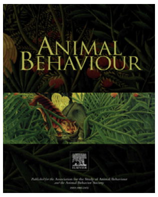
Animal Behaviour 97 (2014) 13–23

Contents lists available at ScienceDirect

Animal Behaviour

journal homepage: www.elsevier.com/locate/anbehav



Walking bumblebees memorize panorama and local cues in a laboratory test of navigation

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ARTICLE INFO

Article history:
Received 13 May 2014
Initial acceptance 10 June 2014
Final acceptance 29 July 2014
Published online
MS. number: 14-00395R

Keywords:
arena maze
guidance by extramaze cues
local cue learning
navigation
panorama learning

Single walking bumblebees, *Bombus terrestris*, were trained in an arena to localize a feeding site using a local cue (blue cardboard) and/or extramaze visual signals, in this case a panorama. The bees reliably chose the local cue in combination with the panorama location. When the local cue and the panorama location were dissociated by rotating the panorama by 90° they preferred the local cue, and they travelled preferentially from the local cue to the quadrant of the panorama location. Training the bees to a location defined only by its spatial relation to the panorama led to a choice preference for the respective quadrant within the first minute of active time, indicating that the panorama was sufficient for spatial guidance although it was not as salient a stimulus as the local cue. The bees steered towards the respective locations from any direction. We interpret our results as evidence for spatial learning with reference to both a local visual cue and a pattern of extramaze signals although the local cue was a more salient stimulus. This laboratory procedure for studying two basic forms of navigation should be useful for future attempts to unravel neural correlates of navigation in a central place foraging insect.

Navigation is an important faculty of highly integrated brain function in a large range of animal species (Shettleworth, 2010a). A comparative approach appears to be a promising strategy to identify corresponding neural processes in animals faced with similar tasks, for example navigating to an important location. Indeed, some fundamental navigational strategies, such as path integration (Collett & Collett, 2000; Mittelstaedt & Mittelstaedt, 1982), mapping to compass values (von Frisch & Lindauer, 1954; Kramer, 1952), panorama matching (Cartwright & Collett, 1982; Collett, 1992; Morris, 1984), vector integration (Cruse & Wehner, 2011; Wehner, 1984) and cognitive mapping (Tolman, 1948), have already been documented for many species including invertebrates (Wiener et al., 2011). Invertebrates solve these tasks with fewer neural resources than most vertebrate species, and so may be suitable models for uncovering the essential components of navigation (Collett & Collett, 2002). Navigation in social insects is particularly impressive since they need to return safely to their nests, forage at unreliable resources and possibly also communicate about locations in the environment (von Frisch, 1967; Seeley, 2011). Given their small brains and the complexity of their homing strategies, it is reasonable to expect that across-sensory integration, learning, motivational switching and appropriate memory retrieval are accessible to neural analysis (Menzel & Giurfa, 2001; Srinivasan, 2010; Wehner, 2003). Laboratory test conditions comparable to those used to study mammals, for example the Morris water maze (Morris, 1984), represent an essential step in this endeavour (Sovrano, Potrich, & Vallortigara, 2013). Although laboratory tests have major limitations in terms of confined space, the absence of compass cues and the lack of extended directional cues (Jacobs & Menzel, 2014), important discoveries have been made about the neural underpinning of navigation in rats, *Rattus norvegicus*, under such rather restricted conditions (Moser, Kropff, & Moser, 2008; O'Keefe & Nadel, 1978).

Flying, foraging Hymenoptera use multiple sensory systems to navigate between their nests and feeding sites. Vision plays an important role in localizing goals over short and long distances (M. Collett, Harland, & Collett, 2002; T. S. Collett & Rees, 1997; Dittmar, Sturzl, Jetzschke, Mertes, & Boeddeker, 2014; Menzel, 2013; Wystrach, Beugnon, & Cheng, 2012). Although these studies provide us with a large body of highly interesting data on behavioural strategies they do not allow us to combine behavioural studies with neural studies. Simplified test conditions that try to incorporate essential components of natural navigation are needed for this. Laboratory studies of navigation use variously designed mazes to determine which environmental stimuli are used and how they are integrated in order to enable goal-directed behaviour (Tolman, Ritchie, & Kalish, 1946). Laboratory methods have already been

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http://dx.doi.org/10.1016/j.anbehav.2014.08.013
0003-3472/

applied to walking insects, but bees have not yet been tested for visual navigation in small laboratory mazes despite the fact that they are known to perform visually guided decisions when walking in confined arrangements (e.g. in a T-maze: Menzel, 1981; Menzel & Greggers, 1985; in galleries: Bisetzki, 1957; Lindauer, 1963).

For the purpose of the present study we developed an arena environment (AE) for indoor visual spatial learning studies in walking bumblebees. The AE was composed of panorama patterns on the walls surrounding the arena and a visual local cue (blue cardboard) against a light grey background. The spatial relationship between the panorama and the local cue could be changed easily by rotating the panorama or displacing the local cue. Single walking bumblebees, *Bombus terrestris*, were trained to localize a feeding site with the help of either both the local cue and the panorama-defined location (henceforth: panorama location) or the panorama location alone. Our AE test system offers new possibilities for future studies aimed at elucidating the neural correlates of basic forms of navigation in insects.

METHODS

Apparatus

The AE had two components: the ground and the dome (Fig. 1a). The ground of the AE consisted of a transparent plastic base with a grey sheet of cardboard on top. The cardboard was divided into two adjacent sections: the training ground and testing ground which could be moved across the plastic base in order to exclude any odour cues produced by the bumblebees during the training session. A transparent vertical plastic frame (31.5 × 31.5 cm and 10 cm high) confined the range of the arena ground. The inner surface of the frame was sprayed with Teflon so that walking bumblebees

