

## RESEARCH ARTICLE

### Do wood ants learn sequences of visual stimuli?

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#### SUMMARY

The visually guided foraging routes of some formicine ants are individually stereotyped, suggesting the importance of visual learning in maintaining these routes. We ask here whether the wood ant *Formica rufa* learns a sequence of visual features encountered at different stages along a route, as reported for honeybees. We trained ants in several simple mazes to follow two alternative routes. Along each two-stage route, the ants first encountered one of two priming stimuli. The identity of the priming stimulus determined which of two choice stimuli was rewarded in the second stage of the route. As stimuli we used ultraviolet and yellow/green light panels, and two black-and-white patterns. Did ants learn to pair each colour with the appropriate black-and-white pattern? Ants learnt readily to discriminate between the two coloured stimuli or between the two black-and-white patterns. They could also pair coloured and black-and-white patterns, provided that the two were presented simultaneously. The ants' behaviour with sequential stimuli varied according to whether the priming stimulus was a coloured stimulus or a black-and-white pattern. When the priming stimulus was coloured, ants seemed to learn the two sequences, but tests showed that their success was probably caused by the after-effects of colour adaptation. With a black-and-white priming stimulus and a coloured second stage stimulus, robust sequential learning could not be demonstrated, although under certain experimental conditions a tiny proportion of ants did acquire the sequence. Thus, ants perform conditional discriminations reliably when priming and choice stimuli are simultaneous, but they usually fail when the stimuli are sequential.

Key words: wood ant, insect navigation, vision, sequence learning, chromatic adaptation, multiple routes, conditional discrimination.

#### INTRODUCTION

Ants can learn long and individually stereotyped foraging routes guided in part by the visual features that they encounter along the route (Collett et al., 1992; Fresneau, 1985; Kohler and Wehner, 2005; Wehner et al., 1996). Visual features are known to control an ant's path in a variety of ways: they can set the compass or panoramically determined direction in which the ant travels (Collett, 2010; Collett and Collett, 2009; Collett et al., 1998; Graham and Cheng, 2009; Wehner et al., 1996); they can act as a beacon and attract the ant's path in their direction (Graham et al., 2003); or they can control the ant's path through image matching (Åkesson and Wehner, 2002; Graham and Collett, 2002; Judd and Collett, 1998; Narendra et al., 2007; Wehner et al., 1996). In each of these cases the ant is responding to the visual features that it currently sees.

In this paper, we are concerned with the added reliability that may result from linking visual features encountered sequentially along a route, such that seeing one feature primes the ant's response to a feature seen later along the route. This ability could help avoid uncertainty when ants have learnt several routes (Beverly et al., 2009; Greene and Gordon, 2007; Sommer et al., 2008) and aim to follow one of them without interference from memories that are relevant to another route (Harris et al., 2005; Wehner et al., 2006).

Evidence that insects learn sequences of visual cues comes from experiments on honeybees (Zhang et al., 1999). Bees were trained to follow two visual sequences within the same complex Y-maze.

At the start of the sequence, bees saw either horizontal or vertical stripes. The orientation of the stripes told bees which stimulus out of two they should approach next. For one sequence, they learnt to link horizontal stripes to a blue stimulus, and the blue stimulus to a black-and-white sectorised disk. The second sequence consisted, successively, of vertical stripes, a green stimulus and a disk of black-and-white concentric rings. At each stage of a sequence, the stimulus to be avoided was that appropriate to the other route. The bees' success at this task shows that they will select and approach one out of two rewarded stimuli according to whether that stimulus was preceded by the previous item in the corresponding sequence.

To explore the ability of wood ants to link together sequentially presented visual stimuli, we used a simplified version of Zhang et al.'s (Zhang et al., 1999) method. Our first step was to find two independent pairs of visual stimuli that ants can easily distinguish. The second step was to arrange these two pairs into two sequences and test whether presenting the first item of a sequence would selectively prime the ant's response to the second item of that sequence.

#### MATERIALS AND METHODS

##### Animals

Wood ant (*Formica rufa* L.) colonies were kept in the laboratory in 550l plastic containers on a 12h:12h day:night cycle and provided with sucrose solution, water and frozen crickets. For a few days before

and during each experiment, the colony from which experimental foragers were taken received a reduced quantity of sugar water. Each experiment began with a group of approximately 25 large foraging ants. These ants were marked individually with spots of coloured paint. After a few days training, we reduced the size of this experimental group to the seven most active ants in order to increase the number of training trials that we could obtain from each ant during a day.

### Maze training

A walkway of wooden dowels (1 cm diameter) was assembled into T- and Y-mazes that were raised 3 cm above the surface of a supporting wooden board. The stems of the Ts and Ys were of variable length, depending on the experiment, and the arms were 22 cm long. Ants walked steadily along the dowel and mostly made no attempt to climb or jump down. Visual stimuli were fixed just beyond the end of each arm and the rewarded and unrewarded stimuli were swapped regularly between the left and right sides of the maze, except in the experiments shown in Fig. 2A and Fig. 4A. Ants were rewarded with sucrose that they drank from a drop in the well of a glass microscope slide placed on a little platform at the end of the dowel in front of the stimulus. The stimuli were just beyond the ants' reach. Both to reduce extraneous cues and to provide a surface for the display of patterns, the T- or Y-mazes were surrounded by a rectangular or a cylindrical arena, with its inner walls (35 or 50 cm high, respectively) covered with black-and-white patterns or with plain black paper.

### Stimuli

To gain access to a sucrose reward, ants had to discriminate between coloured light panels and between black-and-white patterns. Although we refer to the stimuli as coloured stimuli or black-and-white patterns, we were not concerned with the ways in which the ants discriminated the stimuli. Our aim was just to find stimuli that were easy for the ants to distinguish.

### Colour stimuli

Studies on *Formica polyctena* (Menzel and Knaut, 1973) and *Cataglyphis bicolor* (Mote and Wehner, 1980; Paul et al., 1986) suggest that formicine ants are dichromats with ultraviolet (UV) and green colour channels. Accordingly, the colours of the light panels were chosen to be easily discriminable by the ants' UV and green photoreceptors. In our initial experiments (see Figs 1, 2) the coloured stimuli were light bulbs, each enclosed in a light-tight container with a UV or yellow filter covering a window. Later (see Figs 3–9), we changed to diamond-shaped clusters of UV or green light-emitting diodes (LEDs).

