

## Honeybee memory: a honeybee knows what to do and when

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### Summary

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. This study investigated the effect of time of day, task, as well as both parameters simultaneously, as contextual cue(s) in modulating bees' preference for a visual pattern. We carried out three series of experiments to investigate these interactions. The first series of experiments indicated that trained bees can reverse their pattern preference following midday breaks, as well as an overnight break, at the feeder and at the hive. The second series of experiments showed that trained bees are able to reverse their pattern preference in just a few minutes, depending on whether they are going out to forage or returning to the hive. The third series of experiments demonstrated that trained bees can significantly reverse their pattern preference at the feeder and at the hive entrance following midday breaks,

as well as after an overnight break; the bees could also learn to choose different patterns at the feeder and at the hive entrance within each testing period. The training thus imposed a learnt pattern preference on the bees' daily circadian rhythm. This study demonstrates that the bee with a tiny brain possesses a sophisticated memory, and is able to remember tasks within a temporal context. Honey bees can thus 'plan' their activities in time and space, and use context to determine which action to perform and when.

Supplementary material available online at  
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### Introduction

Foraging is essential to a honey bee colony's survival. To forage successfully, a bee has to learn and remember not only the color and shape of flowers that contain nectar and pollen, but also how to get to them (Menzel et al., 1996; Wehner, 2003; Collett et al., 2003). Since the species of flowers that are in bloom, say, in the morning are likely to be replaced by a different species at a different location in the afternoon, the bee needs, and has indeed evolved, an impressive ability to learn and memorize local features and routes, as well as the time of blooming, quickly and accurately. Thus, having found a nectar-bearing flower at a particular time on a particular day, a forager can remember the task and the time at which it was completed, and visit the flower at the same place and time on the following day (von Frisch, 1993; Lindauer, 1960; Gould and Gould, 1988).

During evolution, honeybees have evolved sophisticated sensory systems and well-developed learning and memorizing capacities, the essential mechanisms of which do not differ drastically from those of vertebrates (Giurfa and Menzel, 1997;

Giurfa, 2003; Zhang and Srinivasan, 2004a; Zhang and Srinivasan, 2004b; Dyer et al., 2005). Honeybees also have a time sense, with which they can modulate their response to a local stimulus according to the time of day. Koltermann examined circadian memory rhythm in honeybees, and discovered that honeybees can learn scents or colors in a time-linked process, and remember them in a 24 h cycle (Koltermann, 1971). Circadian systems permit organisms to measure time for adaptively significant purposes (Moore-Ede et al., 1982). Bees 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). 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 behavior, and claimed that time of day or landmarks cannot by themselves elicit the conditioned response, but can control different behaviors (such as image-matching, navigation, timing of motivation to forage) (Menzel

et al., 1996). Work carried out in the laboratory of Robinson (Robinson et al., 1989) has shown that there are molecular processes occurring in the brain that influence the division of labor and biological clocks of social insects. With respect to contextual learning by the honeybee, it has recently been shown that honeybees and bumblebees can learn to modulate their responses to local stimuli according to contextual cues. Collett and Kelber (Collett and Kelber, 1988), for instance, found that honeybees can retrieve the right landmark-memory by the context in which the landmarks are placed. Honeybees can also learn to change their response to a visual pattern according to whether a stimulus provides access to the hive or to the feeder (Gadakar et al., 1995). Colborn et al. examined the contextual modulation of visuomotor associations in bumblebees, and reported that different contextual signals are associated with approaching the nest or approaching the feeder, and that these contextual signals facilitate learnt associations between orientation detection and motor commands (Colborn et al., 1999). The modulation of such contextual cues can help honeybees to recognize landmarks from new vantage points (Zhang et al., 1999; Collett and Collett, 2002). In recent studies, Chittka et al. found that some insects can modulate their response time to perform tasks on a relatively short time scale, depending upon their perceived difficulty and context of a task, in order to make a tradeoff between the speed and accuracy of foraging (Chittka, 2003; Franks et al., 2003; Dyer and Chittka, 2004). Dale et al. demonstrated that honeybees and bumblebees can learn to treat the same visual and olfactory target in different ways in different spatial, temporal or motivational contexts. Such contextual influences are important in allowing honeybees to flexibly adapt to many different situations (Dale et al., 2005).

However, there has been little experimental work investigating bees' abilities to modulate their behavior in response to multiple contextual cues. In the present article, we report that honeybees can learn to simultaneously change their preference for a visual pattern with both the time of day, i.e. whether it is morning or afternoon, and the task at hand, i.e. whether the bee is flying to the feeder or returning to the hive.