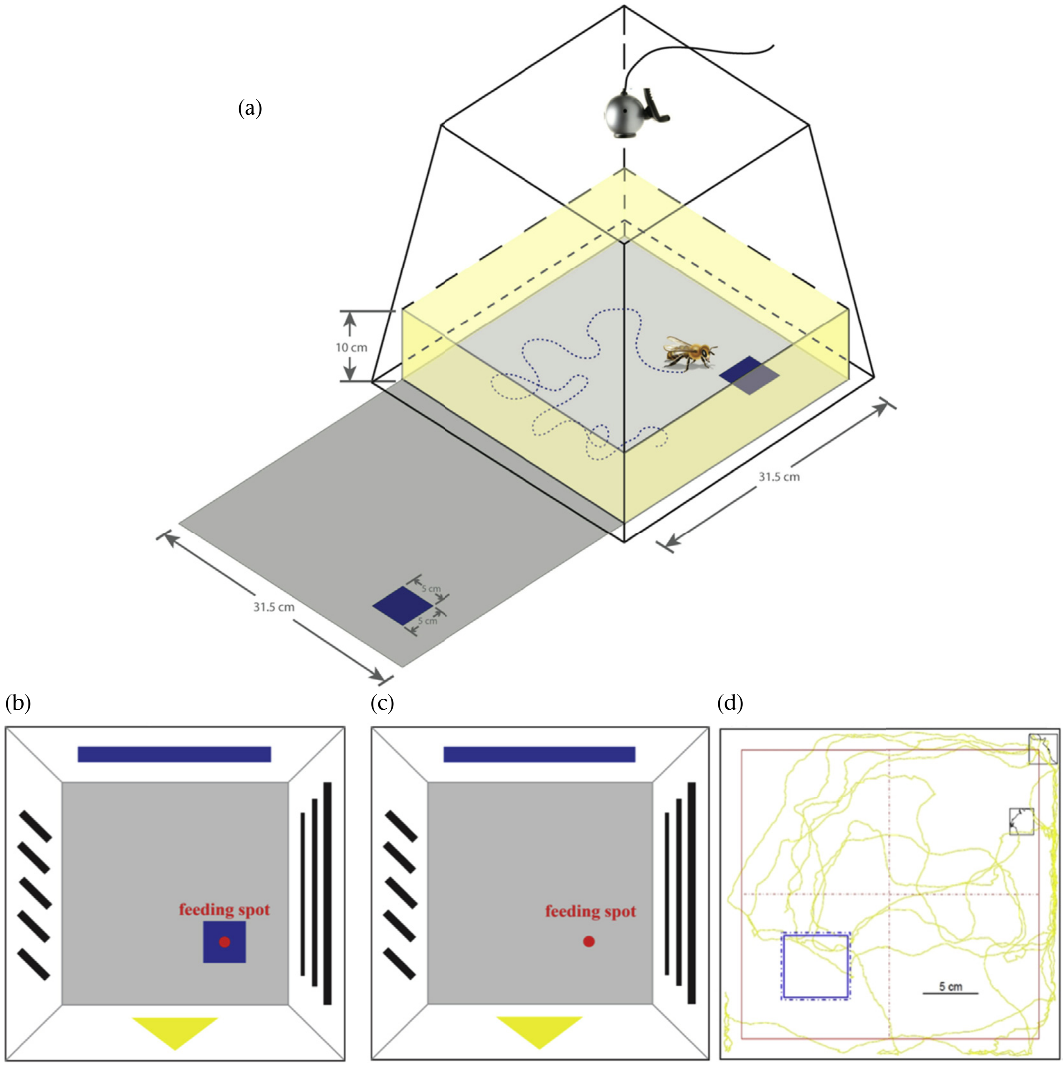

Figure 1. The arena environment (AE). (a) A sketch of the whole AE. The ground of the AE consisted of a plastic base with a sheet of grey cardboard on top that could be moved across the ground. A transparent plastic frame (31.5 × 31.5 cm and 10 cm high, marked by false colour of yellow) confined the range of the arena ground. A local cue (blue cardboard) marked the reward at its centre. A web camera placed beneath the plastic base allowed us to observe the bumblebee as it fed on sucrose solution (reward) from a feeding capillary. (b) Bird's eye view of the AE (local cue on the ground and panorama patterns on the walls) during training condition 1. A video camera mounted on the ceiling recorded the movements of the bee. The box could be rotated by 90° along with the panorama so that the local cue and the location relative to the panorama could be dissociated and the relative (within the AE) and absolute (in reference to the outer world) locations tested. The red point marks the feeding site only in the figure shown here. All bumblebees were trained to the same condition as shown here. (c) The local cue was absent in training condition 2 in which the bees learned to locate the feeding site only with respect to the panorama. (d) Representative trajectory during a test (video No. 2071, first 3 min) in which both the local cue and the panorama indicated the former feeding site (blue box) in the lower left quadrant. This figure also illustrates the different zones of the AE, the escape area (the area between the red line and the black border line), the nonescape area (rest of the ground), the four quadrants of the nonescape area (red dashed lines), resting locations (black boxes) and walking trajectory (yellow lines).

whose one wing was cut could not climb over the frame. In one series of experiments a piece of blue cardboard (5 × 5 cm) was positioned on the training ground as a local cue for the reward site; in another series of experiments no visual cue was presented during training. The distance between the local cue and either edge of its nearest corner was 5.9 cm. A window (1 × 1 cm) was cut into the centre of the local cue, allowing us to observe, via a miniature TV camera, how the bumblebee sucked the sucrose solution from a feeding capillary from underneath the arena. This capillary was connected to a plastic tube outside the arena. Sucrose solution was pressed through the capillary by a syringe (1 ml) controlled by a microdrive. During test conditions the local cue could be presented at any corner of the ground on the adjacent testing ground or it could be absent. The dome, which covered the ground of the AE, was a nontransparent box with an integrated video recording camera on the ceiling, panorama patterns on the inner walls and a symmetrically arranged illumination system. Either white or red LEDs ($\lambda_{max} = 630$ nm) illuminated the AE allowing us to observe and track the bees in the dark too. The visual angle of the flat ceiling of the dome as measured from the centre of the arena ground was 52°. The dome box could be horizontally rotated by 90° showing the panorama at these different angles of rotation. Different spatial relationships between the local cue and the panorama could be generated by presenting the local cue and the panorama at different absolute and relative positions. The direction and strength of the magnetic field were measured frequently. An electronic magnetic force meter and a compass were used to check the intensity and direction of magnetic force on the ground of the AE. No artificial magnetic field was found. The entire space within the dome was perfused with citral odour evenly distributed via a filter paper soaked with the odorant from the middle of the ceiling of the dome box. Citral was selected because it is known to stimulate bumblebees during foraging (Lunau, 1991; Shearer & Boch, 1966).

Animals

Bumblebee colonies were bought from the company Schneckenprofi (Hennstedt, Germany), and were kept in a greenhouse at a temperature of 21–27 °C. Sucrose solution (30%) and pollen powder were provided on a daily basis. Only bees foraging at the feeders were used. All the experimental bees had their right wings cut so that they could only walk on the ground within the range of the frame (Fig. 1a). Each one carried a white dot on its thorax to facilitate video tracking. Before the experiment started the bees were placed separately into wooden cages (10 × 8.5 cm and 6 cm high) for 1 h in the dark. The wooden cages and the AE were kept in a room illuminated with red light (dark for bumblebees) at a temperature of 25–28 °C.

Experimental Procedure

The experiment started when a bumblebee was gently released from the wooden cages into the AE. Each bee was exposed to four phases of different illumination conditions (Fig. 2). First, it was kept for 2 min in the dark (red illumination, dark phase I), then the arena was illuminated with both red and white light (bright phase I). During bright phase I the bee explored the arena and found the sucrose reward. Afterwards, dark phase II was presented before the bee was either further trained in bright phase II (intensive training) or tested. Video recordings (1280 × 1024, 15 fps) were made during all four phases. Only one bee was in the arena during each sequence of training or test phase. When the bee found the sucrose solution (50%) at the centre of the blue local cue it started sucking. We allowed for 2 s of sucking. The interruption of the supply of sucrose solution initiated local search behaviour. Then the sucrose solution was again available for 2 s of sucking. After three reward repetitions in bright phase I, the white light was turned off for 2 min (dark phase II). Afterwards, the white light was turned on again (bright phase II), and the bee was again rewarded. During this reward period the bee could imbibe sucrose solution ad libitum (intensive training). This last part of training ended 3 min after the end of sucking. Then white light illumination was switched off, the bee was put back into the cage, and its cage was placed back in the dark until the next training session on the following day.