The light bulbs used were fluorescent Arcadia Bird Lamps (3.5×8.5 cm), which replicate a daylight spectrum including UVA and UVB. The glass UV filter was transparent for wavelengths between 300 and 417 nm with a maximum between 356 and 375 nm, and the plastic yellow filter transmitted light at wavelengths above 480 nm. The green and UV LED clusters consisted of 70 LEDs formed into a 7×10 cm (width × height) diamond. UV LEDs were 5 mm in diameter, with peak emission at 390 nm, a 30 deg beam and a maximum intensity of 2 mW sr<sup>-1</sup>. Green LEDs were 5 mm in diameter, with peak emission at 536 nm, a 20 deg beam and a maximum intensity of 19.7 mW sr<sup>-1</sup>.

The overhead high-frequency fluorescent lights were off during colour priming experiments. After we had established that these lights did not interfere with the ants' ability to discriminate the stimuli, the fluorescent lights were switched on during the pattern priming experiments.

### Black-and-white patterns

The black-and-white patterns were printed on white A4 paper using a laser printer (HP LaserJet P3005 PS). For experiments in which a colour cued the black-and-white pattern, the patterns were glued to card so that they could be swapped between the arms of the maze. A black–white (BW) or white–black (WB) oblique edge formed the boundary between equal areas of black and white. Each pattern was 68×35 cm (width × height) (see Fig. 1B, Fig. 2). The edge was oriented at 10 deg to the vertical and was tilted clockwise from the vertical for the BW pattern and anti-clockwise for the WB pattern. The second pair of patterns consisted of a vertical or horizontal grating of 8 cm wide black-and-white stripes, which covered either half or the entire inner wall of a cylindrical arena (50 cm high, 55 cm diameter) (see Figs 3–9). Viewed from the centre of the arena, the stripes were 16.7 deg wide and should be resolved easily by the ants' eyes (e.g. Zollikofer et al., 1995).

### Data collection and statistical analysis

Initially, we were uncertain whether ants would reach the end of the dowel without reversing direction. We put a mark halfway between the junction and the end of the arm and scored whether ants that passed the halfway mark continued to the end of the arm. In 98.5% of trials, ants that crossed the halfway point continued to the end. Because reversals were so rare, we scored a trial as correct or incorrect according to whether, on its first try, an ant reached the end of the rewarded or unrewarded arm, respectively. If the ant was incorrect, it was moved to the start of the maze and its second and subsequent attempts were not scored.

The data in Figs 1–4 and 6–9 are presented in the form of cumulative learning curves. The abscissa ('Training trials') gives the total number of training runs completed. The ordinate ('Correct choices') represents the cumulative sum of trials completed without mistake. In these figures, the dashed line indicates a random choice between the two stimuli, with steeper data curves showing a preference for the correct stimulus. All figures, with the exception of Fig. 4A, Fig. 6B, Fig. 7B and Fig. 9, show the results of the individual ants who learnt the imposed task, using the criterion that the proportion of correct trials during the whole training period was greater than chance at a significance level of  $P < 0.05$  (binomial test). We specify for each experiment how many ants did and did not learn according to this criterion.

When learning was slow or did not occur, ants often exhibited side or colour preferences. We then adapted the training schedule to attempt to overcome the ants' bias by inserting extra trials in which the opposite side or other colour was rewarded. If this procedure did not induce learning, it had an impact on the cumulative choice curve and pushed the curve below the random choice level, as shown in Fig. 4A and Fig. 6B. The number of ants contributing to the scores on each trial shown in Figs 1–4, 6 and 7 drops during the course of an experiment because some ants ceased foraging and some disappeared.  $\chi^2$  values for 2×2 contingency tables were used to assess the group performance of all the ants trained for the experiments. This statistic included all the trained ants, regardless of whether they fulfilled the learning criterion.

## RESULTS

### Colour priming of black-and-white patterns

#### Stimulus discrimination

Our initial requirement was to find two pairs of stimuli that could be used to test the ants' ability to learn a sequential task. The few ant species that have been tested are dichromats with peaks of sensitivity in the UV and yellow–green parts of the spectrum [e.g.

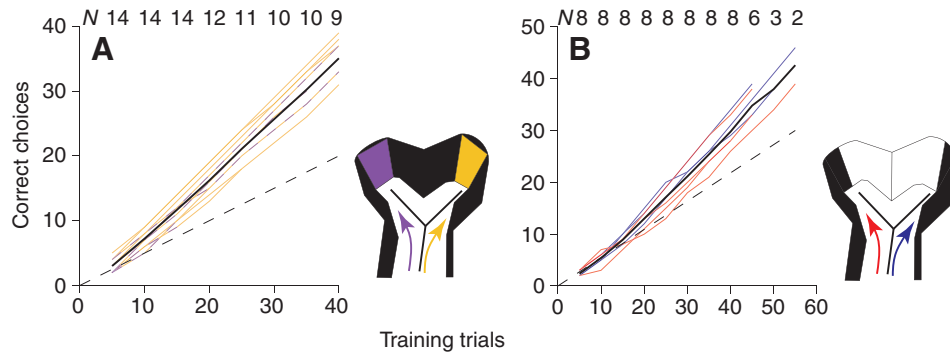


Fig. 1. Colour and pattern discrimination. (A) Yellow/UV discrimination. Individual cumulative learning curves of nine ants trained to approach a yellow light (solid yellow lines) and five ants trained to approach a UV light (dashed purple lines) when given the choice between yellow and UV. Solid black line, mean performance over both groups (14 ants); dashed black line, chance level. (B) Black–white pattern discrimination. Cumulative learning curves of three ants trained to approach BW (blue lines) over WB, and five ants trained to prefer WB (red lines). Solid black line, mean performance over both groups (eight ants); dashed black line, chance level. Ants’ scores are pooled over successive blocks of five trials. *N*, number of ants contributing to each block of trials. Insets: experimental setup.

*Formica polyctena* (Kiepenheuer, 1968; Menzel and Knaut, 1973) and *Cataglyphis bicolor* (Mote and Wehner, 1980; Paul et al., 1986)]. We asked first whether ants could learn to approach a rewarded yellow light rather than an unrewarded UV light or *vice versa*. One group of ants was rewarded when it approached the yellow stimulus and a second group was rewarded on reaching the UV stimulus. All 14 ants trained in the Y-maze learnt rapidly to approach either the yellow (nine ants) or the UV (five ants) light according to which colour led to food ( $\chi^2=260.98$ , d.f.=1,  $P<10^{-5}$ ; Fig. 1A). As our aim was only to find stimuli that could be distinguished, we made no attempt to control the relative intensities of the two colours and the ants’ behaviour could have been driven by differences in the lights’ apparent luminosity and chromaticity.

The second pair of stimuli consisted of black-and-white edges of opposite polarities (see Materials and methods) and had already been used successfully in an earlier experiment (Harris et al., 2005). The acquisition curves shown in Fig. 1B indicate that ants can associate both the WB pattern (three ants) and the BW pattern (five ants) with food. Ants took slightly longer to learn to discriminate

these spatial patterns than they did the coloured light panels. But all eight ants learnt the association between pattern and reward ( $\chi^2=113.93$ , d.f.=1,  $P<10^{-5}$ ).