## Materials and methods

### General

The experiments were conducted during the Australian summer at the Australian National University and during the following German summer at Wuerzburg University. The research project commenced in November 2004 of the Australian summer season, and was carried out in an all weather bee flight facility (AWBFF) at the Australian National University's Research School of Biological Sciences. The facility consists of a modified glasshouse in which the internal temperature is regulated by a computer to maintain  $24.0 \pm 5^\circ\text{C}$  during the day and  $17.0^\circ\text{C}$  at night, with a relative humidity of  $\sim 45\%$ . The experimental hive and a Y-maze setup were located in the AWBFF; the distance between the hive and the Y-maze was 8 m (Movie 1 in supplementary material). Further

experiments were carried out in the following German summer season during July and August 2005 at the Bee Station of the Wuerzburg University, where an observation hive and a Y-maze setup were maintained in two small huts; the distance between the two huts was 24 m (Figs S1–S2 in supplementary material). About 20 foraging bees (*Apis mellifera* L.) were marked individually at the beginning of each experiment, and trained to visit a feeder with a  $0.5 \text{ mol l}^{-1}$  sugar solution in the Y-maze. Bees entering the Y-maze were trained to choose one of two patterns (termed positive) which indicated the position of the feeder. Bees returning to the hive also had to choose between two patterns to access the hive, as only the entrance behind the positive pattern was open during training (Fig. S3 and Movie 2 in supplementary material). During the test periods, both entrances were open. Thus, bees could choose to access the hive through either entrance. An experimental bee had to learn two tasks in one foraging trip, i.e. making a choice in the maze to receive sugar water and then another choice to enter the hive. During training, the positions of the positive and negative patterns at the feeder and the hive were regularly swapped every 30 min, so that the bees could not use position as a cue to either find the feeder or access the hive. Similarly, the positions of the positive and negative patterns were interchanged every 10 min in the middle of testing periods. During the midday break, the visual patterns at the feeder and the hive were removed; the feeder was moved to outside the maze, and both entrances of the hive were opened. The bees could therefore continue to visit the feeder located in the front of the maze, and access the hive through either of the two entrances.

### Visual stimuli

The visual patterns ( $18 \text{ cm} \times 18 \text{ cm}$ ) were printed on a color laser printer (Fuji Xerox Document Centre C360 PS color printer). Gratings oriented at  $45^\circ$  versus  $135^\circ$  in blue/white (see the inset in Fig. 1), sectors versus rings, vertical versus horizontal gratings in black/white or blue/white (see the insets in Fig. 2, Fig. 4 and Fig. S4 in supplementary material) and blue versus yellow color patterns (see Fig. 3) were used for different experiments.

### Training and testing procedures

The present study comprised three series of experiments, and training and testing procedures are described separately as follows:

#### Series 1

We investigated whether honeybees can modulate their preference for a visual pattern with the time of day.

Experiment 1 of this series was carried out during the Australian summer in the AWBFF at the Australian National University. Here, a pair of blue/white gratings oriented at  $45^\circ$  or  $135^\circ$  to the horizontal was used at the feeder and the hive. In our experimental setup, the choice of the  $45^\circ$ -oriented grating allowed a forager bee access to a feeder and entry to the hive in the morning. In the afternoon, however, a  $135^\circ$ -

oriented grating at the feeder as well as the hive became the positive pattern.

Experiment 2 of this series was carried out during the German summer at Wuerzburg University. In Experiment 2, the visual patterns were black/white sectors *versus* rings at the feeder, and the black/white vertical *versus* horizontal gratings at the hive. The sectors pattern at the feeder and the vertical grating at the hive were positive patterns in the morning. However, the rings at the feeder and the horizontal gratings at the hive were positive patterns in the afternoon. The tests commenced after 3 days of training for each experiment, and lasted for 3 days.

### Series 2

We examined whether honeybees can reverse their pattern preference with the task at hand, i.e. foraging or returning home. Two experiments were conducted during the Australian summer in the AWBFF at the Australian National University. The yellow pattern was the positive pattern at the feeder, but the blue pattern was the positive pattern at the hive in Experiment 1. The blue pattern at the feeder and the yellow pattern at the hive became the positive patterns in Experiment 2. The tests, that commenced after 3 days of training for each experiment and lasted for 3 days, were conducted at both the feeder and the hive, one in the morning at 11:00 h and another in the afternoon at 16:00 h for 20 min, over 3 days.

### Series 3

We examined whether honeybees can simultaneously change their preference for a visual pattern with both the time of day, and the task at hand. The experiment was carried out at the Bee Station of the Wuerzburg University. In our experimental setup, the choice of a 'horizontal grating' stimulus allowed a forager bee access to a feeder, whereas a 'vertical grating' stimulus allowed entry to the hive in the morning. In the afternoon, however, a vertical grating at the feeder and a horizontal grating at the hive became the positive patterns. In order to facilitate learning this complex task, horizontal and vertical gratings in blue/white were used at the feeder, and gratings in black/white were used at the hive entrance during the training period and the learning test. Three days of trainings were followed by 3 days of learning tests (see Training and learning tests for experimental Series 3 in supplementary material). After the learning tests, the bees were trained to more complex tasks, in which horizontal and vertical gratings in black/white were used at the feeder as well. This was followed by 'critical' tests, which lasted for another 3 days. In the critical tests, the visual patterns at the feeder and the hive were the same, i.e. black/white gratings. The bees were trained continuously between each critical test.