Each bee was trained in this way on 4 consecutive days always at the same time of day. The location of the local cue containing the reward did not change during the training sessions. On the 5th day dark phase II and bright phase II were modified for a test. The feeding capillary was removed and the ground cardboard was carefully moved over to the test ground without moving the plastic frame. Depending on the specific demands on test conditions, the position of the local cue was changed and/or the upper part of the arena (panorama) was rotated by 90°. Thus the absolute and/or relative spatial relations between the local cue and the panorama were changed. In total, four different kinds of tests were done. First, we applied two cue-present tests: In test 1, the local cue was moved to the lower left quadrant and the panorama was rotated by 90° clockwise; therefore the feeding place was changed not in relation to the cue and the panorama but in the absolute sense relative to the outer surrounding. In test 2, the local cue was moved to the upper left quadrant and the panorama was rotated by 90° clockwise leading to a spatial dissociation between local cue and panorama. In cue-absent tests, depending on whether the bees were trained to the local cue or not, we applied another two tests: the bees were trained in the presence of the local cue but were tested in its absence (test 3); and the local cue was removed during both the training and the test sessions (test 4). In tests 3 and 4 the panorama was rotated by 90° clockwise from the training condition leading to the same condition in which only the panorama indicated the feeding site.

Definition of Behavioural Terms

Bumblebees showed a tendency to walk along the border of the AE (thigmotactic behaviour) or to stop walking for some time. To analyse their searching within the AE we excluded the thigmotactic

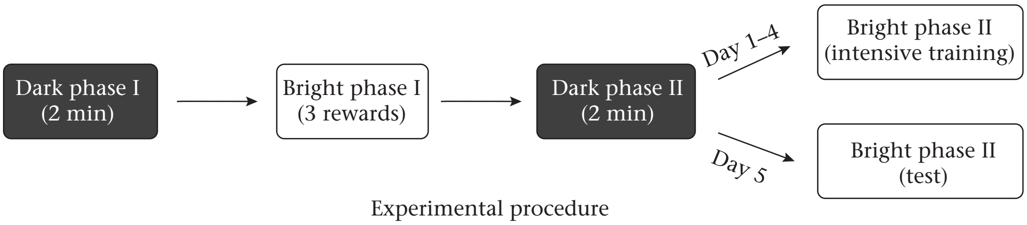

Figure 2. Flow chart of the experimental procedure. Video recordings could be carried out even in the dark phases because the AE was illuminated with red LED light. During the bright phases the AE was illuminated with white LED light. Training was performed during the first 4 days, and tests were carried out on day 5.

and resting behaviours (Fig. 1d). We use the term ‘escape’ for the thigmotactic behaviour and defined the ‘escape area’ by a distance of 2 cm (1/16th the width of the arena) along the frame of the AE. Resting behaviour (‘rest’ time) was defined by a period ≥6 s during which a bee did not walk out of any area of 2.5 × 2.5 cm (see Fig. 1d, ‘rest areas’ are marked by small black boxes). Thus ‘active time’ is the time bees spent walking outside the escape area and not resting (Appendix Fig. A1).

Graphical Display and Statistics

To display the cumulative active time distribution in the AE we calculated a normalized heat map. The nonescape area (Fig. 1d, the area in the red line box) was divided equally into 60 × 60 hexagons. In each hexagon area, a group of bees accumulatively spent a certain amount of active time. For normalization the total active time per area was divided by the number of bees and normalized such that only integer numbers resulted (each value was multiplied by 10). In the figures, this normalized value is depicted in false colours ranging from blue to yellow for each hexagon.

The independent samples *t* test was applied since the values were derived from independent samples. The values were extracted from the trajectory of each bee, for example active time in a particular area, number of crossings through a border line, direction from frame to frame (expressed in radians) and the walking distance. We evaluated only the first 2 × min of active time during the 15 min test period in all tests since all trained bees ($N = 36$) reached 2 min active time. Customized programs were written in R (R Core Team, 2014). SPSS Statistics 19 was used for these calculations.

RESULTS

Bumblebees Learn a Location in the AE

Naïve bumblebees had a tendency to inspect the local cue; they spent more active time in the local-cue quadrant than naïve bees that had not been provided with a local cue in the same quadrant (Fig. 3f). Bees trained to the local cue and the panorama (Fig. 3a) searched more in the quadrant marked by the local cue and the panorama (Fig. 3c, e, f; Supplementary videos 1, 2). The distributions of active times of 13 naïve and nine trained bees are shown as heat maps in Fig. 3d and e, respectively. For the statistical analysis we evaluated the first 3 min of active time and included only bees that reached at least 2 min of active time in each test session. The trained bees spent a significantly longer time in the quadrant with the local cue than naïve bees during the first 2 min of active time (Fig. 3f), indicating that the bees learned the feeding site marked by the local cue and the panorama. The trained bees did not visit the other three quadrants more frequently than naïve bees (data not shown).