Given that ants had little difficulty in learning these colours and patterns independently of each other, we then asked whether ants could link patterns to colours. Can they learn to approach one of the black-and-white patterns contingent upon which colour they had seen previously?

Sequence learning

Ants were trained in a long Y-maze, which consisted of two chambers (see Fig. 2A). In the first chamber, ants walked along a 20 cm length of dowel straight towards a single illuminated filter, which in some trials was UV and in others yellow. The filter was just above the dowel and the ants went under the stimulus and along a 4 cm unlit black corridor to reach the second chamber. The corridor between the chambers ensured that the two stimuli were not seen together. In the second chamber, the dowel bifurcated with a BW pattern at the end of the left arm and a WB pattern on the right (see Fig. 2A).

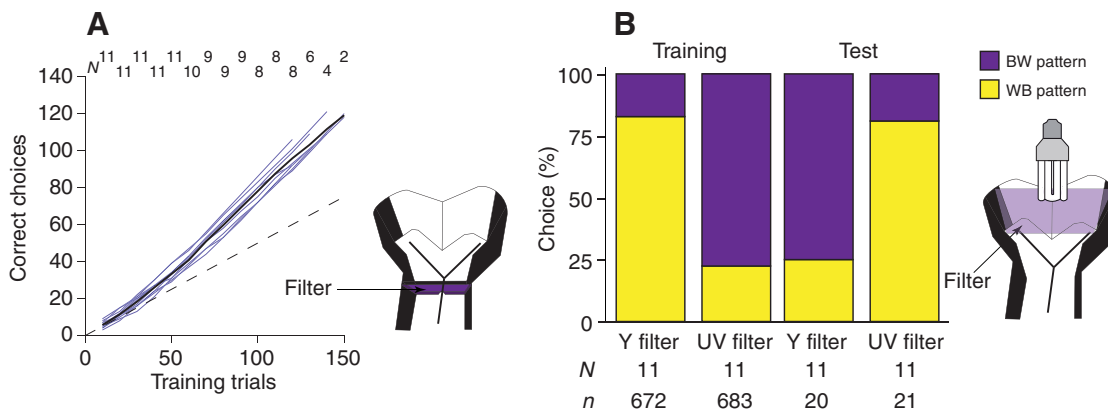


Fig. 2. Colour priming of pattern. (A) Individual cumulative learning curves (blue lines) of 11 ants trained to approach the BW pattern at the end of the left arm when the filter was UV and the WB pattern at the end of the right arm when the filter was yellow. Each of the filters was illuminated with a broad-spectrum Arcadia bird lamp (see Materials and methods). Choice frequency is pooled over both conditions within successive blocks of 10 trials. Solid black line, mean group performance (11 ants); dashed black line, chance level. (B) Choices with UV and yellow filters during training and in afterimage test. In training (replot of data from A), ants chose the correct pattern after seeing either yellow or UV filters. In tests with the choice chamber lit from above by yellow or UV filtered light (inset), the ants’ choices were complementary to those in training. *N*, number of ants; *n*, number of training or test trials.

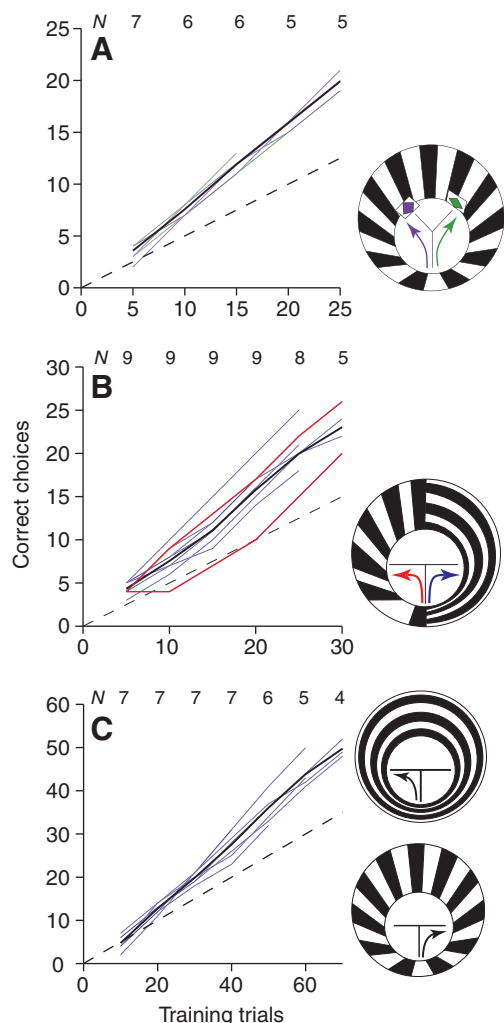


Fig. 3. Discrimination of colours and stripe orientations. (A) Colour discrimination. Individual cumulative learning curves of three ants trained to approach green over UV LEDs (green lines), and four ants trained to UV (purple lines). Solid black line, mean performance over both conditions (seven ants). (B,C) Discriminating between horizontal and vertical black-and-white gratings. (B) Individual cumulative learning curves of two ants trained to approach a vertically striped half-cylinder (red lines) and seven ants trained to approach a horizontally striped half-cylinder (blue lines). Solid black line, performance of both groups (nine ants). (C) Individual cumulative learning curves of seven ants trained to turn right when the whole cylinder is lined with vertical stripes and left when stripes are horizontal (blue lines). Solid black line, performance pooled over both conditions. Choices in all panels are pooled over successive blocks of five trials. Dashed black lines in all panels indicate the chance level.

The ants' task was to approach the BW pattern after seeing the UV light and the WB pattern after seeing the yellow light. All 11 trained ants learnt the task ( $\chi^2=494.33$ , d.f.=1,  $P<10^{-5}$ ; Fig. 2A). But is this result real evidence that ants have learnt a sequence?

A possible alternative explanation is that the few seconds that ants took to walk through the first chamber were enough to adapt their UV or green colour channel and, consequently, the white-lit black-and-white stimuli in the second chamber were tinged with a negative afterimage. The negative afterimage would give the patterns a complementary UVish appearance after exposure to yellow light and tint the patterns yellowish after exposure to UV.

Specific adaptation of UV and green receptors has been induced in ants (Menzel and Knaut, 1973) and chromatic adaptation affecting colour perception has been shown in honeybees (Dittrich, 1995; Hori et al., 2006; Neumeyer, 1981). Little is known about the temporal parameters of adaptation in ants, but in bees chromatic adaptation can be completed within a few seconds (Dittrich, 1995) or can take up to 5 min (Neumeyer, 1981), depending on the strength of the stimulus and details of the behavioural assays. It is therefore possible that the several seconds that the ants spent in the first part of the maze lit by yellow or UV light was enough to influence the ants' choice in the second chamber. Did ants learn the sequence or did they perform correctly because of the effects of adaptation, which coloured the black-and-white patterns?