In the experiments of Series 1 and 3, training in the morning started at 09:30 h, and lasted for 3 h, and the afternoon session started at 14:30 h, and lasted for 3 h as well. The tests were carried out four times a day: 09:30 am, i.e. early morning; 11:30 h, i.e. late morning; 14:30 h, i.e. early afternoon; and 16:30 h, i.e. late afternoon. The mid-day break lasted from

12:30 h to 14:30 h. The terms MF1 and MH1 (or MF2 and MH2) denote the early morning (or late morning) tests at the feeder and the hive, and AF1 and AH1 (or AF2 and AH2) denote the early afternoon (or late afternoon) tests at the feeder and the hive, respectively.

### Data analysis

During tests, the first choices of bees at the feeder and the hive were recorded. We performed analyses of variance (ANOVA) across all repeated tests for individual bees and for each type of experimental condition using the statistical software SYSTAT. Thus, 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. 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 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.). We used nonlinear regression to approximate the average choice frequency with reference to the specified patterns in Series 1 and 3.

To check whether the task at hand affects pattern preference in Series 2, we carried out statistical tests for each experiment to check for significant differences in performance at the feeder and the hive. To check whether 'time of day', i.e. morning or afternoon affects pattern preference in Series 1 and 3, we carried out the following four statistical tests to check for significant differences in performance: (MF1 *vs* AF2), (MH1 *vs* AH2), (MF2 *vs* AF2) and (MH2 *vs* AH1). To check the same for 'task', in Series 3, the following four tests were done: (MF1 *vs* MH1), (MF2 *vs* MH2), (AF1 *vs* AH1) and (AF2 *vs* AH2).  $\chi^2$  tests were used for all of these comparisons.

### Checking bias at the hive and the feeder

As a control, side bias counting was carried out usually in the morning before the experiments started, to check whether the trained bees had developed any side bias in their choices. In the bias counting, the bees' choice performance was measured while two identical visual patterns were presented at the hive or the feeder.

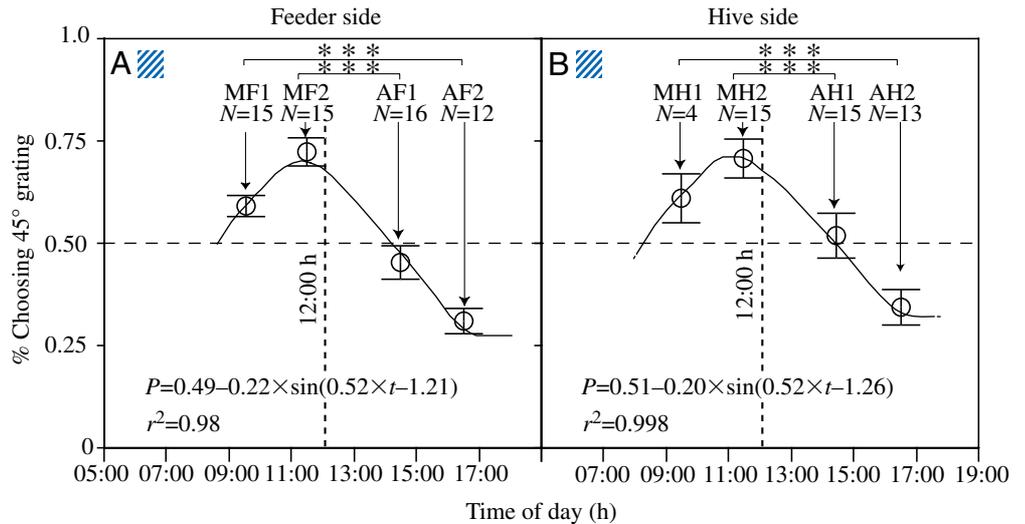
## Results

### Series 1

Honeybees can learn to reverse their preference for a visual pattern with the time of day, i.e. morning or afternoon.

The first experiment in this series was carried out in the AWBFF at the Australian National University. The trained bees were tested in all of eight different types of test (MF1, MH1, MF2, MH2, AF1, AH1, AF2 and AH2). The results of this experiment, shown in Fig. 1, revealed that the bees

Fig. 1. Results of experimental Series I carried out in the AWBFF (indoor) at the Australian National University. A pair of blue/white gratings oriented at 45° or 135° to the horizontal was used at the feeder and the hive. The results indicated that trained bees can reverse their pattern preference following midday breaks, as well as an overnight break, both at (A) the feeder and (B) the hive. The modulation of the average choice frequency, with reference to 45° grating can be approximated very well by a sinusoidal curve with a frequency of 0.52, i.e. a period of 12 h. The correlation coefficient are 0.98 for the feeder and 0.998 for the hive, respectively. Terminology for the test types:



M, morning; A, afternoon; H, hive; F, feeder; 1, early; 2, late. \*\*\* $P < 0.001$ . See text for further details.