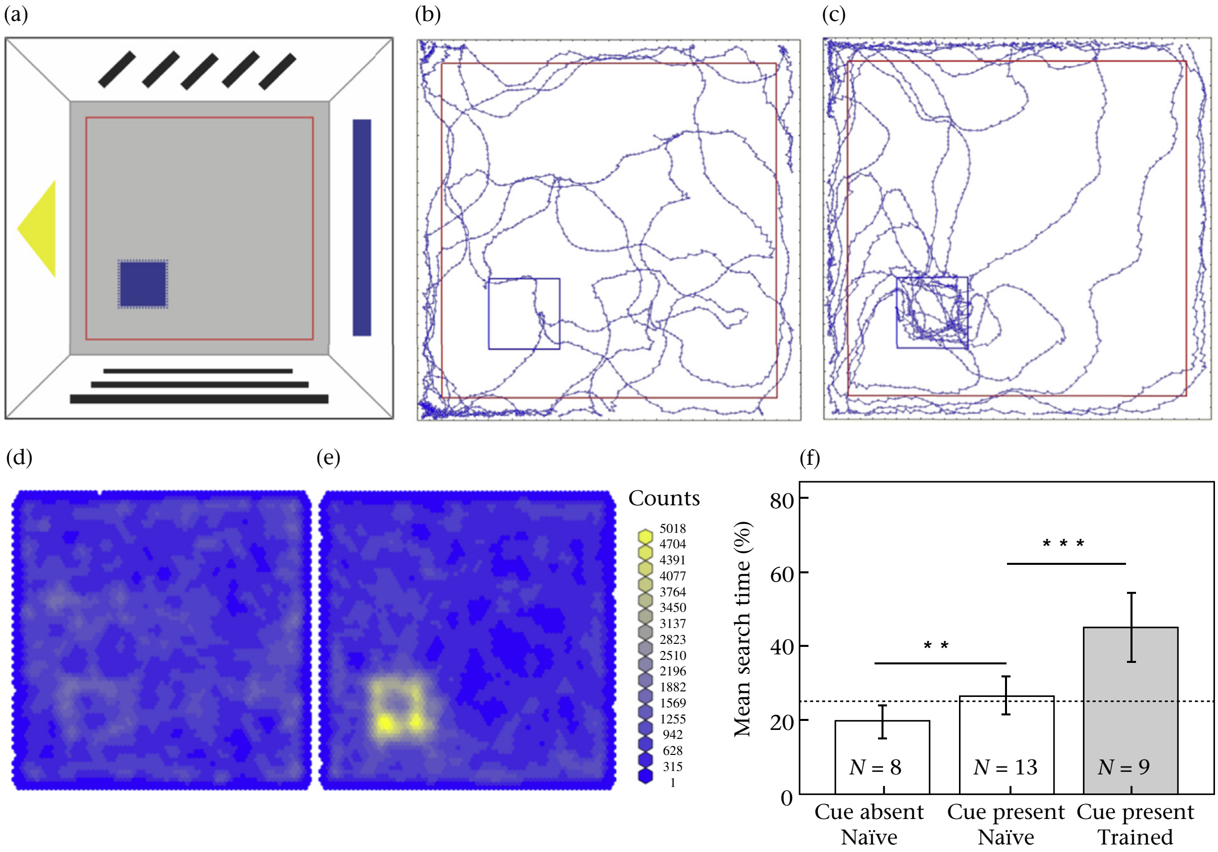

Figure 3. Learning of the local cue and the panorama. (a) The local cue was positioned in the lower left corner during testing. Its location was also defined by the spatial relations to the panorama. (b) Representative trajectory of a naïve bee with the arrangement shown in (a). (c) Representative trajectory of a bee trained to this arrangement during a test situation in which the local cue and the panorama were unchanged. Red boxes in (a), (b) and (c) mark the border of the escape area. (d) Heat map of 13 naïve bees for the same arrangement. (e) Heat map of nine trained bees in the corresponding test situation. (f) Cumulative active time spent in the quadrant with the local cue that was rewarded during the training session. Cue absent: test situation in which the local cue was absent; cue present: test situations in which both the local cue and the panorama locations were similar to the training situation (before and after training). Independent samples *t* tests: $^{***}P < 0.001$; $^{**}P < 0.01$. Error bars: SD. The dashed line shows the chance line of 25%. The sample size of each group is given in each column.

Dissociation Between Local Cue and Panorama Locations

We next asked whether the bees learned only the local cue or also the panorama location. Bees were trained in the same way as before, experiencing a fixed spatial relationship between the local cue and the panorama (Fig. 1b). This spatial relationship was changed during this test by dissociating the local cue and panorama locations (Fig. 4a). First we analysed the proportion of active time spent in the local-cue quadrant, and found that the trained bees spent significantly more time in the quadrant with the local cue (upper left quadrant) than naïve bees during first 2 active minutes (Fig. 4e, f) corroborating the findings reported above (Fig. 3). No significant difference was found between the active times of naïve and trained bees for the quadrant characterizing the panorama location (Fig. 4g). Thus either the bees did not learn the panorama location or the high salience of the learned local cue overshadowed the choice of the panorama location.

We noticed that bees shuttled back and forth between the local cue and the panorama location during dissociation tests. Therefore, we analysed next the directional components of their walking trajectories under these training and test conditions. The test arrangement was the same as shown in Fig. 4 with a dissociation of 90° (Fig. 5). All the bees were analysed that set out from the local cue at least twice within the 2 min active time. We found that trained bees steered from the local cue to the panorama-related quadrant significantly more frequently than naïve bumblebees (Supplementary videos 3, 4). Frequently, bees walked multiple times between these two locations (Supplementary video 4).

As a control we analysed the directional component of trajectories departing from the local cue when both the local cue and the panorama indicated the same place but both were rotated by 90° clockwise compared with the training situation (see Fig. 3a). The local cue and the panorama location were placed in the upper left quadrant similar to the condition in Fig. 5a for the local cue. No preference was found for trajectories departing from the left upper quadrant to the lower left quadrant (Fig. 5c). This result supports our conclusion that the behaviour of trained bees in the dissociation test (Fig. 5b) indicates visual orientation to the panorama. Thus, after dissociation between the local cue and panorama locations, trained bees recognized the panorama and located the site of the reward relative to the panorama. This effect was rather small and not seen in the spatial distribution of active time (Fig. 4) indicating that the highly salient local cue overshadowed the searching behaviour in the AE.

Bumblebees Learn the Feeding Location in Relation to the Panorama

Next we addressed the question whether the location indicated only by the panorama can be learned. To answer this question, we applied two sets of training and test conditions: (1) bumblebees trained to the local cue and the panorama, and tested without the local cue; (2) bumblebees trained and tested without a local cue.

Bumblebees trained with a local cue

Fig. 6a shows the test condition after the bees were trained with a local cue, Fig. 6b shows the trajectory of a naïve bee and Fig. 6c that of a trained bee. Trained bees spent significantly more active time in the panorama-defined quadrant than naïve bees (Fig. 6d, e, f). This result indicates that after training with the local cue, bees learnt and recognized the feeding place in reference to the panorama only. Note that the trained quadrant was chosen less than 25%

