternoon). Then, we averaged the ratios for all learning tests and also for all transfer tests in the morning and afternoon. The results are presented in Fig. 2. During the learning tests in the morning, the vast majority of visits ( $0.96 \pm 0.01$ ) from 16 tests were recorded at maze B ( $n=16$ , total visits  $N=559$ ); whereas only a small number of bees (total 22 visits in 16 tests) approached maze A. These

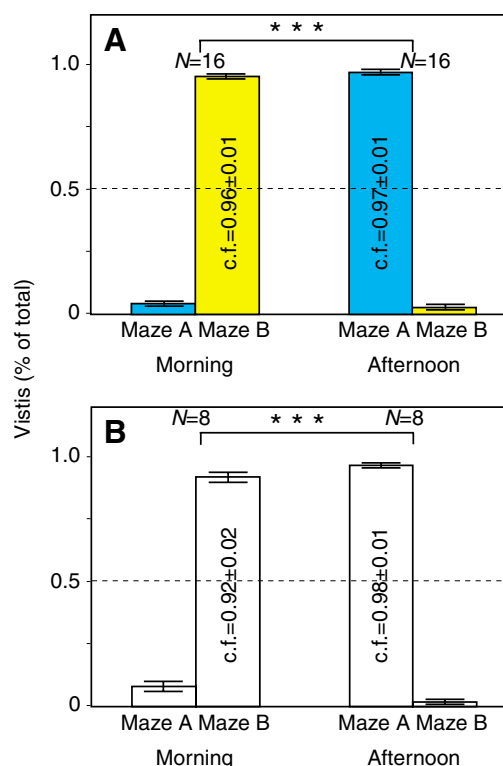


Fig. 2. Trained bees change their preference to visit Maze A or Maze B from morning to afternoon. (A) Percentage of total visits at Maze A and B in the learning tests with yellow gratings in the morning at maze B and blue gratings in the afternoon at maze A; (B) percentage of total visits at Maze A and Maze B in the transfer tests with black and white gratings.  $N$ , number of repetitions of tests; values are means  $\pm$  s.e.m. See text for further details.

bees mostly confined their visit to a quick fly-over, only occasionally entering the maze. In the afternoon training, an equally large proportion of visits ( $0.97 \pm 0.01$ ) from 16 tests were recorded at maze A ( $n=16$ , total visits  $N=770$ ; Fig. 2A), whereas a very small number of bees (total 17 visits in 16 tests) visited maze B. Here too, most visits were confined to a quick fly-over, with only a few bees entering the maze. The bees clearly preferred maze B in the morning, and changed their location preference to maze A in the afternoon ( $t$ -test,  $d.f.=30$ ,  $P<0.001$ ).

The transfer test conditions (black patterns at mazes A and B) did not affect the bees' location preference. During the morning transfer tests, most visits ( $0.92 \pm 0.06$ ) from 8 tests were recorded at maze B ( $n=8$ , total visits  $N=124$ ); whereas only a small number of bees (total 10 visits in 8 tests) approached maze A. In the afternoon transfer tests, most visits ( $0.97 \pm 0.03$ ) from 8 test times were recorded at maze A ( $n=8$ , total visits  $N=440$ ; Fig. 2B), whereas a very small number of bees (total 13 visits in 8 tests) visited maze A. These bees visited the wrong maze, i.e. maze A in the morning and maze B in the afternoon and confined their visit to a quick fly-over, only occasionally entering the maze. The bees still preferred maze B in the morning, and reversed their preference to maze A in the afternoon ( $t=39.1$ ,  $d.f.=14$ ,  $P<0.001$ ).