modulated their preference to the 45°-oriented grating according to the time of day, both at the feeder (Fig. 1A) and at the hive (Fig. 1B). In the morning, the bees significantly preferred the 45°-oriented grating at the feeder (MF1 and MF2 in Fig. 1A;  $P < 0.001$ ) and at the hive (MH1 and MH2 in Fig. 1B;  $P < 0.001$ ). Their pattern preference gradually changed to 135° around midday, and reduced to the random choice level of 0.50 in the early afternoon test at the feeder (AF1 in Fig. 1A;  $P > 0.3$ ) and at the hive (AH1 in Fig. 1B;  $P > 0.90$ ). However, pattern preference was significantly changed to the 135° grating in the late afternoon test, at the feeder (AF2, in Fig. 1A;  $P < 0.001$ ) and at the hive (AH2, in Fig. 1B;  $P < 0.001$ ). When we compare the average choice frequency in favor of the 45° grating between MF2 and AF1 at the feeder, or MH2 and AH1 at the hive, the results show that the bees' pattern preference has significantly changed in the time between the late morning and the early afternoon tests ( $P < 0.001$ ). Similar conclusions can be drawn when we compare the performance in the late afternoon test (AF2 at the feeder, in Fig. 1A and AH2 at the hive, in Fig. 1B) to the following early morning test (MF1 at the feeder, in Fig. 1A and MH1 at the hive, in Fig. 1B). The trained bees significantly reversed their pattern preference from the 135° grating to 45° grating after an overnight break.

The modulation of the average choice frequency in favor of the 45° grating can be approximated very well by a sinusoidal curve with a frequency of 0.52, i.e. a period of 12 h. The correlation coefficient for the sinusoidal curve is 0.98 at the feeder and 0.998 at the hive.

The second experiment in this series was carried out at the Bee Station in Germany. The bees were trained to simultaneously (i) choose the sectors pattern at the feeder to obtain a reward, and the vertical grating to access the hive in the morning; and (ii) the rings pattern at the feeder and the horizontal grating at the hive in the afternoon. The trained bees were tested in all of eight different types of test (MF1, MH1,

MF2, MH2, AF1, AH1, AF2 and AH2). The results of this experiment, shown in Fig. 2, revealed:

(i) The average choice frequency for the positive pattern in the all of eight different types of test, except the MH1 test (Fig. 2C), is significantly different to the random choice level of 0.50 ( $P < 0.001$ ). (Fig. 2A,B,D–H). The average choice frequency in the early morning test at the hive (MH1) was  $0.56 \pm 0.06$  ( $P > 0.03$ ,  $N = 14$ ).

(ii) When we compare the average choice frequency between the early morning and the late afternoon tests, i.e. the MF1 (Fig. 2A) and AF2 tests (Fig. 2B) at the feeder, or MH1 (Fig. 2C) and AH2 tests (Fig. 2D) at the hive, we find that the bees' pattern preference has significantly reversed in the time between the late afternoon test and the early morning test ( $P < 0.001$ ) after an overnight break.

(iii) When we compare the performance between the late morning test and the early afternoon test, i.e. MF2 (Fig. 2E) and AF1 (Fig. 2F) at the feeder, or AH2 (Fig. 2G) and AH1 (Fig. 2H) at the hive, we find that the bees' pattern preference has significantly reversed from the late morning test to the early afternoon test after the midday break ( $P < 0.001$ ).

### Series 2

Honeybees can learn to reverse their preference for a visual pattern depending on the task – foraging or returning home.

The experiments for this series were carried out in the AWBFF at the Australian National University's Research School of Biological Sciences. In the tests of the first experiment in this series, the bees showed a strong preference for the yellow pattern at the feeder with a statistically significant difference from the random choice level ( $0.93 \pm 0.03$ ,  $N = 11$ ,  $P < 0.001$ ), but for the blue pattern at the hive ( $0.86 \pm 0.03$ ,  $N = 11$ ,  $P < 0.001$ ; Fig. 3A). In the second experiment of this series, the bees showed a preference for the blue pattern at the feeder ( $0.75 \pm 0.03$ ,  $N = 8$ ,  $P < 0.001$ ), but for the yellow pattern