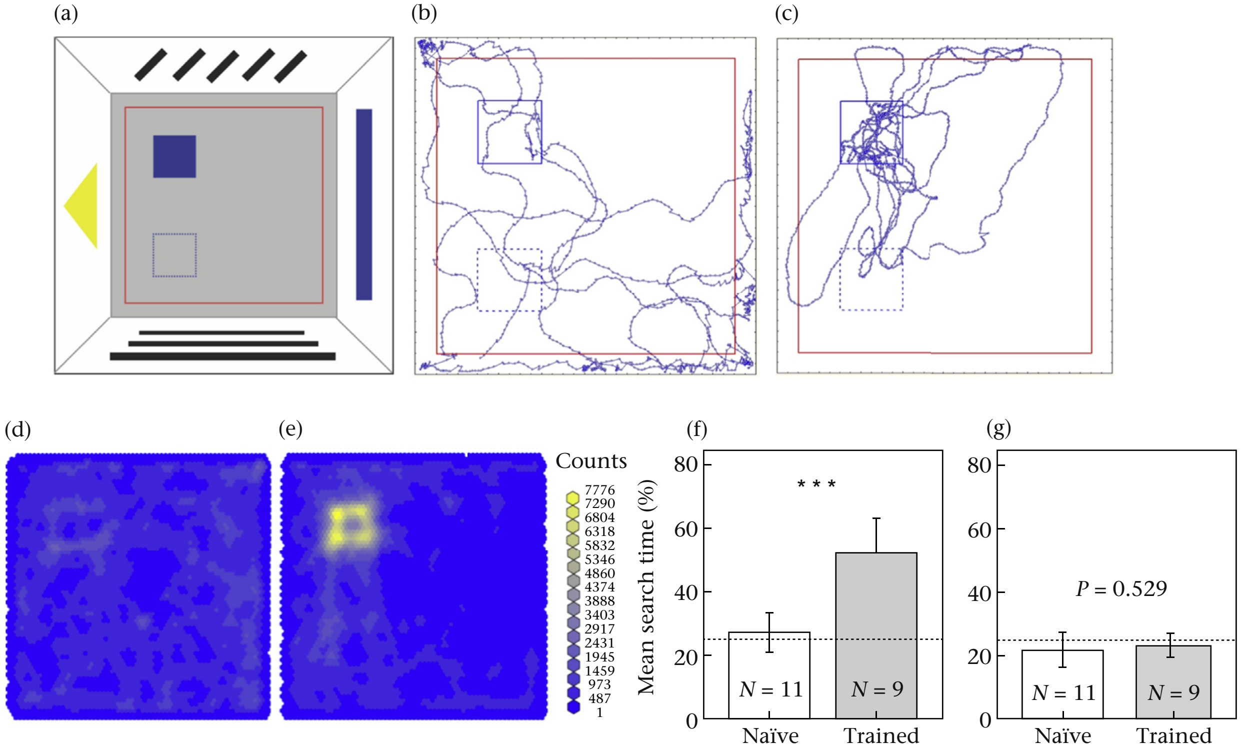

Figure 4. Dissociation of the local cue and the panorama location. (a) During the test the local cue was moved to the upper left corner and the panorama location stayed the same as during training. (b) Representative trajectory of a naïve bee with the arrangement shown in (a). (c) Representative trajectory of a bee trained to the same arrangement. Red boxes in (a), (b) and (c) mark the border of the escape area. (d) Heat map of 11 naïve bees. The local cue was presented in the upper left corner. (e) Heat map of nine trained bees. The local cue was presented in the upper left corner and the panorama location at the trained place (lower left corner). (f) Cumulative active time spent in the quadrant with the local cue. (g) Cumulative active time spent in the quadrant of the panorama indicated location. Independent samples *t* tests: $^{***}P < 0.001$. Error bars: SD. The horizontal dashed lines in (f) and (g) show the chance level of 25%. The sample size of each group is given in each column.

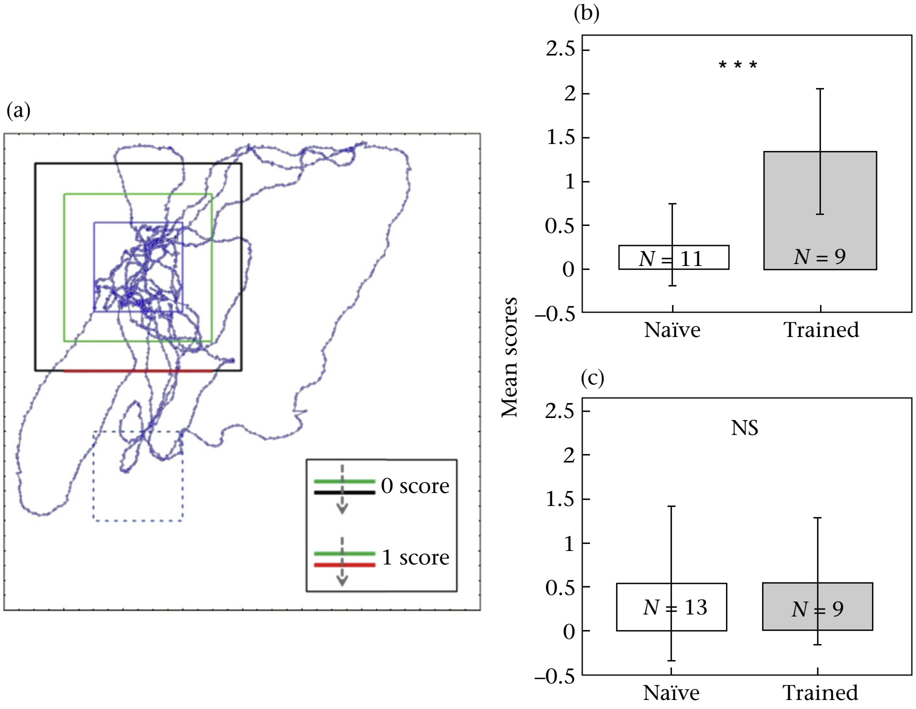

Figure 5. Directional components of outbound trajectories from the local cue during the dissociation test. (a) Local cue and panorama locations are dissociated by 90°. The lines around the local cue (blue square) define an inner box (green, length of sides 4 cm) and two outer boxes (black and red lines, length of sides: 13.8 cm; the length proportion of red:black is 5:23). Trajectories crossing the green lines and then the black or red lines were counted as departing from the local cue. Those crossing the red line were given a score of 1 since they indicate a departure of the local cue area towards the panorama location (dotted lines of a square in the lower left quadrant). Trajectories crossing the black lines scored 0. Only the first and second departures of each bee were evaluated. Thus the score of a single bee could be 0, 1 or 2. (b) Mean departure scores for the dissociation of 90°. (c) Mean scores of outbound trajectories of naïve and trained bees under test conditions in which both the local cue and panorama location coincided in the upper left quadrant. Independent samples *t* tests: $^{***}P < 0.001$. . Error bars: SD. The sample size of each group is given in each column.

by naïve bees indicating that the panorama induced some preference for quadrants other than the trained one.

Bumblebees trained without a local cue

Then we asked whether the location indicated by the panorama only can be learned if the local cue was never shown to the bumblebees during training. To this end the bees were trained and tested without the local cue (Fig. 7a). Since the control test was the same as for bees trained with a local cue, we used the same group of naïve bumblebees as the control group here. Fig 7b shows the trajectory of a naïve bee and Fig. 7c that of a trained bee. Trained bees did not spend significantly more active time in the panorama-defined quadrant than naïve bees although there was a tendency towards more active time in the panorama location (Fig. 7d, e, f). However, when we analysed only the first minute of active time, a significant difference between naïve and trained groups was found (independent samples *t* test: $P < 0.05$). The training effect of the panorama only is obviously less stable leading to an extinction effect from the first to the second minute of active time. Taken together, the results shown in Figs. 6 and 7 document that bumblebees learned the location relative to the panorama alone irrespective of whether there was a local cue present during the learning process or not.

Search Strategies of Naïve and Trained Bumblebees

Since the location of the feeding site as signalled by both the local cue and the panorama is learned, bees may apply different search strategies when aiming towards the local cue or the panorama location. The local cue might be seen by the bee from a certain distance from all directions guiding the bee directly towards it. The average height above ground of the eyes of a medium-sized bumblebee is 3.5–4.5 mm (N. Jin, T. Landgraf, S. Klein, & R. Menzel, personal observation) and, since the visual angle of an ommatidium in its compound eye is close to 2°, one can estimate that the bee sees the 5 × 5 cm large local cue at a distance of 5–6 cm. This means that the bee can see the local cue at any position within the quadrant with the local cue. We analysed the trajectories of both naïve and trained bees but did not detect any obvious turning towards the local cue when a bee entered this quadrant. Recognition of the panorama location may require a matching strategy allowing the bee to aim into the appropriate location by multiple sequential steps of body alignment. Such search runs may become straighter when getting closer to a better match. Furthermore, bees might have learned the appearance of the panorama from a vantage point leading to a kind of snapshot memory acquired at a particular area. Such a possibility is favoured by the fact that two sides of the local cue faced the open space whereas the other two were close to the frame. In this case one might expect patterns of trajectories and body alignments that may indicate systematic searches for least mismatch between the actual and the remembered snapshot. We analysed all the trajectories of our experiments according to these hypotheses, quantifying the locations of turns in relative and absolute relation to either the local cue or the panorama location, but did not find any systematic pattern. This negative result may depend on the rather limited dimensions of our arena and the relatively large size of the local cue.

