

Development of a behavioural assay to examine magnetosensitive behaviour in bumblebees

Viktor Nilsson
April 2013

Master thesis
Supervisor: Rachel Muheim
Department of Biology
Lund University



Development of a behavioural assay to examine magnetosensitive behaviour in bumblebees

Viktor Nilsson
April, 2013
Master thesis

Supervisor: Rachel Muheim
Department of Biology
Lund University

*"Vision is the art of seeing the invisible."
Jonathan Swift*

Summary: The magnetic field surrounding the Earth has repeatedly been shown to be used by various animals for navigation purposes. There is not yet any published data on the existence of a magnetic sense in a bumblebee. I here present the development of a method suitable for examining the existence of a magnetic sense in the buff-tailed bumblebee (*Bombus terrestris*) by using a four-armed maze located in a magnetic coil system. I also present some preliminary results that lend support to the hypothesis that bumblebees can indeed sense magnetic fields and orientate according to them.

Keywords: bumblebee, *Bombus terrestris*, magnetoreception, orientation, magnetic coil.

Introduction

The Earth is surrounded by a magnetic field with its poles relatively close to the geographic poles. Animals from several classes have shown behavioural capabilities to sense and make use of this magnetic field for navigation purposes. Birds are the most thoroughly studied organisms; Keeton (1971) showed magnetic orientation in pigeons and Wiltschko and Wiltschko (1972) demonstrated a magnetic compass in the European robin (*Erithacus rubecula*). Salamanders (Phillips, 1986) from the group of amphibians, mammals such as mice (Muheim et al., 2006) and bats (Holland et al., 2006), and several insects such as honeybees (*Apis mellifera*) (Walker and Bitterman, 1989b) and fruitflies (*Drosophila melanogaster*) (Phillips and Sayeed, 1993), are some other animals that have been shown to be capable of magnetoreception. The only published indications of a possible magnetic sense in

bumblebees come from a study by Chittka et al. (1999), who suggested that the ability of bumblebee workers (*Bombus impatiens*) to navigate in total darkness could depend on a magnetic compass.

The animal most closely related to the bumblebees that has been examined in view of a magnetic sense is the European honeybee. Honeybees dancing on horizontal wax comb orientate along the cardinal magnetic directions; on a normal vertical comb they orientate relative to gravity (Lindauer and Martin, 1977). Also, when honeybees dance on a vertical comb, they make small consistent errors in direction relative to gravity, known as *Mißweisung*, that disappear when the ambient magnetic field is cancelled (Martin and Lindauer, 1977).

There is also published data indicating that honeybee swarms build newly established wax combs relative to the ambient magnetic field, continuing building in the same direction as in the mother colony (De Jong, 1982), a phenomenon that might be referred to as magnetic alignment, which is the spontaneous alignment of an animal along the surrounding magnetic field. Honeybees can also learn to associate local magnetic anomalies with a reward (Walker and Bitterman, 1985), and their threshold for detection is around 260 nT, but as low as 26 nT for some individuals (Walker and Bitterman, 1989b). The intensity of the geomagnetic field at the surface of the Earth varies from 25 000 to 65 000 nT, depending on location (Intermagnet, 2013). Thus, the threshold for the honeybee's ability to detect differences in field strength typically lies at less than 1% of the ambient field.

Bumblebees belong to the order Hymenoptera, to which also ants belong. Ants of the species *Formica pratensis* were found to be able to use the directional information of an artificial magnetic field to find their way to a reward in a four-armed maze (Çamlitepe et al., 2005). Riveros and Srygley (2008) showed, by displacing leaf-cutter ants (*Atta colombica*) and then changing the magnetic field, that they use a magnetic compass to orientate. For a further introduction to magnetoreception in eusocial insects, see Wajnberg et al. (2010).

The exact mechanism for magnetoreception in animals has not yet been proven, but there are two main hypotheses: (1) A compass based on tiny particles of magnetite (Fe_3O_4) in the body; the movements of which are sensed by nerve endings and relayed to the nervous system (Kirschvink et al., 2001), or (2) a compass based on interactions between the magnetic field and radical pairs formed when light hits light-sensitive cryptochromes (*Cry*) in the eye or other light-sensing parts of the body (Ritz et al., 2002). There is published data to support both hypotheses, thus it is likely that they both exist. In *Drosophila*, it has been noted that knock-out of the *Cry* gene causes loss of magnetoreceptive behaviour, which is reinstated when the gene is

rescued (Gegeer et al., 2010). There are also numerous examples of magnetic orientation dependent on the wavelength of light, as reviewed in Phillips et al. (2010). On the other hand, there is also some evidence for a magnetite-based compass, such as the reversal of the magnetic orientation in honeybees after a re-magnetizing pulse (Kirschvink and Kirschvink, 1991). Evidence for the magnetite hypothesis is reviewed in Kirschvink et al. (2001).