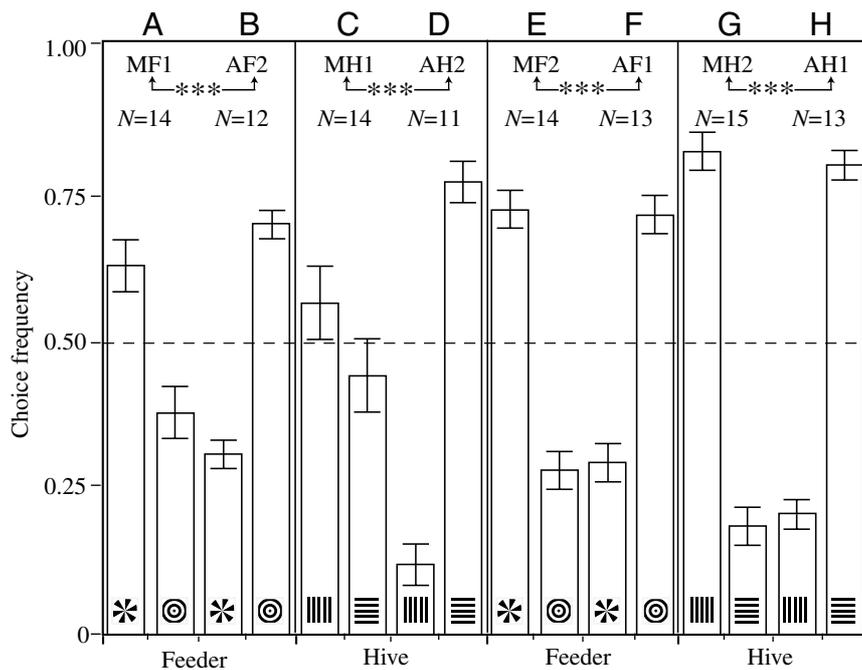


Fig. 2. Results of Series I experiments carried out at the Bee Station (outdoors) at Wuerzburg University. The visual patterns were black/white sectors versus rings at the feeder, and black/white vertical versus horizontal gratings at the hive (patterns shown at the base of each bar). The results showed that trained bees can reverse their pattern preference following midday breaks, as well as an overnight break, both at the feeder and at the hive. Terminology for the test types: M, morning; A, afternoon; H, hive; F, feeder; 1, early; 2, late. \*\*\* $P < 0.001$ . See text for further details.

at the hive ( $0.58 \pm 0.05$ ,  $N=8$ ,  $P < 0.05$ ) (Fig. 3B). Thus, the trained bees could significantly reverse their preference for a visual pattern within a time interval of about 2 min on average, which included the flight time between the feeder and the hive and the time of drinking sugar solution on the feeder.

Series 3

Honeybees can change their preference for a visual pattern simultaneously with the time of day as well as task at hand.

In these learning tests, blue/white gratings were used at the feeder, while black/white gratings were used at the hive. The learning tests were carried out on the third day after training commenced. All eight tests were carried out. The results showed that preference for the positive pattern was significantly better than the random choice level of 0.50 in the learning tests (Fig. S4 in supplementary material). The critical

test commenced after the learning tests had finished – now, the visual patterns at the feeder side and the hive side were the same, i.e. black/white gratings. The results showed that preference for the positive pattern was significantly better than the random choice level of 0.50 in all critical tests, namely MF1, MF2, AF1, AF2 ( $P < 0.001$ , Fig. 4A) and MH1, MH2, AH1 and AH2 ( $P < 0.001$ , Fig. 4B). Whereas the average choice frequency at the feeder was in favor of the horizontal grating in the late morning test (MF2, Fig. 4A), it reverted to the vertical grating in the early afternoon test (AF1, Fig. 4A), following the midday break ( $P < 0.001$ ). At the hive entrance, the average choice frequency favored the vertical grating in the late morning test (MH2, Fig. 4B), but reverted to the horizontal grating in the early afternoon test (AH1, Fig. 4B) after the midday break ( $P < 0.001$ ). The trained bees therefore significantly reversed their pattern preference at the feeder and the hive entrance following midday breaks ( $P < 0.001$ ). Similar conclusions can be drawn when we compare the performance at the feeder in the early morning test (MF1, Fig. 4A) and the late afternoon test (AF2, Fig. 4A), and also at the hive when we compare the performance in the early morning test (MH1, Fig. 4B) and the late afternoon test (AH2, Fig. 4B). The trained bees significantly reversed their pattern

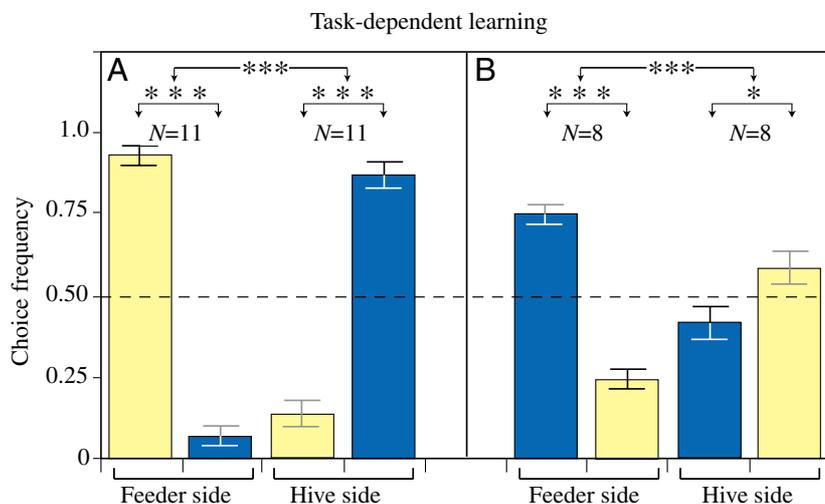


Fig. 3. Results of Series 2 experiments, carried out in the AWBFF at the Australian National University, which demonstrated that trained bees are able to reverse their pattern preference in just a few minutes, depending on whether they are flying out to forage or returning to the hive. \*\*\* $P < 0.001$ , \* $P < 0.05$ . See text for further details.