#### Afterimage test

A rough simulation of an afterimage occurring after adaptation to a coloured light can be achieved by illuminating the black-and-white patterns with either UV or yellow light. If the ants were exploiting afterimages during training, they should, when placed directly in the second chamber, prefer the BW pattern when the second chamber was illuminated with yellow light and the WB pattern when it was illuminated with UV light, the converse of the pairing during training.

In tests, an ant was placed directly into the second chamber, with a UV or a yellow filter placed under the white Arcadia lamp lighting the chamber (diagram in Fig. 2B). Tests were given with either the yellow or the UV filters, either after a training trial with the yellow filter or after a training trial with the UV filter, making a total of four test types. Individual ants were tested once with each type of test (except for one ant that was tested only once). In 15 out of 20 tests (75%,  $P=0.02$ ) with the yellow filter, the ants approached the BW pattern, and in 17 out of 21 tests (81%,  $P=0.003$ ) with the UV filter, ants chose the WB pattern (Fig. 2B, 'Test' histograms). The stimuli seen on the preceding training run had no obvious influence on the ants' choices. In 22 out of 41 tests, ants chose the same pattern as on the previous training run, and in the remaining 19 tests they chose the other pattern. These test results suggest that chromatic adaptation occurred in the first chamber during training and that its consequences controlled the ants' behaviour in the second chamber. The ants' performance in tests is close to the inverse of that during training (Fig. 2B). The 'Training' histograms in Fig. 2B show the data from Fig. 2A pooled across all the training trials of all 11 ants.

The fact that ants may exploit afterimages in order to take the correct route does not, of course, mean that they are incapable of sequential learning. To see whether ants can link information sequentially, we reversed the order of the stimuli, presenting patterns of vertical or horizontal black-and-white stripes in one chamber to prime the ants' choice between green and UV LED lights in a second chamber. The striped patterns are less likely to induce static afterimages because the stripes move across an ant's retina as the ant traverses the priming chamber.

#### Pattern priming of coloured stimuli

Because we used different colours and different black-and-white patterns in the pattern-priming experiments, we had to check that ants could distinguish between the colours and between the black-and-white patterns.

#### Discrimination of colour stimuli

Two groups of ants ( $N=4$  and 6) were trained in a Y-maze to discriminate between the green and UV LED arrays (see Materials and methods). The Y-maze was placed in an upright cylinder with the inner wall lined with black-and-white vertical stripes (Fig. 3A).



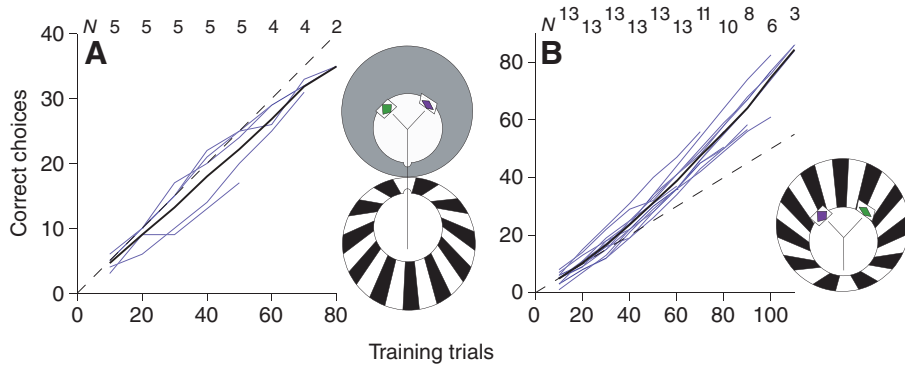


Fig. 4. Stripes priming colours. (A) Sequential training. Performance of five ants rewarded for approaching green LEDs on the left after seeing vertical stripes and for approaching UV LEDs on the right after encountering horizontal stripes. No learning occurs. (B) Simultaneous task. Thirteen ants learnt to approach green LEDs when the choice cylinder displays vertical stripes and UV LEDs when the choice cylinder shows horizontal stripes. In both panels, individual cumulative curves show choices pooled over both conditions within blocks of 10 trials and solid black lines indicate mean group performance; dashed black lines, chance level; *N*, number of ants.

Both groups learnt to approach the rewarded LED array ( $\chi^2=240.88$ , *d.f.*=1,  $P<10^{-5}$ ; Fig. 3A). At an individual level, seven ants learnt to discriminate between the arrays. The remaining three ants that did not fulfil the learning criterion (see Materials and methods) chose correctly on 70% of the training trials.

#### Discrimination of stripe orientation

The ants' ability to discriminate between vertical (V) and horizontal (H) gratings was tested in two experiments, using a T-maze placed inside a cylinder lined with black-and-white stripes (Fig. 3B). For the first experiment, half of the inside wall of the cylinder was covered with H stripes and half with V stripes. The arms of the T were directed at the midpoints of the two striped areas and ants were released individually at the stem of the T. One group of ants was trained to approach V stripes (five ants) and a second group was trained to approach H stripes (seven ants). The sides of the V and H stripes were regularly switched. The ants as a group learnt to discriminate between the two stripe patterns ( $\chi^2=88.4$ , *d.f.*=1,  $P<10^{-5}$ ) and the individual cumulative learning curves of the nine ants, which performed significantly better than chance, are plotted in Fig. 3B.

For the second experiment, the entire cylinder wall displayed either V or H stripes on any given trial. The ants had to turn left in the T-maze if the stripes were H, and right if the stripes were V (Fig. 3C). Seven out of nine ants learnt to make the correct turn and the group as a whole performed correctly ( $\chi^2=96.68$ , *d.f.*=1,  $P<10^{-5}$ ).

#### Sequential training

The ants were trained in a Y-maze that passed through two cylinders (Fig. 4A). The first cylinder, which contained the stem of the maze, primed the ants with V or H stripes. The second cylinder containing the Y-maze gave ants a choice between UV and green. This cylinder was lined with black paper. The green LED array was always at the end of the left arm and was rewarded when the first cylinder had V stripes. The UV LED array was at the end of the right arm and was rewarded when the stripes were H. Even after 80 training trials, the five trained ants showed no evidence of linking the stripe orientation seen in the first cylinder with their choice of colour in the second cylinder ( $\chi^2=3.1$ , *d.f.*=1,  $P=0.078$ ; Fig. 4A). At this point the experiment was stopped.