It might at first not seem very important for a non-migrating insect such as a bumblebee to use a magnetic sense, and it probably doesn't play an as important role as vision or olfaction, but given the difficult task of finding the way back to a small nest from a distance of several kilometers, any help that a magnetic compass could provide should be valuable, especially if the cost of magnetoreception is not very high in terms of weight and energy expenditure.

In my project I examined whether buff-tailed bumblebees (*Bombus terrestris* L. 1758, sv. *mörk jordhumla*; Fig. 1A) have a magnetic sense. I first tried to establish a suitable method for studying magnetosensitive behaviour. I investigated whether bumblebees can be trained to learn to orientate relative to a magnetic direction to find a reward by letting a magnetic field point towards the reward.

Methods

Colonies of bumblebees (*B. terrestris*) were purchased from Koppert B.V. (Berkel en Rodenrojs, The Netherlands) through their Swedish distributor. Each colony consisted of a nest with about 150–200 worker bees in a ventilated plastic box. Three different colonies were used for the experiments, with no apparent differences in bumblebee behaviour between the colonies. A feeder with sugar solution attached to the nest box provided food for the colony. It was left in place during experiment 1, but was removed from experiments 2-5.

Magnetic fields were produced by a system of electromagnetic copper coils arranged in a cube system according to Merritt et al. (1983). There were 4 coils in each direction, one direction being north-south, one east-west, and one vertical. The size of the cube was $2 \times 2 \times 2$ meters. The four coils of each direction were connected in series, and produced a magnetic field by being fed a 12 volt direct current from a car battery, regulated with an electronic box to make calibration of the field strength possible.

By switching on electrical currents to the different coils, natural strength magnetic fields were created, pointing in the direction of choice, i.e., the

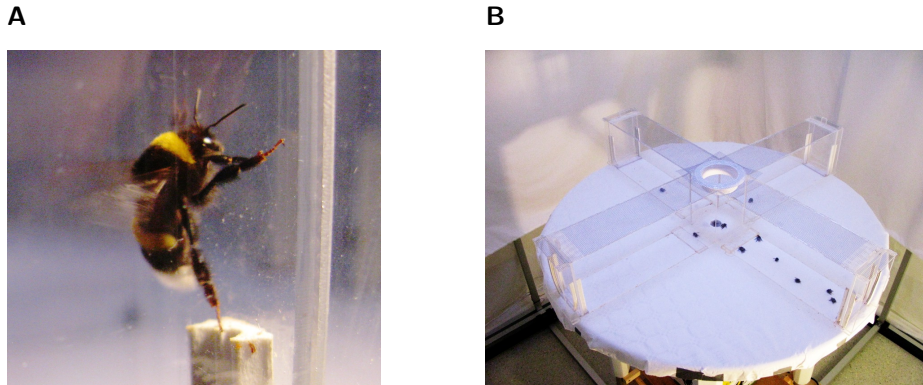


Figure 1: (A) Buff-tailed bumblebee (*Bombus terrestris*). (B) The orientation maze, located inside the magnetic coil system.

magnetic north pole was pointing either towards geographic north, east, south or west. The north-south coils were wound with double wire to enable neutralisation of the natural surrounding north-south field using one of the wires, while still allowing the other one to be used for creating an artificial north or south field. This also has the advantage that always at least two coils were switched on during the experiments, reducing the risk that the animals could be comparing artificial versus natural fields, or using artifacts such as heat or vibration as orientation cues. The vertical coils were used to manipulate the vertical component of the ambient field. In the present experiments, the vertical component was amplified by approximately 1 200 nT, since the natural magnetic field in the laboratory building was somewhat weaker than the outdoor surrounding magnetic field. Magnetic field strength and direction were measured several times and calibrated using a flux-gate magnetometer (Model 539; Applied Physics Systems, Sunnyvale, CA, USA). For everyday use, a common compass (Silva, Johnson Outdoors Inc., St. Racine, USA) was used to ensure that the coil system was working. Outside of testing hours, the colonies were kept in a vertical magnetic field with no horizontal component.