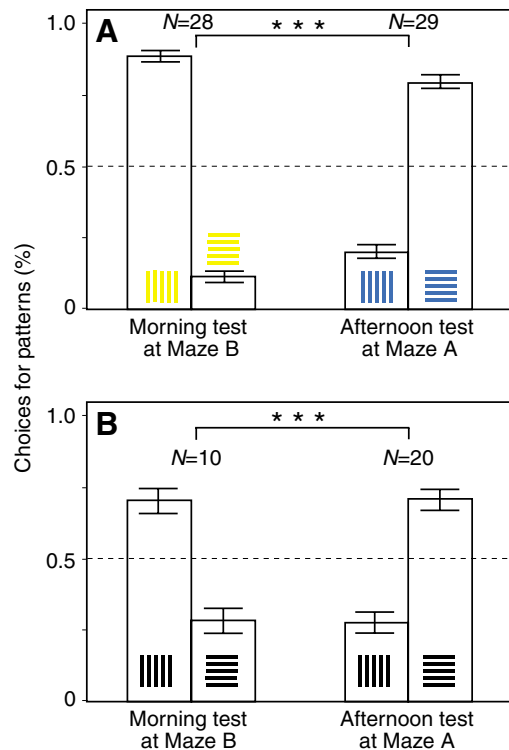


Fig. 3. (A) Results of the learning tests at mazes A and B. The tested bees significantly reversed their pattern preference from the yellow vertical grating in the morning to the blue horizontal grating in the afternoon. (B) Results of experiment 1, the transfer tests with black patterns at mazes A and B. The tested bees significantly reversed their pattern preference from the vertical grating in the morning to the horizontal grating in the afternoon. *N*, number of individual bees attending the test; values are means  $\pm$  s.e.m. See text for further details.

*The bees learned to choose the yellow vertical grating at maze B in the morning, and the blue horizontal grating at maze A in the afternoon*

The learning tests were conducted to ensure that the bees had a constant learning level throughout the experimental period. Once again, the bees had to fly to maze B in the morning, and choose the yellow vertical grating there. In the afternoon, they had to forage at maze A, and choose the blue horizontal grating. The results are shown in Fig. 3A.

Analysis of variance showed no significant differences in the performances of individual bees on all testing days either in the morning (ANOVA, d.f.1=5, d.f.2=84,  $F=1.14$ ,  $P=0.362$ ) or the afternoon sessions (ANOVA, d.f.1=13, d.f.2=75,  $F=0.787$ ,  $P=0.672$ ) at mazes A and B. When the bees visited maze B in the morning during training (Fig. 3A), they chose the yellow vertical grating in most of the visits ( $0.88 \pm 0.02$ ,  $N=28$ ). In the afternoon at maze A (Fig. 3A), their choice frequency for the blue horizontal grating was  $0.80 \pm 0.02$  ( $N=29$ ). Results of ANOVA tests are as follows: d.f. (interaction)=7,  $F=0.573$ ,  $P=0.778$ ; d.f. (time)=1,  $F=853.6$ ,  $P<0.001$ ; d.f. (repeated tests)=1,  $F=1.737$ ,  $P=0.102$ . All the Bonferroni post tests showed performance in the morning and in the afternoon are significantly different. Thus, they learned to reverse their pattern preference between morning and

afternoon. The transfer tests were started with this performance as a baseline.

*Experiment 1*

*The bees could distinguish black patterns at mazes A and B*

Analysis of variance showed no significant differences between the performances of individual bees on all testing days either in the morning (ANOVA, d.f.1=6, d.f.2=25,  $F=1.584$ ,  $P=0.193$ ) or in the afternoon (ANOVA, d.f.1=14, d.f.2=44,  $F=0.52$ ,  $P=0.908$ ). When the bees were tested for transfer to black gratings at mazes A and B (Fig. 3B), they chose the vertical grating at maze B in the morning in most of the visits ( $0.74 \pm 0.04$ ,  $N=10$ ). In the afternoon at maze A, the choice frequency for the horizontal grating was  $0.72 \pm 0.04$  ( $N=20$ ). Results of ANOVA tests are as follows: d.f. (interaction)=2,  $F=0.05$ ,  $P=0.949$ ; d.f. (time)=1,  $F=75.12$ ,  $P<0.001$ ; d.f. (repeated tests)=2,  $F=2.257$ ,  $P=0.113$ . All the Bonferroni post tests showed that performances in the morning and in the afternoon are significantly different. The bees significantly reversed their pattern preference from vertical in the morning to horizontal in the afternoon, even without the colour cue, choosing a grating orientation according to the time of day (when) and the maze location (where).