This experiment was designed so that the coloured lights had permanent positions within the maze, thus allowing the ants to form two types of associations. Surprisingly, not only did the ants fail to form sequential links between the priming striped pattern and the coloured light, but they also did not associate a left or right turn with the pattern. Bumblebees have been shown to succeed at a task that is formally similar, in which they must turn left or right depending

on the colour that they have previously viewed (Chittka, 1998), although it takes them approximately 100 training trials to perform well. Ants forage in the experimental maze less frequently than bees, which reappear for a new trial every 5 to 10 min. Ant training is especially slow when several ants are trained in parallel. As always, it is possible that ants might have learnt the sequence with more training. But the rapidity with which ants can learn routes in the laboratory (Graham et al., 2003) and in their natural habitat (Sommer et al., 2008) suggests that very slow acquisition of associations is unlikely to be an important part of their normal navigational learning.

To dissect where the difficulty in sequence learning might lie, we first tested whether the ants can link the same patterns and LED stimuli when they are presented simultaneously rather than sequentially.

#### Simultaneous training

The ants were trained in a Y-maze within a single cylinder. The cylinder walls were on each trial lined with either V or H stripes and ants had to choose between the arms that led to the UV or green LED array according to the orientation of the stripes (Fig. 4B). Approaches to the green LED array were rewarded when the stripes were V, and approaches to the UV LED array were rewarded when the stripes were H. LED arrays were frequently swapped between arms. Considered as a group, the 19 trained ants learnt to associate stripe orientation and colour ( $\chi^2=257.44$ , *d.f.*=1,  $P<10^{-5}$ ). The individual cumulative learning curves of 13 ants, which performed correctly at an individual level, are plotted in Fig. 4B. The six ants that failed to learn were correct on  $62\pm 21\%$  trials after 60 training trials. They may have been below par in other ways as well, as by trial 70 they had all disappeared.

Are ants successful in choosing both UV and green LED arrays correctly? Fig. 5A shows, separately for each ant that learnt, the proportion of correct choices for each of the two stimulus pairs. The two colours attract roughly the same proportion of correct choices with a hint of a preference for UV. This slight preference for UV is also seen in the other panels of Fig. 5, which show a similar breakdown for other experiments.

Thus, ants can link the two stimuli (stripes and coloured light) together provided that they are presented simultaneously. To test whether this easily learnt simultaneous task can facilitate the acquisition of a related sequential one, we trained another group of ants to learn the two tasks in parallel.

#### Mixed simultaneous and sequential training

The ants were trained on the same Y-maze as in the earlier sequential task, but they received two types of training trials in a pseudorandom order. In some trials, simultaneous information was provided by having

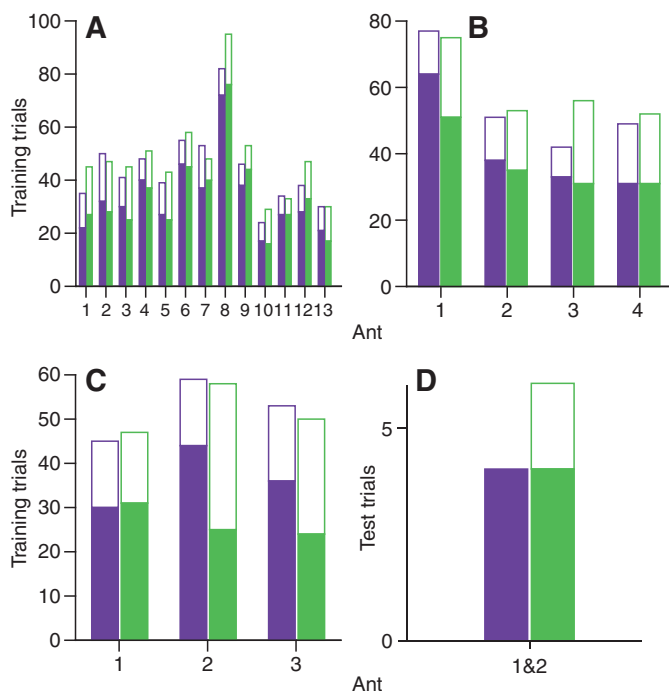


Fig. 5. Colour choices of individual ants during successful simultaneous and sequential training. (A) Proportions of correct choices of UV and of green by 13 ants that were trained to associate colour and pattern simultaneously (Fig. 4B). Filled and unfilled bars represent the correct and incorrect choice, respectively, when the UV (purple) and green (green) stimuli were rewarded. (B) Similar plot for individual ants trained in the simultaneous task shown in Fig. 6A. (C) Similar plot for individual ants that were categorised as learning to link coloured LED array to stripe orientation during sequential trials (Fig. 8C, left panel). (D) Similar plot for two ants in sequential trials when tested on their ability to choose the correct stripes without making a turn (Fig. 8C, right panel)

the walls of both cylinders lined with stripes of the same orientation (Fig. 6A). In other trials, the task was purely sequential: the first cylinder had either V or H stripes and the second cylinder was always lined with diagonal stripes (Fig. 6B). Diagonal stripes make the two cylinders somewhat similar, but do not cue which colour the ants should approach. In both sets of trials, V stripes were paired with the green LED array and H stripes were paired with the UV LED array. Colour stimuli were regularly swapped between the arms of the maze.

We scored the ants separately on these two tasks. Four out of five ants learnt the simultaneous task (group performance:  $\chi^2=60.28$ , d.f.=1,  $P<10^{-4}$ ). Fig. 5B gives separately for each successful ant the proportion of correct choices for each of the two stimulus pairs. The proportion of trials on which individual ants chose green or UV LED arrays correctly are roughly similar for the two colours.

The memories acquired on trials with simultaneous information did not help the ants to learn the sequence (group performance:  $\chi^2=1.46$ , d.f.=1,  $P=0.23$ ; Fig. 6B). Performance on sequential trials remained at or below chance throughout training. Both in Fig. 4A and in Fig. 6B, the cumulative choice curve makes it appear that ants tended to choose the wrong stimulus. This tendency is likely to be a joint product of a prior preference coupled with a training procedure that tried to overcome this bias (see Materials and methods).

In this experiment we know that ants attended to colours and stripes in the second cylinder. Nonetheless, they did not seem to learn anything about the same stripes in the first cylinder.

#### Training to stripes and coloured stimuli in sequence

One reason why ants in the previous experiment might have failed to learn the sequence is that when they were in the first cylinder they had no immediate reason to attend to the stripes on the wall and so might have ignored the stripes' orientation. To avoid this possibility, we forced ants to choose a path in the first cylinder that depended on the orientation of the stripes. They could only enter the second cylinder and decide between the colours after they had chosen correctly in the first cylinder. Another reason for failure in the previous experiment might have been that the diagonal stripes in the second cylinder disrupted the memory of the stripe orientation seen in the first cylinder. This possibility was avoided by lining the second cylinder with plain black paper.