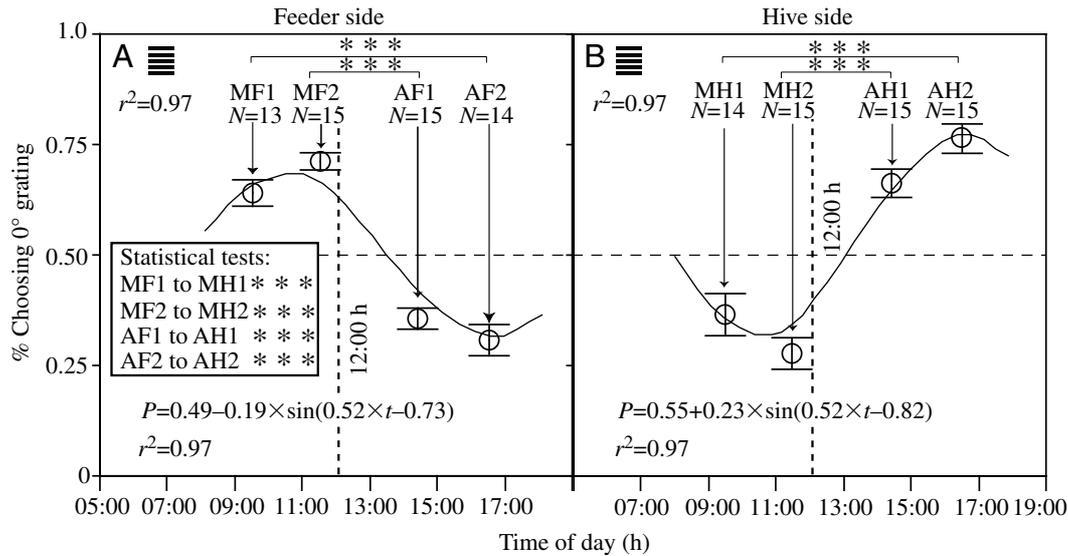


Fig. 4. Results of the critical tests in Series 3 experiments carried out at the Bee Station at Wuerzburg University. The visual patterns were black/white horizontal *versus* vertical gratings at the feeder and at the hive. However, the horizontal grating at the feeder and the vertical grating at the hive were the positive patterns in the morning, whereas the vertical grating at the feeder and the horizontal grating at the hive became the positive patterns in the afternoon. Results for one pattern are given. The results showed that trained bees significantly reverse their pattern preference (A) at the feeder and (B) at the hive entrance following midday breaks, as well as after an overnight break. The trained bees also had a significantly different pattern preference at the feeder and at the hive entrance within each testing period. The modulation of the average choice frequency, with reference to the horizontal grating could be approximated very well by a sinusoidal curve with a frequency of 0.52, i.e. a period of 12 h. The correlation coefficient was 0.97 for both the feeder and the hive. However, the phase of the sinusoidal curve at the hive was shifted 180° with reference to the feeder. See text for further details. Terminology for the test types: M, morning; A, afternoon; H, hive; F, feeder; 1, early; 2, late. \*\*\* $P < 0.001$ .

preference at the feeder and the hive entrance after an overnight break ( $P < 0.001$ ).

The modulation of the average choice frequency in favor of the horizontal grating can also be approximated very well by a sinusoidal curve with a frequency of 0.52, i.e. a period of 12 h. However, the phase of the sinusoidal curve at the hive was shifted 180° in reference to the feeder. The correlation coefficient for the sinusoidal curve is 0.97 at the feeder and the hive. When we compared average choice frequencies at the feeder to those at the hive entrance within each testing period, i.e. early morning (MF1 in Fig. 4A to MH1 in Fig. 4B), late morning (MF2 in Fig. 4A to MH2 in Fig. 4B), early afternoon (AF1 in Fig. 4A to AH1 in Fig. 4B) and late afternoon (AF2 in Fig. 4A to AH2 in Fig. 4B), the bees were found to have reversed their pattern preference significantly ( $P < 0.001$ ) (see inset table of statistical tests in Fig. 4A). Thus, the bees had learnt to choose a different pattern, depending on whether they were foraging or returning home.

#### Consistency of performance between individual bees

We conducted ANOVA to check the homogeneity of the data before pooling and averaging individual bees' performances. However, a question that remains is whether the test results indicate a collective performance, driven by only a few bees that outperformed the others. Therefore, we compared the performance of individual bees in all types of tests in the critical test of Series 3. Table 1 summarizes the performance

history of six individual bees; these bees were involved in all eight types of tests throughout the duration of the experiment. Table 1 convincingly demonstrates consistent performance between individual bees, in which all of the individuals reversed their pattern preference with time, when comparing MF1 to AF2, MH1 to AH2, MF2 to AF1 and MH2 to AH1; and also with the task, when comparing MF1 to MH1, MF2 to MH2, AF1 to AH1 and AF2 to AH2. Therefore, we can safely say that individual bees can reverse their pattern preference according to the time of day and the task, i.e. foraging or returning home.