*Experiments 2–4 at maze C*

Maze C had a neutral position between the training mazes A and B (Fig. 1), and the bees had never foraged in this maze before. To avoid any further learning at maze C during the transfer tests, the testing time was kept short, so that each bee did not visit more than three times in each testing session. Moreover, the transfer tests were followed by at least 30 min of normal training at mazes A and B.

*Experiment 2*

*Honeybees can apply the learnt rules from mazes A and B to a new location within a temporal context*

In the first transfer experiment at maze C, the bees were tested with the usual training patterns, but with the location cue excluded. They encountered yellow gratings in the morning and blue gratings in the afternoon. Analysis of variance showed no significant differences between the performances of individual bees on all testing days either in the morning (ANOVA, d.f.1=5, d.f.2=15,  $F=1.334$ ,  $P=0.303$ ) or in the afternoon (ANOVA, d.f.1=7, d.f.2=6,  $F=0.918$ ,  $P=0.55$ ). The results of this experiment are shown in Fig. 4A. The choice frequency for the yellow vertical grating in the morning was  $0.94 \pm 0.02$  ( $N=18$ ), while the choice frequency for the blue horizontal grating in the afternoon was  $0.73 \pm 0.06$  ( $N=9$ ). Results of ANOVA tests are as follows: d.f. (interaction)=1,  $F=2.736$ ,  $P=0.108$ ; d.f. (time)=1,  $F=220$ ,  $P<0.0001$ ; d.f. (repeated tests)=1,  $F=0.283$ ,  $P=0.598$ . All the Bonferroni post tests showed that performances in the morning and in the afternoon are significantly different. They reversed their pattern preference in the same (neutral) location, choosing a grating according to the time of day (when) and the pattern colour (what).

*Experiment 3*

*Honeybees can use colour cues alone to make a correct decision at maze C within a temporal context*

In the next experiment at maze C, the bees were tested for

colour preference by presenting blue and yellow vertical gratings in the morning, and blue and yellow horizontal gratings in the afternoon. Thus, we excluded the pattern orientation and the maze location cues. Analysis of variance showed no significant differences between the performances of individual bees on all testing days either in the morning (ANOVA, d.f.1=9, d.f.2=12,  $F=0.625$ ,  $P=0.756$ ) or in the afternoon (ANOVA, d.f.1=7, d.f.2=8,  $F=0.170$ ,  $P=0.985$ ). The results of this