Analysing the cumulative turning angles as a measure of the straightness of walking, we found that bumblebees trained with the local cue walked significantly straighter and faster than naïve bees (Fig. 8a, e), whereas bees trained without the local cue (Fig. 8d,

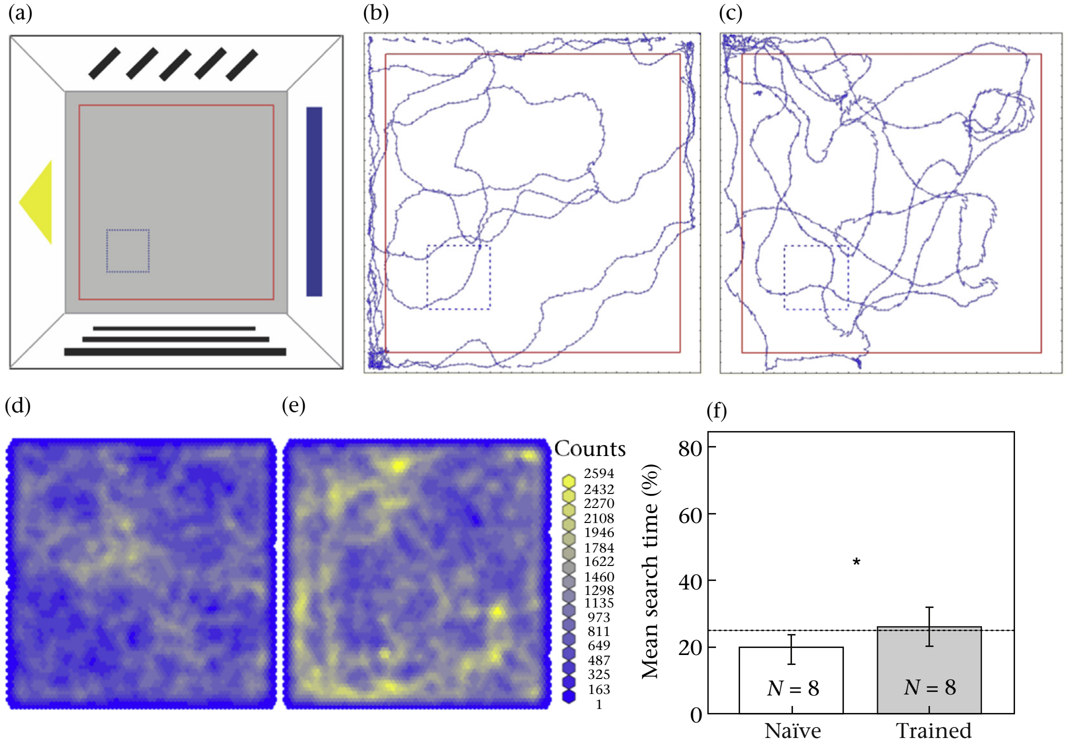

Figure 6. Learning of the panorama location together with the local cue and tested without the local cue. (a) Bird's eye view of the AE in test conditions without the local cue. (b) Representative trajectory of a naïve bee with this arrangement. (c) Representative trajectory of a trained bee. (d) Heat map of active time spent by eight naïve bees in the AE without the local cue. (e) Heat map of active time spent by eight trained bees. The learned panorama location was in the lower left quadrant. In (a), (b) and (c) the red boxes mark the border of the escape area; the blue dashed line box marks the feeding location. (f) Comparison of active time spent by naïve and trained bees in the quadrant of the panorama location during the 3 min of active time. Independent samples *t* tests: $^*P < 0.05$. Error bars: SD. The horizontal dashed line shows the chance line of 25%. The sample size of each group is given in each column.

h) walked significantly less straight and more slowly than naïve bees. Bumblebees trained with the collaborative local cue and panorama but tested with 90° dissociated locations of local cue and panorama location showed no significant difference in their turning angles and walking distances (Fig. 8b, c, f, g). The respective values in the latter test condition lie between those in the two former test conditions. Thus trained bees changed their search behaviour differently depending on the signals they used for localizing the feeding place in the arena indicating that both training and test patterns influenced their navigating strategies.

DISCUSSION

Central place foragers such as bees and ants localize the nest and feeding sites with the help of multiple cues allowing them to steer towards the respective goal along straight flight paths or idiosyncratic walks (Collett et al., 2002; Collett & Zeil, 1988; Kohler & Wehner, 2005; Wehner, 2003). Local cues and further distant landmarks are learned during exploratory behaviour (Capaldi et al., 2000; Zeil, 2012) or during training to a feeding site (Philippides, Baddeley, Cheng, & Graham, 2011; Wystrach, Schwarz, Schultheiss, Beugnon, & Cheng, 2011). How the spatial memory of bees and ants is organized is still an open question (Collett, 2005; Collett & Graham, 2004; Jacobs & Menzel, 2014; Wiener et al., 2011) partially because no direct access to the neural substrates exists so far. We prepared for such an attempt by setting up a laboratory test procedure that aimed to capture at least some components of the natural navigation task. In particular we established test conditions that made it possible to test learning of local cues and extramaze (panorama) cues. Such test conditions have been very helpful in elucidating essential components of the neural substrates of navigation in laboratory mammals (e.g. Morris, 1984; O'Keefe & Nadel, 1978). Local cues and extramaze cues are coded differently in the mammalian brain leading to navigation performances that require the hippocampus in reference to extramaze cues and no hippocampus dependence for local cue reference (Morris, Garrud, Rawlins, & O'Keefe, 1982).

Flying honeybees, *Apis mellifera*, localize a feeding place by referring to both local cues and extramaze cues even if they fly into or within a small box (Dittmar, Egelhaaf, Sturzl, & Boeddeker, 2011; Dittmar et al., 2014; Sovrano et al., 2013), a Y-maze (Giurfa, Zhang, Jenett, Menzel, & Srinivasan, 2001) or a T-maze (Menzel, 2009). Walking honeybees, too, were found to navigate to extramaze cues including the sun azimuth (Bisetzki, 1957). We switched to walking bumblebees because preliminary experiments with honeybees indicated that restraining them from flight by cutting or gluing one or both wings induced rather strong escape behaviour. Bumblebees, too, spent some time trying to escape from the arena, but explored the open space of the arena actively and learned a local cue as a rewarding place well. As we have shown here, they learned the location of a feeding place in reference to both a local visual cue (blue cardboard) and extramaze cues (the panorama). Their search motivation and choice behaviour were facilitated by a weak and evenly distributed background of floral odour inside the test box corroborating observations with flying bumblebees in a greenhouse (Lunau, 1991). Not surprisingly, their search behaviour for a feeding place and learning were less consistent than those of flying bumblebees that return to the colony and to the test station after delivering their sample in the colony. Both choice motivation and resistance to extinction were, therefore, reduced under our test