For this experiment the complete maze consisted of three cylinders (Fig. 7). The central cylinder displayed either H or V stripes and contained a T-maze. Each arm of the T led to a Y-maze in one of two flanking cylinders, with a UV LED array at the end of one arm of the Y and a green array at the end of the other arm. The ant started each trial in the central cylinder and had to turn left when the stripes were H and right when the stripes were V. The stem of the T was regularly swapped between the two start positions (marked 'S' in Fig. 7, with the dashed line showing the stem), so that a left (or right) turn could bring the ant to either of the two side cylinders. A gap in the dowel prevented access to an incorrectly chosen side cylinder. In each side cylinder, the green arm was rewarded when the stripes were V and the UV arm was rewarded when the stripes were H. As before, green and UV LEDs were regularly swapped

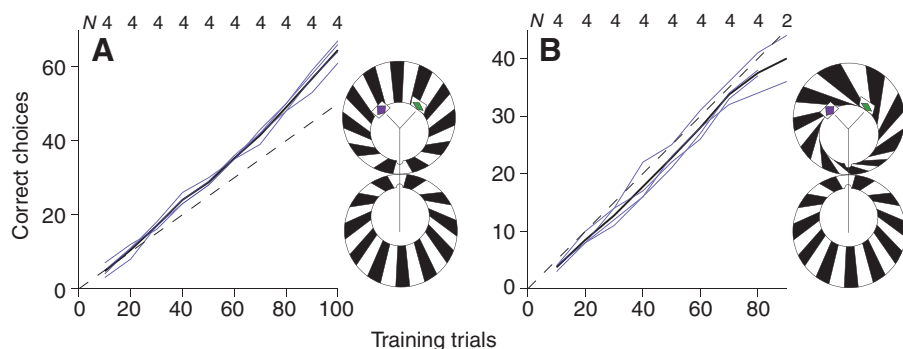


Fig. 6. Mixed simultaneous and sequential training. (A) Individual and mean cumulative learning curves of four ants during simultaneous training trials. (B) The performance of the same ants in sequential training trials during which no learning occurred. In both panels, choices are pooled over both conditions within blocks of 10 trials; dashed black lines, random (chance) level; N, number of ants.

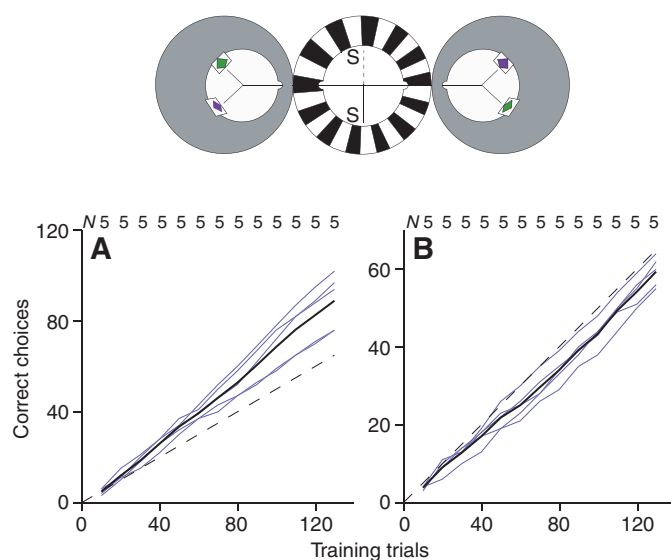


Fig. 7. Training to stripes and colour. (A) Individual and mean cumulative learning curves of five ants on the first part of a task in which they were trained to turn left or right according to whether stripes in central cylinder were vertical or horizontal. The start point (S) was regularly rotated through 180 deg. The ants learnt this part of the task. (B) The performance of the same ants on the second part of the task, in which they had to approach green or UV LEDs in one of the flanking cylinders. The correct choice of colour depended on the orientation of the stripes that they had seen in the central cylinder. Ants performed at chance on this part of the task. In both panels, choices are pooled over both conditions within blocks of 10 trials; dashed black lines, chance level; *N*, number of ants.

between left and right arms of the Y-mazes. We scored the ants' performance both in the central cylinder where they turned left or right according to stripe orientation and in the side cylinders where they chose between the colours.

In Fig. 7A we show the performance of five ants that successfully learnt to turn correctly in the striped central cylinder ( $\chi^2=101.98$ , d.f.=1,  $P<10^{-5}$ ). However, although we continued training for more than 100 trials, these ants did not learn to choose the rewarded colour in the side cylinders ( $\chi^2=1.87$ , d.f.=1,  $P=0.17$ ; Fig. 7B). Their performance did improve slightly towards the end of the experiment, and we cannot discount the possibility that, had we been able to continue training, they might eventually have learnt to choose colours correctly.

#### Training to stripes and colours seen simultaneously and sequentially

To give the ants one last chance to display an ability to learn sequences, we combined the methods of the last two sections. In an attempt to speed up training, we switched to a setup with two connected cylinders. The ants started in a cylinder lined with H or V stripes. To reach the second cylinder they had to turn left or right according to the orientation of the stripes (Fig. 8A and Fig. 9A). Because ants had to turn left or right in the first cylinder and still reach the same place, the position of the initial part of the dowel (shown by the two Ss in Figs 8 and 9) was switched whenever the orientation of the stripes was changed.

Once in the second cylinder they chose between the UV and green LED arrays. The wall of the second cylinder was either lined with the same stripe pattern as the first cylinder (simultaneous training trials, Fig. 8B and Fig. 9B) or with black paper (sequential training

trials, Fig. 8C and Fig. 9C). At the start of training, most trials were simultaneous. Sequential trials were introduced as training progressed. The proportion of sequential trials gradually increased to 100%. This increase is shown for each ant at the bottom of Fig. 8A and Fig. 9A by the increasing density of vertical tick marks, each of which represents a sequential trial.

Out of 24 ants, 10 lasted long enough to be given 100 or more sequential training trials, comparable with the amount of sequential training in the previous experiment. There was considerable individual heterogeneity and data from this group are separated according to whether (Fig. 8) or not (Fig. 9) an individual showed some sequential learning. Three of the 10 ants learnt the sequence (Fig. 8C), according to our criterion of  $P<0.05$  over all training trials (see Materials and methods). But after the significance level was adjusted with the Sidák–Dunn correction for multiple comparisons (Sokal and Rohlf, 1995), the scores of only one ant can be considered to show significant learning ( $P<0.0051$ ).

Fig. 5C shows the individual scores of these three ants for the two stimulus pairs. The ant that showed robust learning was equally correct in its choice of green and UV LED arrays. The other two ants chose UV when it was rewarded on sequential trials, but showed random choice for the rewarded green LED array.