## Discussion

### Training imposes pattern preference on the circadian rhythm

The results of Series 1 and 3 clearly demonstrate that a daily rhythm with sinusoidal variance of a period of 24 h exists in the honeybee, if we take into account the cessation of foraging activity for 12 h overnight (Koltermann, 1971; Kaiser, 1988; Moore, 2001; Bloch and Robinson, 2001; Sauer et al., 2004).

Is it possible that this result is an artifact of averaging the performances of trained bees? How does the behavior of individual bees compare with this pattern? To clarify this point, we carefully analyzed the performance of individual bees; the results are summarized in Table 2, which shows that a large percentage of individuals varied their behavior in four successive periods. In Series 1 at the feeder and at the hive,

Table 1. Summary of the performance of six individual bees

Test	Performance	Bee no.					
		BY	RW	WY	Y/	YW	YY
MF1	HG	13	16	12	11	18	8
	VG	3	10	5	5	6	7
	% of HG	81%	62%	71%	69%	75%	53%
MH1	HG	5	5	3	4	4	2
	VG	7	11	9	7	13	9
	% of HG	42%	31%	25%	36%	24%	18%
MF2	HG	17	19	21	21	22	10
	VG	7	10	8	9	7	6
	% of HG	71%	66%	72%	70%	76%	63%
MH2	HG	4	4	6	4	5	5
	VG	17	12	14	18	20	7
	% of HG	19%	25%	30%	18%	20%	42%
AF1	HG	5	7	8	5	6	7
	VG	7	9	15	20	8	13
	% of HG	42%	44%	35%	20%	43%	35%
AH1	HG	16	15	18	18	13	16
	VG	7	3	4	6	7	5
	% of HG	70%	83%	82%	75%	65%	76%
AF2	HG	6	5	8	6	8	10
	VG	18	14	19	19	12	11
	% of HG	25%	26%	30%	24%	40%	48%
AH2	HG	14	20	15	16	9	20
	VG	5	0	4	4	4	4
	% of HG	74%	100%	79%	80%	69%	83%

HG (or VG) denotes choice frequency for the horizontal gratings (or vertical gratings); % HG denotes percent choice frequency for the horizontal grating.

most individuals increased their preference for the specified pattern in period 1; this reduced to 0.50 or less in period 2 during the midday breaks, decreased further in period 3, and rose again to higher than 0.50 in period 4. A similar modulation of the choice frequency among individuals can also be observed in Series 3, with the difference that the direction of change at the feeder and at the hive within each period is reversed. Using nonlinear regression, we were able to fit a sinusoidal curve to the average choice frequencies of trained bees with reference to the specified pattern evaluated in the four daily tests. The tests were ordered around a daily cycle of 12 h,

therefore giving a frequency of 0.52 to the sinusoidal curve. The results of the approximation show that the modulation of the average choice frequency in favor of a specified pattern in the four daily tests can be approximated very well by a regular sinusoidal pattern (Figs 1 and 4).

In period 1 and period 3, the average choice frequency increased with reference to the specified positive pattern (note: the positive pattern was reversed in the afternoon). It could be caused by a difference in the choice-reversal rate of individual bees after overnight and midday breaks, as well as due to continuous training after the early morning and early afternoon

Table 2. Changes of individual preference for a specified pattern in four successive periods

Experiment	Feeder				Hive			
	Period 1 (09:30–11:30 h)	Period 2 (11:30–14:30 h)	Period 3 (14:40–16:30 h)	Period 4 (16:30–09:30 h)	Period 1 (09:30–11:30 h)	Period 2 (11:30–14:30 h)	Period 3 (14:40–16:30 h)	Period 4 (16:30–09:30 h)
Series 1	↑ 92.9%	↓ 92.9%	↓ 85.7%	↑ 100%	↑ 71.4%	↓ 78.6%	↓ 78.6%	↑ 86.7%
Series 3	↑ 78.6%	↓ 100%	↓ 100%	↑ 78.6%	↓ 78.6%	↑ 100%	↑ 85.7%	↓ 92.9%

The symbol ↑ denotes an increase in choice frequency in favor of the specified pattern; ↓ denotes a decrease in choice frequency; a downward double-arrow denotes a tendency towards the specified pattern; an upward double-arrow denotes a tendency towards the opposite preference.

tests. However, the 09:30 h test was commenced after an overnight break without re-training, and the 14:30 h test was commenced after midday breaks without re-training. Therefore, the performance level in these two tests could be only set by the biological rhythms. The zero-crossing point of the sinusoidal regression curve, predicting bees' pattern preference reversed at the feeder and at the hive, coincides with the midday and overnight breaks (Figs 1 and 4).

A large percentage of individuals followed this trend (Table 2), as the experimental bees switched their preference from one pattern to the other, as dictated by their internal clock. We suggest that our training was able to impose the modulation of pattern preference on the biological rhythms of individual bees.