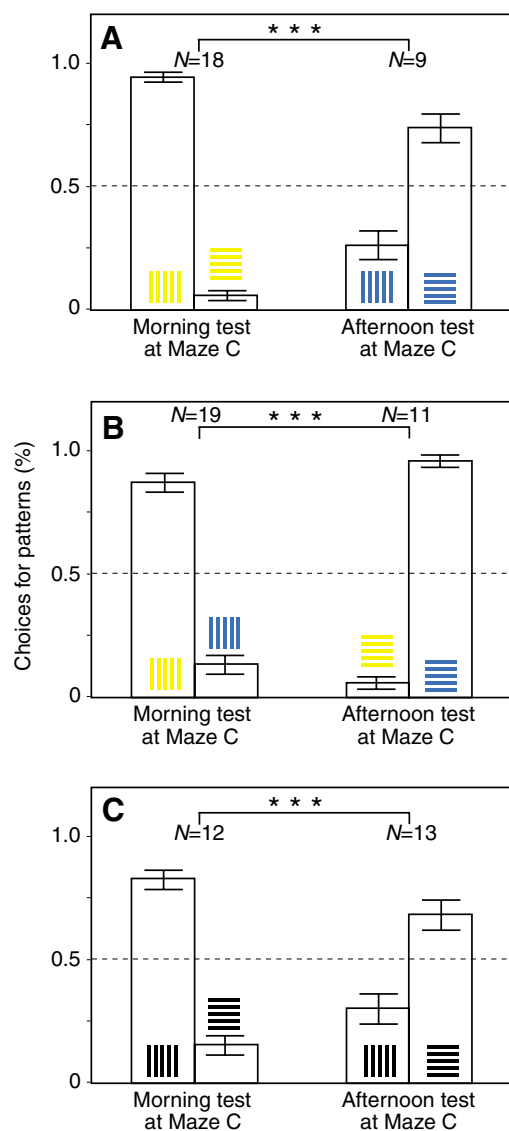


Fig. 4. Results of experiments 2–4, the transfer tests at maze C. (A) Experiment 2: yellow and blue training patterns. The bees significantly reversed their pattern preference from the yellow vertical grating in the morning to the blue horizontal grating in the afternoon at the neutral location. (B) Experiment 3: yellow and blue patterns in the same orientation. The bees significantly reversed their colour preference from yellow in the morning to blue in the afternoon at the neutral location. (C) Experiment 4: black patterns. The bees significantly reversed their pattern preference from the vertical grating in the morning to the horizontal grating in the afternoon.  $N$ , number of individual bees attending the test; values are means  $\pm$  s.e.m. See text for further details.

experiment are shown in Fig. 4B. The choice frequency for the yellow grating in the morning was  $0.87 \pm 0.04$  ( $N=19$ ). In the afternoon, the bees preferred the blue horizontal grating over the yellow horizontal grating, with a choice frequency of  $0.95 \pm 0.02$  ( $N=11$ ). Results of ANOVA tests are as follows: d.f. (interaction)=1,  $F=2.827$ ,  $P=0.102$ ; d.f. (time)=1,  $F=344.5$ ,  $P<0.0001$ ; d.f. (repeated tests)=1,  $F=3.646$ ,  $P=0.065$ . All the Bonferroni post tests showed performance in the morning and in the afternoon are significantly different. The bees were able to reverse their colour preference from yellow in the morning to blue in the afternoon in a neutral location, basing their decision in the maze on the temporal context.

#### Experiment 4

*Honeybees can use orientation cues without colour cues, to make a correct decision at maze C within a temporal context*

In the last experiment of this series, the location and colour cues were excluded by presenting black horizontal and vertical gratings at maze C (Fig. 4C). The bees had to choose a grating orientation according to the time of day. Analysis of variance showed no significant differences between the performances of individual bees on all testing days either in the morning (ANOVA, d.f.1=10, d.f.2=8,  $F=0.539$ ,  $P=0.822$ ) or in the afternoon (ANOVA, d.f.1=12, d.f.2=10,  $F=1.638$ ,  $P=0.221$ ). In the morning, the bees preferred the vertical grating ( $0.84 \pm 0.04$ ,  $N=12$ ). In the afternoon, they significantly reversed their pattern preference to the horizontal grating ( $0.69 \pm 0.06$ ,  $N=11$ ). Results of ANOVA tests are as follows: d.f. (interaction)=1,  $F=1.631$ ,  $P=0.209$ ; d.f. (time)=1,  $F=52.4$ ,  $P<0.0001$ ; d.f. (repeated tests)=1,  $F=0.243$ ,  $P=0.625$ . All the Bonferroni post tests showed that performances in the morning and in the afternoon are significantly different. The bees could reverse their pattern preference solely within a temporal context; no other cue was present to influence the bees' choices for the horizontal or vertical patterns. The same was true for the experiments with sector and ring patterns (see Fig. S2 in supplementary material).