All 10 ants that had more than 100 sequential training trials learnt to turn correctly in the first cylinder (Fig. 8A, Fig. 9A, individual significance level  $P<10^{-5}$ ). The ants' correct direction could have resulted from learning a spatial cue to the position of the entrance to the second cylinder or from linking the direction of turn to the orientation of stripes. To test whether the ants had linked turn direction to stripe orientation, the two possibilities were put into conflict. For each stripe orientation, the starting piece of dowel was placed so that a correct turn cued by the stripes would take the ant away from the entrance to the second cylinder. In these tests, ants mostly turned in the direction cued by the stripes (combined data from Fig. 8A and Fig. 9A yields 82 correct choices in 111 test trials,  $P<10^{-5}$ ).

As a group, all 10 ants also learnt to link stripes and colours during simultaneous training ( $\chi^2=25.93$ , d.f.=1,  $P<10^{-4}$ ). Six out of the seven ants that failed to acquire the sequence did learn to link the two stimuli when they were presented simultaneously (Fig. 9B). Only one of the three ants that learned the sequential task (Fig. 8B) performed successfully during simultaneous trials. The failure of the other two ants shown in Fig. 8B may have occurred because the emphasis on sequential training resulted in too few simultaneous trials.

In sequential trials, both the orientation of the stripes and the ants' direction of turn in the central cylinder give cues to the correct colour. To test whether ants could choose correctly without turning in the central cylinder, we removed the start dowel on some trials and placed the ants on the straight piece that led into the second cylinder. The ant that learnt the sequence most robustly and one other ant were tested. Unfortunately, these tests seemed to disrupt the ants' performance during subsequent training so that we were limited to five tests per ant, after which training broke down. On these 10 tests, ants chose correctly eight times ( $P=0.054$ ; Fig. 8C, right panel), leading to the tentative conclusion that a small proportion of ants may link the rewarded colour in the second cylinder to the stripe orientation in the first.

#### DISCUSSION

We began this study with the aim of exploring the details of sequence learning in ants. Instead, it turned out to be hard to demonstrate that ants do link sequences of visual stimuli. Our conclusion is that ants probably find it genuinely difficult to link sequences and that their

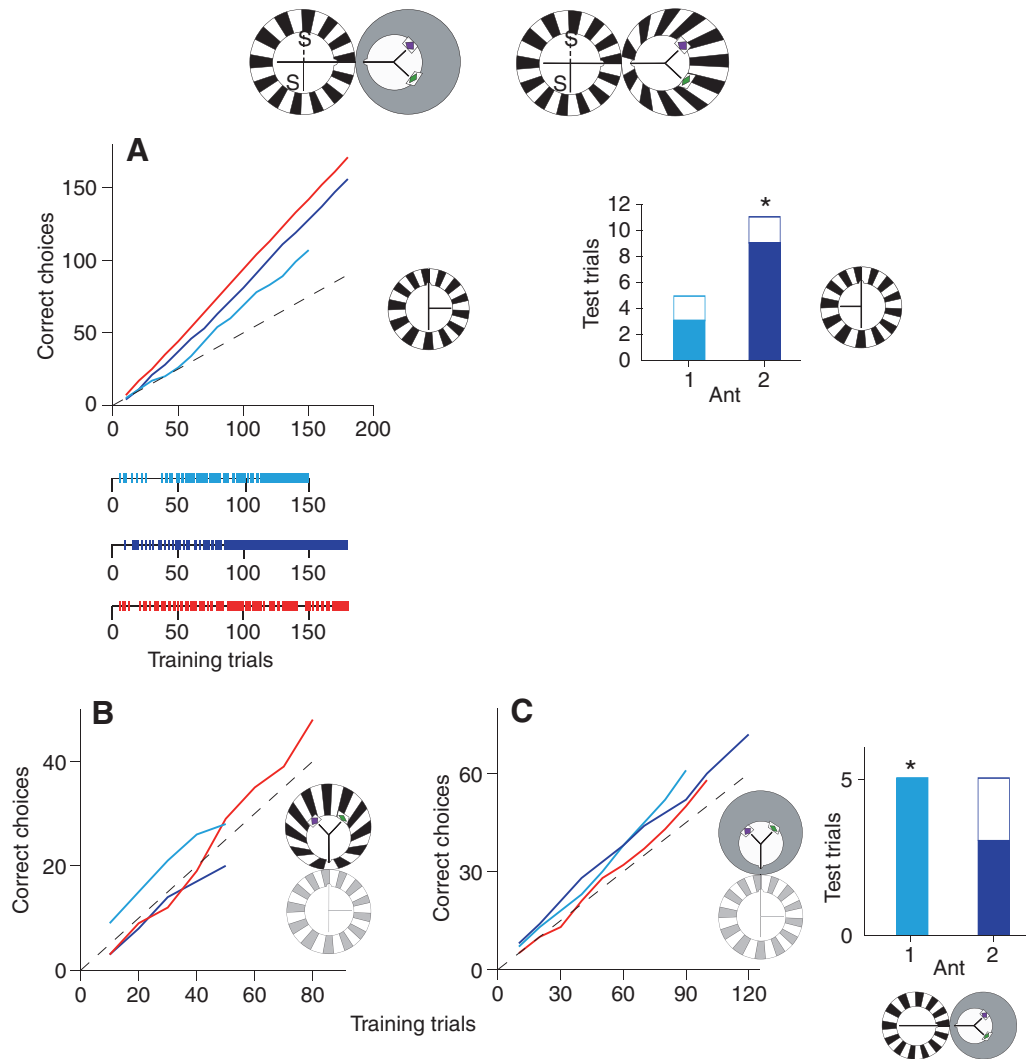


Fig. 8. Training to stripes and colour in mixed simultaneous and sequential mode. (A) Cumulative learning curves of three ants in the first cylinder showing that they learn to turn left or right according to stripe orientation. The figure is explained more fully in the section Training to strips and colours seen simultaneously and sequentially. Two of these ants were tested to see whether the stripes guided their turn or whether they turned correctly because they headed towards a specific place (data in bar chart). There was sufficient data from one ant to show that stripe orientation controlled its direction of turn ( $*P < 0.05$ ). Ants after reaching the second cylinder chose between UV and green LED arrays according to stripe orientation. For simultaneous training, the same stripe orientation was present in both cylinders. For sequential training, stripes were only presented in the first cylinder. The vertical ticks in the coloured panels below the learning curves show for each individual the occurrence of sequential trials within the training sequence. (B,C) Cumulative learning curves of the same ants in simultaneous (B) and sequential (C, left panel) training trials. (C, right panel) Performance of two ants, when tested during sequential training with no opportunity to turn in the first cylinder. One ant chose the correct colour as cued by stripe orientation in five out of five tests; the second ant chose correctly in three out of five tests. In the bar charts, filled bars correspond to predicted correct choices and unfilled bars correspond to incorrect choices. Individuals are identified by the same colour across all panels.

failure is not likely to be just a result of inadequate experimental methodology. Indeed, our method of training ants to follow two virtual routes has been reported to work successfully in honeybees (Srinivasan et al., 1998; Zhang et al., 1999). We suspect we may have uncovered a basic and revealing limitation in what these ants can learn easily, but there remains the possibility that different methods might show that sequential learning falls within their normal repertoire.