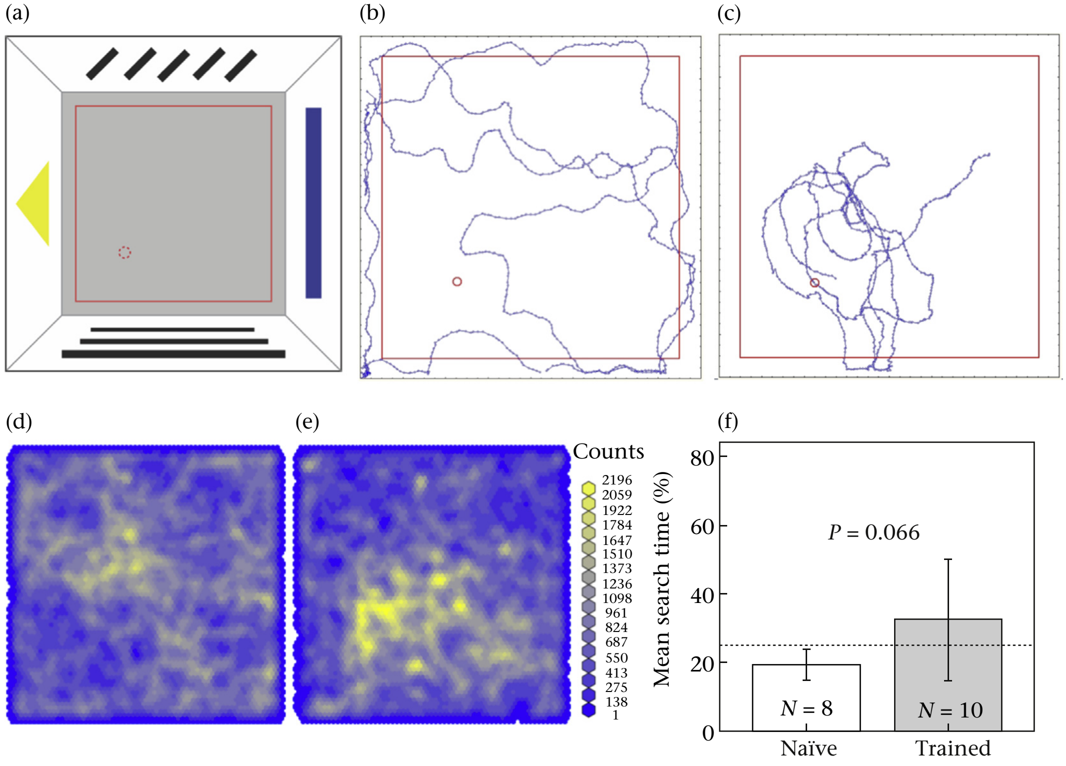

Figure 7. Learning of the panorama location without a local cue. (a) Bird's eye view of the AE under the training and test conditions without a local cue. (b) Representative trajectory of a naïve bee with this arrangement. (c) Representative trajectory of a bee trained to this arrangement during a corresponding test situation. In (a), (b) and (c) the red boxes mark the border of the escape area. The red open dot marks the location of the opening of the feeding tube which can be seen by the bee only when it is right above it. (d) Heat map of active time spent by eight naïve bees in the AE without a local cue. (e) Heat map of active time spent by 10 trained bees. The learned panorama location is in the lower left quadrant. (f) Comparison of active time spent by naïve and trained bees in the quadrant of the panorama location during the first 2 min of active time. Independent samples *t* tests. Error bars: SD. The horizontal dashed line shows the chance line of 25%. The sample size of each group is given in each column.

conditions. We evaluated only the first 2 min of active time from each 15 min test session, and exposed each bee to only one training or test session per day. Furthermore, the bees in our test conditions could not use a route memory as was found to be important for resolving ambiguities between cue-related and panorama-related localizations (Dittmar et al., 2014).

Naïve bumblebees distributed their walking activities unevenly across the four quadrants of the arena, a phenomenon particularly obvious when no local cue was present (Fig. 7). This result indicates less attraction of the quadrant that housed the feeding place during training, most likely because of an effect of the panorama. In dissociation tests, the preference of trained bumblebees for the learned local cue was found to be stronger than that for the panorama location resisting demotivation caused by the unrewarded visits and extinction learning for longer test periods. This finding indicates that the local cue is a more salient stimulus than the features of the panorama defining the food location. It will be interesting to manipulate the patterns of the panorama, add depth to panorama features and embed these in compass cues, for example the sun compass. Since the local cue in our experiments could only be seen within the respective quadrant we expected an obvious turn towards the cue when the bee reached the visual catchment area, as observed in wood ants (Durier, Graham, & Collett, 2004) and in freely flying bees, for example when they are trained to a colour target in a Y-maze (Giurfa et al., 2001). This was not the case in our experiments, possibly indicating that the bee may be well informed about the spatial relations between its current position and the intended goal either by a working memory that stores recent segments of the walking trajectory (path integration mechanism) or by connecting local cue and panorama information, or both. This finding may indicate that both forms of memory are intimately connected.

Learning of the panorama location was demonstrated in our experiments by two findings: (1) bees preferred outbound directions from the local cue to the panorama location in a dissociation test, and (2) bees chose the panorama location when no local cue was available. The choice of the panorama location was not initiated by an obvious body turn towards that location as observed in wood ants (Lent, Graham, & Collett, 2010). It is thus unlikely that image matching of the panorama pattern is connected with retinal alignment. However, the database for this conclusion is rather limited because of the small scale of the arena. The possibility that the bees learned the panorama pattern from a particular vantage point and used the learned pattern for image matching is contradicted by the behaviour of the bees in the dissociation test. In these experiments bees were trained to the compound of local cue and panorama location, and exposed to test conditions in which the two locations were separated by 90°. The bee walked from the local cue to the panorama place (Fig. 5) following a sequence of views not experienced during training. During training the bees reached the visual catchment area of the local cue from the open space, thus from a 180° segment which they did not experience after the local cue and panorama locations were rotated by 90°. It is thus unlikely that either the local cue or the panorama pattern was learned at defined vantage points. Further evidence comes from the walking characteristics during tests (Fig. 8). Neither the straightness of the search walks nor their interruptions indicate possible locations where snapshot memories may have been established. These findings favour the interpretation of Wystrach, Mangan, Philippides, and Graham (2013) who argued that the information