To negate the possibility that the bees had an unexpected preference for the vertical pattern, all experiments at the mazes A and B and the black pattern transfer test at maze C were repeated with a different group of bees and a set of central symmetric sector and ring patterns. The results are shown in Figs S1 and S2 in the supplementary material.

#### Control tests

Control tests were conducted to ensure that the honeybees did not develop a preference to a particular side of the maze. In these tests, the decision cylinders carried the same visual patterns on both sides, and no food reward. The bees' decisions for the left or right side were monitored.

At maze A, the choice frequency for the right side was 51.7%, while that for the left side was 48.3%. Thus, the bees did not have a preference for a particular side of maze A ( $2 \times 2$  McNemar test, d.f.=1,  $P=0.961$ , see Fig. S3a in supplementary material). At maze B, the bees chose the right side in 52.8% of the visits. The left side was chosen in 47.1% of the visits. There was no significant difference in the bees' choices for the left or the right side of maze B ( $2 \times 2$  McNemar test, d.f.=1,  $P=0.936$ , see Fig. S3b in supplementary material).

## Discussion

### *Foraging at multiple feeding sites*

The bee may simultaneously retain several different mnemonic constellations, each specifying different locations, with different sensory characteristics, and providing food at different times (Gallistel, 1990). In our experiments, during training, the bees learned to choose the yellow vertical grating at maze B in the morning, and the blue horizontal grating at maze A in the afternoon (Figs 1, 2). The very low error rate in this training indicates that visiting two or more feeding places at fixed times is an easy task for foragers, and might be a common strategy in honeybees.

The aim of experiment 1 was to investigate whether honeybees can still find the correct pattern shape, without the colour cue. The bees extracted the orientation information of the coloured patterns, and applied it to the novel black patterns (Fig. 3A,B). The same was true for the sector and ring patterns (see Fig. S3b in supplementary material). They showed a clear preference for the correct pattern at the respective maze and time of day.

### *Foraging at a novel feeding site*

In all the transfer tests at the neutral maze C (experiments 2–4), the location cue was excluded. This set-up further allowed us to artificially remove all other cues, except the temporal cue, as required. Under these conditions, the bees had to rely on their time-sense when deciding between visual patterns that possessed only partial features, such as colour or shape.

The aim of experiment 2 was to investigate whether honeybees can transfer learnt rules to a novel location. The data show that the bees recalled the rewarded patterns according to the pattern colour and the time of day, independent of the location cue (Fig. 3A). In this case, the colour and the time could prime the visual memory of shape, i.e. choosing the vertical grating if they saw yellow, and the horizontal grating if they saw blue, or choosing the vertical shape in the morning and the horizontal shape in the afternoon. The next experiment was carried out to check the importance of pattern colour.

Experiment 3 was conducted to check whether honeybees can transfer previously formed time–colour associations to a novel location. The high performance of the bees in this experiment (Fig. 4B) shows the importance of the colour cue in decision-making. As the pattern orientation always suggested a positive pattern, the bees had to decide on a colour according to the time-frame. The bees recalled the correct colour according to the time of day from their memory.

In experiment 4, we tried to determine if honeybees can transfer previously formed time–shape associations to a novel location. The lower performance in this experiment (Fig. 4C), compared to the colour experiment (Fig. 4B), shows that this task was more difficult for the bees. However, they still significantly reversed their pattern preference between morning and afternoon. The location and the colour cue were not necessary for the bees to make a correct decision in this test. While the shape cues (orientation of gratings) certainly made the learning process easier, the bees successfully extracted and memorized the rule ‘choose the vertical grating in the morning and the horizontal grating in the afternoon’, and recalled it to solve the task, independent of the pattern colour, and without a

location context. The same is true for the additional experiments with sector and ring patterns (see Fig. S3c in supplementary material).