Although ants could discriminate between stripe orientations and between coloured stimuli, they mostly failed to link a particular colour stimulus to a particular stripe orientation when there was a short temporal separation between seeing the stripes and seeing the colours. In contrast, ants had no difficulty in pairing a particular

colour with a particular stripe orientation when the stripes and colours could be seen at the same time. Weak evidence for sequence learning in a small minority of ants was obtained only when we provided a scaffold for learning. The scaffolding had two parts. The ants' attention was drawn to the orientation of stripes in the first part of the route. The contingency between orientation and colour was then emphasised by presenting orientation and colours together on some training trials. Even with this help, ants rarely developed an association between stripes and colours in the two parts of the route, and when they did it was fragile.

A related effect was found in bumblebees. After bees were trained to link two pairs of stimuli with each pair viewed simultaneously, the learnt pairing persisted when the stimuli were viewed



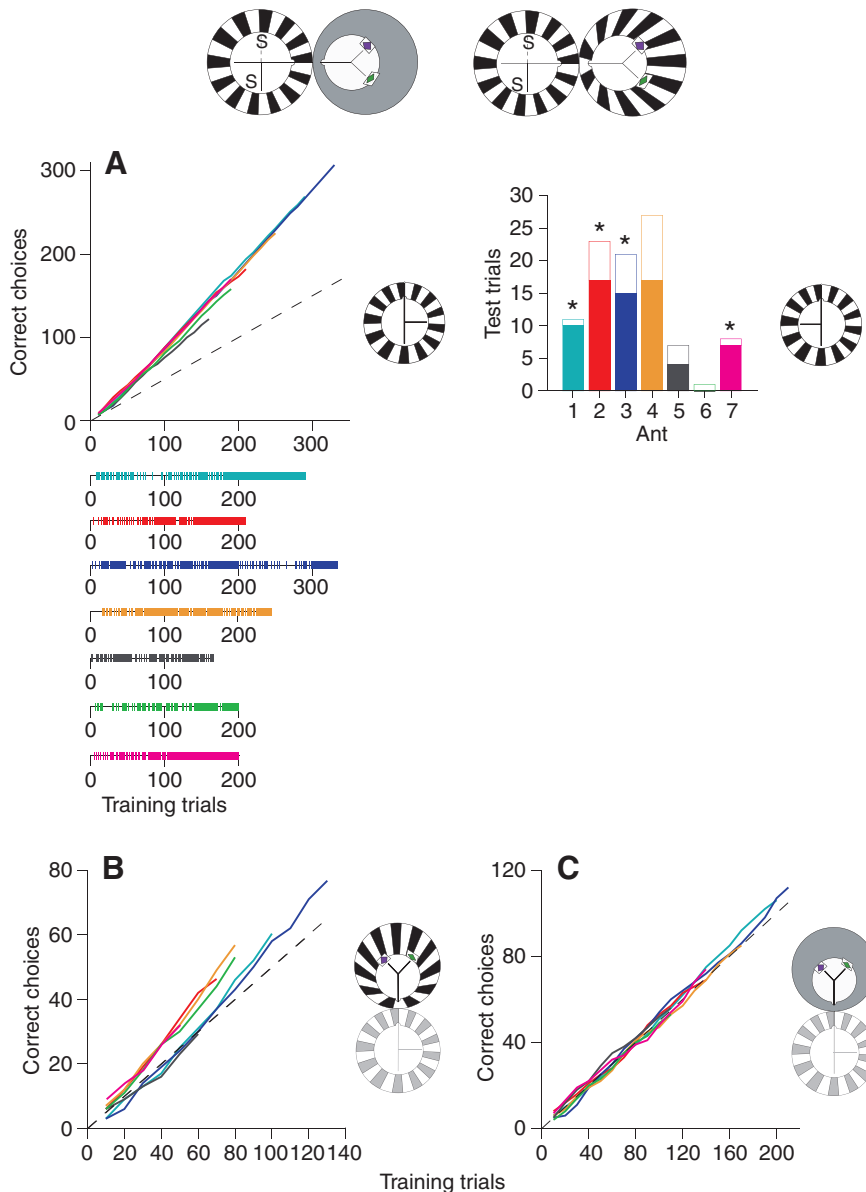


Fig. 9. Ants that did not learn the sequence when given combined simultaneous and sequential training. (A–C) Data are shown as in Fig. 8, except that these ants did not learn the sequence ( $\chi^2=1.91$ , d.f.=1,  $P=0.17$ ) and so were not given the final test.

sequentially (Fauria et al., 2000). Bumblebees are also similar to ants in that they exhibit strong simultaneous but weak sequential effects between pairs of visual stimuli (Dale et al., 2005).

What is it that makes sequential learning hard? Is the brief interval between seeing the stripes and the colour too long (Menzel, 2009)? Because stripe orientation and turn direction can become strongly associated (see e.g. Fig. 9A), some trace of the conjunction of stripe orientation and turn direction must somehow be reinforced during the route. But the orientation trace by itself might have decayed to be too weak to combine with a much stronger immediate colour trace in the second cylinder.

These experiments highlight the difficulties of establishing an animal's cognitive capacities by testing whether they can learn to solve a somewhat artificial task. Had we just examined the priming of black-and-white patterns by coloured stimuli and not tested for the effects of colour adaptation, we might have concluded erroneously that ants can learn visual sequences. In fact, it is simpler to suppose that the ants behaved appropriately because they were exploiting the after-effects of colour adaptation. But this simpler

account does not exclude the possibility that ants may also have learnt a sequence, so we continued by analysing the priming of colour by spatial pattern. Here the danger is one of a false negative – concluding prematurely that ants cannot learn sequences.

Some support for the validity of our findings can be found in other recent studies. Ants will join and continue a route when shifted to an arbitrary place along it (Kohler and Wehner, 2005), indicating that knowledge of a visual sequence is not essential for route following. Other signs that wood ants may not link visual cues along a route come from experiments in which wood ants switched very rapidly from retrieving visual memories appropriate to one route to retrieving those appropriate to another route, with no apparent interference between the two routes (Lent et al., 2010).

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