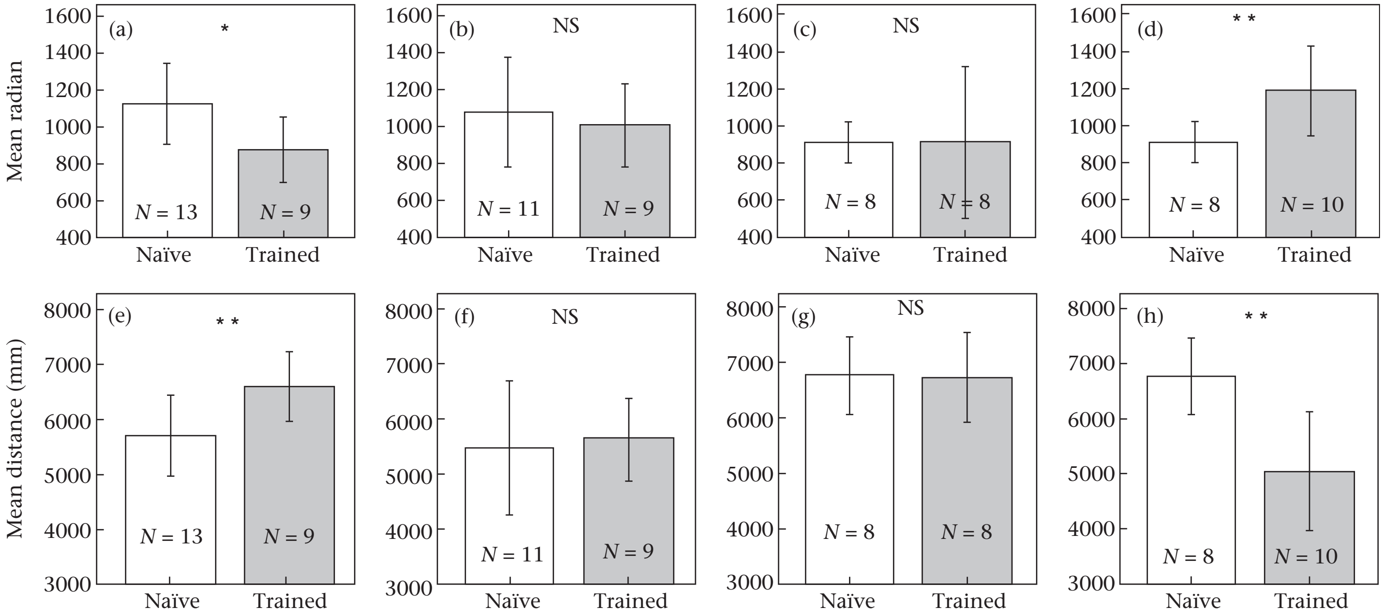

Figure 8. Comparison of walking characteristics between trained and naïve bumblebees in four test conditions: (a, e) trained and tested with both cues; (b, f) trained and tested with both cues but with the two cues dissociated during the test; (c, g) trained with both cues and tested without the local cue; (d, h) trained and tested without the local cue. (a, b, c, d) Straightness of search walks as measured by cumulative turning angles in radians. (e, f, g, h) Search activity as measured by the total walking distance in 2 min active time. Independent samples *t* tests: $^{**}P < 0.01$; $^{*}P < 0.05$. Error bars: SD. The sample size of each group is given in each column.

about panorama cues is collected from many experienced views and stored in one kind of spatial panorama memory.

Localization by extramaze cues was also demonstrated for *Drosophila* (Foucaud, Burns, & Mery, 2010; Ofstad, Zuker, & Reiser, 2011) and the cockroach, *Blatella germanica* (Durier & Rivault, 1999; Matsumoto & Mizunami, 2004; Mizunami, Weibrecht, & Strausfeld, 1998; Moore, ReaganWallin, Haynes, & Moore, 1997). Targeted genetic silencing of small subsets of cells in the ellipsoid body but not of the mushroom body of the *Drosophila* brain indicated a loss of spatial memory (Ofstad et al., 2011). The role of the ellipsoid body in *Drosophila* navigation is supported by the finding that ring neurons of this structure play a causal role in spatial working memory (Neuser, Triphan, Mronz, Poeck, & Strauss, 2008). In contrast, bilateral lesions in the area of the mushroom body of the cockroach compromised spatial behaviour. So, which central structures of the insect brain might be involved in spatial memory and whether different processes of navigation might be controlled by different central brain structures is still unclear. While complex (but not simple) visual tasks require a functional mushroom body in *Drosophila* (Liu, Wolf, Ernst, & Heisenberg, 1999; Ren, Li, Wu, Ren, & Guo, 2012; Tang & Guo, 2001), the ellipsoid body, as part of the central complex, is thought to be concerned with high-order motor control (Kuntz, Poeck, Sokolowski, & Strauss, 2012; Strauss, Hanesch, Kinkelin, Wolf, & Heisenberg, 1992). Thus it is possible that high-order sensory and motor performances are processed in the mushroom body and the ellipsoid body, respectively. In honeybees the mushroom body integrates across all sensory modalities, receives input from the reward system and stores visual and olfactory memories (Menzel, 2012). It is therefore likely that sensory integration across sensory modalities as in the case of panorama-related navigation may involve the mushroom body. It is conceivable that more direct sensory-motor links as required for learning and recognizing the local cue may not depend on the mushroom body. So far a separation of neural structures essential for local cue and panorama localization has not been addressed in insects and will be the goal for further studies applying the training and test procedures developed here for the bumblebee.

Conclusion

Two basic strategies of visual navigation, guidance by a local cue and by the pattern of the panorama, were studied in walking bumblebees using this laboratory set-up. The choice of the combined cues (local and panorama related) was highly reliable. Although the dominance of the local cue overshadowed the preference of the panorama location in a dissociation test, learning of the panorama location could still be proven. First, bees preferred to walk from the local cue to the panorama location after having been trained to both local cue and panorama when the two cues were dissociated. Second, the panorama location was preferred if no local cue was available during training although the effect was much weaker than the choice of the local cue.

Acknowledgments

Dr. Julien Colomb gave us important suggestions when programming the routines for analysing our data. Ruth Bartels helped in constructing the experimental set-up and improving the experimental methods. We are grateful to Anne Carney for correcting the English. N.J. was supported by a stipend of the China Scholarship Council.

Supplementary Material

Supplementary material associated with this article is available, in the online version, at http://dx.doi.org/10.1016/j.anbehav.2014.08.013.

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APPENDIX

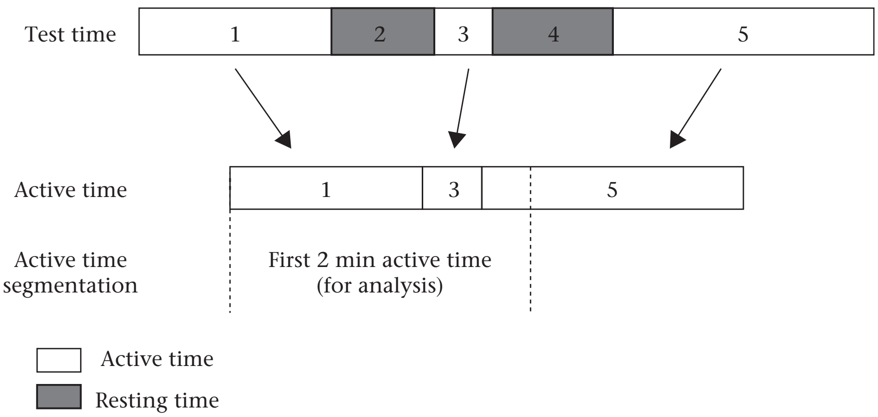

Figure A1. Definition of active time. The test time includes the duration of a bee being active or resting. To analyse the searching process, we omitted the resting times. The analysis of active bees is based on the first 2 min active time (dashed lines).