Our findings were very consistent, in spite of the fact that experiments were carried out in different environmental conditions. The experiments began at the Australian National University, and were then repeated at the Wuerzburg University. We obtained the same results, in spite of the experiments being carried out both outdoors and within the indoor bee flight facility, which has a controlled ambience. There is relatively little UV light within the indoor bee flight facility, because the Perspex roof blocks most of it. The consistent results obtained indoors and outdoors indicate that the honeybees did not need to use polarized sunlight as a cue to change their preference according to time. However, there are slight differences in the results of the indoor and outdoor experiments. The bees gradually changed their pattern preference between the morning and the afternoon, immediately following a midday break in the indoor experiment of Series 1. In the case of the outdoor experiments, however, the bees significantly reversed their pattern preference immediately following a midday break in Series 3. A possible reason is that bees could not use polarized light and the exact sun position as cues in the indoor experiment, but were able to do so in the outdoor experiments of Series 3. However, as two different hives were used in the indoor and outdoor experiments, we cannot yet conclusively state that the presence of polarized light was responsible for the difference in the performance of the indoor and outdoor bees. Further experiments are required to clarify this matter.

#### *The complexity of honeybee memory*

In the experiments of Series 3, honeybees had to simultaneously change their preference for a visual pattern with both the time of day, as well as the task at hand. To date, this is the most complex task that honeybees in our laboratories have been trained to perform, since we started to explore this insect's cognitive capabilities at the Australian National University's Research School of Biological Sciences in 1996. In this task, the honeybees had to retrieve the exact memory for the decision not only by means of a single visuo-spatial input, but also using their biological clock and motivation as cues. In other words, the bees had to remember their responses to visual patterns within the context of the task to be performed, as well as the current time coordinates. In order to facilitate the

learning of this very complex task, we adopted the method used by Collect and his colleagues: they trained bumblebees to discriminate between horizontally and vertically oriented gratings of black/white, in order to reach the feeder, and between different diagonally oriented gratings to gain access to their nest. Once the bees had been trained on these two tasks, they could rapidly learn new discrimination tasks, where they had to distinguish between horizontal and vertical gratings at both sites. Whereas they still approached the horizontal grating to reach food, they now had to choose a vertical grating in order to return to their nest (Colborn et al., 1999; Cheng, 2005). In our experiments, we introduced the additional contextual cue of color difference at the feeder and the hive during the training phase and learning tests: the bees had to approach a blue horizontal grating to reach the feeder, and a black vertical grating to access their hive in the morning, and then reverse their preference at both sites in the afternoon. The trained bees learned these tasks well, and performed equally well in the learning tests. Afterwards, the bees were only briefly trained to the new, more complex tasks, following which the critical tests were carried out, where horizontal and vertical gratings of black/white were used at both sites. The bees performed very well in the critical tests, and could simultaneously change their preference for a visual pattern with both the time of day, and the task at hand.

#### *An individual bee knows whether it is foraging or returning home*

Foraging behavior in honeybees can be modulated by juvenile hormone (Huang and Robinson, 1995), by demand for food in the colony (Seeley, 1997; Seeley and Visscher, 2004) or by a worker's nutritional state (Toth et al., 2005). Our experiments have demonstrated that individual bees can quickly reverse their pattern preference between an outward foraging flight and the return trip back to the hive. Thus, the forager knows what to do during both events. In our experiments, the distance between the hive and the feeder was very short, being only 8 m in the indoor experiments at the Australian National University and 24 m in the field experiments at the Wuerzburg University. The flight time between the hive and the feeder was a few seconds for the indoor experiments and 20 s for the field experiments on average. Each forager remained on the feeder drinking sugar solution for about 90 s. The experimental foragers could therefore change their pattern preference in about a few minutes, as their status changed from foraging to returning home. What could be happening in honeybee's brain during this short period of time? Colborn et al. (Colborn et al., 1999) proposed that different contextual signals are associated with approaching the nest or approaching the feeder, and that these contextual signals facilitate learnt associations between orientation detectors and motor commands. Dyer examined the relation between motivation and vector navigation in honeybees, and found that the resetting of the path integration vector can be influenced by motivational cues associated with food deprivation (Dyer et al., 2002). If this is indeed the case,

the brain dopamine level could be operating as a subsecond modulator of food seeking (Roitman et al., 2004). It might even be that the motivations of foraging or returning home act as contextual cues, which can modulate the decisions available to a bee. Further changes in a bee's status, such as an empty or a full stomach before and after obtaining a reward, could act as triggers that change a bee's behavior.

The processes of learning and memory are undoubtedly more sophisticated in primates and mammals than in insects, but there seems to be a continuum in these capacities across the animal kingdom, rather than a sharp distinction between vertebrates and invertebrates. The abilities of an animal seem to be governed largely by what it needs in order to pursue its lifestyle, rather than whether or not it possesses a backbone (Zhang and Srinivasan, 2004a; Zhang and Srinivasan, 2004b). The properties of learning and memory in insects have been shown to be well suited to the requirements of the tasks that they have to perform (Cheng and Wignall, 2006; Lynn et al., 2005). The present research demonstrates that the honeybee possesses a complex memory capable of memorizing tasks within a time schedule. Honeybees can 'plan' their activities in time and space, and use context to determine which action to perform and when.

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