The results confirm that bees would be able to forage from different kinds of flowers, at different times of the day, at the same feeding location. They could also select a particular kind of flower when visiting a new feeding location, recalling the memory of the most profitable flower species for a particular time of day.

These results cannot be an artefact caused by learning during a test, because it takes 20–30 visits for a honeybee to learn a geometric pattern (Zhang and Srinivasan, 1994; Zhang et al., 2004). The transfer test periods were kept short, so that each bee could make a maximum of three foraging trips per testing session. In addition, the 10 min testing period was followed by at least 30 min of normal training, which would also prevent a possible learning effect (Fischer, 1957).

### *Context learning*

Contextual cues offer the possibility of treating the same stimulus in two or more different ways, thereby enabling the animal to interact more flexibly with its environment. In the case of the honeybee, contextual cues are essential for efficient foraging. When visiting a patch of flowers, the bees can decide on a profitable flower within the temporal context, i.e. choose the yellow flower in the morning and the blue flower in the afternoon, even when exploring new foraging territories.

Pattern memory can be primed by two locations, in which a bee that is trained to recognize one pattern at one site and another pattern at a second site will choose A+ over B– at site A, but B+ over A– at site B (Menzel et al., 1996). Bees’ memories can also be primed by the surrounding panorama, which includes spatial and colour contextual cues (Cheng et al., 1986; Collett and Kelber, 1988; Collett et al., 1997; Dale et al., 2005). A familiar nectar scent, encountered at the hive entrance before departing, can trigger specific route memories that expedite navigation to one of two different food sources (Reinhard et al., 2004). The limit of memorisable scent–feeder associations seems to be two; to distinguish three different feeder–scent combinations the bees need additional cues (e.g. colours) (Reinhard et al., 2006).

Finally, recent experiments have shown that honeybees are able to change their visual pattern preference in the presence of two cues: the time of day and the task at hand (Zhang et al., 2006).

In our present experiment, location cues were excluded by testing the bees at a neutral position (in maze C), the task at hand was always foraging, and the only reliable rule for the bees was the time–colour or the time–shape association. The bees chose the correct patterns at maze C in two different experiments, at a level significantly different from random choice. This indicates that bees can still find the correct visual pattern according to the time of day, when all other available cues are excluded.

### *The honeybees’ internal clock*

Animals ranging from bees to rats routinely record the time of day at which they have a noteworthy experience and make use of this record to time their subsequent behaviour (Gallistel,

1990). Bees need a precise time-sense to compensate for the movement of the sun in their dance language, which is performed in the total darkness of the hive (Lindauer, 1960). Some 'marathon dancers' perform the recruitment waggle dance for hours, without leaving the darkness of the hive, accurately indicating the direction of a food source with respect to the sun's azimuth at any time of the day or night (Chalifman, 1950; Lindauer, 1954; Wittekindt, 1955). It was suggested that bees are innately informed of certain general spatial and temporal features of solar movement (Dyer and Dickinson, 1994). Bees can synchronize their behavior with daily floral rhythms, foraging only when nectar and pollen are at their highest levels. At other times, they remain in the hive, conserving energy that otherwise would be exhausted on non-productive foraging flights (Moore, 2001).

The greenhouse used in our experiments was covered by an opaque PVC sheet, which was similar to the materials used to cover an extension of our All Weather Bee Flight Facility (AWBFF). We have discussed the effect of UV and polarized light on circadian rhythms in the honeybee in a previous publication (Zhang et al., 2006). However, we still measured the illumination spectrum in the greenhouse where the present experiments were carried out, and found that UV light was tremendously reduced to undetectable levels at the maze areas. In addition, the bees' performance was not affected by large changes in the weather. The weather record for the region shows that our experiments were sometimes carried out under complete cloud cover. Even then, the bees could still perform as usual. The consistent results obtained under different weather conditions indicate that the honeybees did not need to use the sun's position, UV or polarized sunlight as cues to change their preference according to time.

It is not clear, however, if the honeybee has a time-sense governed by a circadian rhythm, connecting a specific memory to a certain phase in the 24 h-cycle, or if it is also capable of measuring the elapsed time between two events. Interval-timing has been shown in vertebrates (Richelle and Lejeune, 1980; Gallistel, 1990; Babb and Crystal, 2005), and recently in an invertebrate from the same family as the honeybee, the bumblebee *Bombus impatiens* (Boisvert and Sherry, 2006). Further experiments are planned to investigate the question of interval timing in the honeybee. If honeybees show this ability, their memory of 'what', 'where' and 'when' could fulfil the behavioural criteria for episodic-like memory in nonhuman animals, as shown in the food-caching scrub-jay *Aphelocoma coerulescens* (Clayton and Dickinson, 1998). Our experiments have shown that the honeybee links together the elements of circadian time (when during a day), place (where), and colour and pattern characteristics (what) in an integrated fashion. This is akin to episodic-like memory, except that the temporal element is circadian time, instead of interval timing. We have named this kind of memory circadian timed episodic-like memory.

#### Cue ranking

In the learning tests (Fig. 3A and supplementary material Fig. S1a), the bees reached an average performance (morning and afternoon sessions) of 83% correct choices. Setting this as a baseline, and comparing it with the other tests where one or

several contextual cues were taken out, we can compare the difficulty of the transfer tests, and thus determine the relative importance, to the bees, of the different cues.

In the transfer tests at maze C, the bees reached their best average performance of 91% in experiment 3, the colour discrimination task (Fig. 4B). The performance in the morning showed no significant difference between the learning test and the transfer test ( $t=0.258$ , d.f.=45,  $P>0.8$ ); however, in the afternoon, the performance in this transfer test was even better than that of the learning test ( $t=3.6$ , d.f.=38,  $P>0.001$ ), regardless of the missing location and pattern orientation cues. Thus, colour seems to be the most important visual cue for honeybee choice behaviour. These findings are consistent with previous reports that honeybees learn a new colour after about five visits, whereas they normally require 20–30 visits to learn a pattern (Zhang and Srinivasan, 1994).

Using the training patterns at maze C in experiment 2, the bees performed about the same as in the training tests at mazes A and B (83%, Fig. 4A). There was no significant difference in performance between the learning test and the transfer test in the morning ( $t=2.01$ , d.f.=44,  $P>0.05$ ) and in the afternoon ( $t=1.25$ , d.f.=36,  $P>0.20$ ). Here, the only missing cue was the maze location. This cue seems to have had almost no effect on the bees' choice performance in small scale navigation, when other contextual cues were available. The results of this transfer test demonstrate that pattern colour and the time of day were enough to allow a baseline level of performance at a new location.

When the colour cue and the location cue were both taken out in experiment 4, the bees' average performance was reduced to 72% (Fig. 4C and supplementary material Fig. S2). The performance in the morning was slightly worse in the transfer test than in the learning test, but not significantly so ( $t=1.10$ , d.f.=38,  $P>0.2$ ). However, in the afternoon, the performance in the transfer test was slightly worse than that of the learning test ( $t=2.1$ , d.f.=40,  $P<0.05$ ). The results indicate that the shape cue is more difficult for the bees to use than the colour cue. Pattern orientation or, in nature, the shape of different flowers, is thus more important than location for the bees' choice behaviour once they have reached their feeding site. The bees clearly used the former to distinguish between the patterns in all experiments except the colour discrimination task (where pattern orientation was unavailable).

Applying these findings to the natural situation, we can say that the colour and shape of flowers are the most important visual cues used by bees to choose between different flower species. When visiting different feeding sites, or different patches of flowers, they can recall their memory of the most rewarding species in conjunction with the time of day, and thus find the most profitable food source even at a new location.

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