The magnetic coil system was draped in a fabric with interwoven thin silver-plated copperwires (Swiss Shield NATURELL™ Spoerry 1866 AG, Flums, CH), creating a Faraday cage that shielded electromagnetic radiation in the radio frequencies (RF) with an attenuation of ≈ 47 dB at 1.415 MHz, which is the most important frequency with respect to disturbance of magnetoreception (Henbest et al., 2004). RF fields have earlier been shown to disrupt magnetoreception in birds (Ritz et al., 2004) and cockroaches (Vácha et al., 2009). Measurements of RF fields were taken inside the cage using a digital spectrum analyzer (N9340B; Agilent Technologies, Santa Clara, CA, U.S.A.), primarily measuring frequencies from 1 to 10 MHz. In this range, all RF fields were below measurement threshold (<10 nT). The only detectable

RF fields were measured when the probe was in direct proximity to a cable used in experiments 4 and 5 to power LED lights under the table, fed by a switched-mode power supply (see below). These fields were in the range of a few nanovolts, but there were no measurable fields in the flight cage itself or in the test arena.

Tests and training were performed in a 4-arm maze placed centrally in the magnetic coil. The maze was placed on a circular plastic table with a hole in the middle acting as an entrance from below. The walls of the maze were made from transparent acrylic plastic, and the maze was covered in dark plastic netting. The length of each arm was 37 cm, the height 15.5 cm and the width 9.5 cm. At the end of each arm a plastic box could be inserted by lifting a sliding door; the box covered the full width of the arm and 4.5 cm in height. Each box had a centrally placed entrance (\varnothing 2.5 cm) facing the arm, where bumblebees could enter and find pollen, sugar, salt, or nothing, depending on the particular experimental situation. These boxes could be taken out and moved around. Different boxes were used for training and testing, so that boxes used in tests had never contained pollen, sugar or salt. The inner walls and ceiling of the magnetic coil were covered in white cotton fabric to make the surroundings as homogeneous as possible, to avoid topographic biases.

Light for the testing arena was provided from a centrally placed custom-made lamp in the ceiling of the magnetic cage, with trichromatic LEDs as light source and an opaque Plexiglas sheet scattering the light. A camera was mounted centrally in the lamp facing the arena from above. Six UV LEDs with peak wavelength of 375 nm (T5F37; Seoul Semiconductor Co. Ltd., Gyeonggi-do, Korea) were also initially housed in the same lamp, until we found that the Plexiglas blocked all light in the UV spectrum. Four of the UV diodes were therefore moved and attached symmetrically along the periphery of the lamp, so that the UV light reached the arena unfiltered. UV light is widely believed to be important for bumblebee navigation (Koppert, no date), but research indicates that the UV light itself might not be very important for visual learning or target finding, but rather that relatively constant spectral lighting conditions are more important, with or without UV light (Dyer et al., 2004). Light intensity was measured using a light power meter (IL1400A; International Light Inc., Peabody, MA, U.S.A.). The intensity was 8.8 mW/cm² in the center of the maze, and 6.3 mW/cm² at the end of each arm.

The light spectrum was measured with a spectrometer (USB2000-VIS-NIR; Ocean Optics Inc., Dunedin, FL, U.S.A.) with a range of 350-1000 nm, and also with another spectrometer going further down to the UV (brand unknown). The spectrum was measured primarily to confirm the presence or

absence of UV light. Outside of testing and training hours, diffuse ambient light was provided by a 40 W incandescent light on a 12/12 hour light/dark cycle. The experimental setup was placed in a specially designated windowless lab, where the controlled ventilation was set to low.

The bees were always trained with the reward in the magnetic north direction. Since the maze was inside the cube of electromagnetic coils, it was possible to neutralize the natural magnetic field, and substitute it with a similar field in any other direction. During training, the geographic direction of the food reward was regularly and randomly changed between the arms of a four-armed maze (a '+'-maze; Fig. 1B). The direction of the magnetic field was changed accordingly, always keeping the relative orientation of the food reward to the magnetic field direction constant. If the bumblebees have a magnetic compass and use it, they should consequently learn to associate the food reward with the trained magnetic field direction and not with the geographic location of a specific arm. To test whether the bumblebees learned the task, I recorded their directional choice in a test situation without a reward, but in the same maze and setup.

Testing sessions were recorded using a digital video surveillance system from ABUS (TVHD40000 camera custom fitted with a wide-angle lens, TVHD80000 video recorder; ABUS August Bremicker Söhne KG, Wetter, Germany). The video system was always turned on during training sessions. Tests were performed before noon, except on a few occasions. A recent paper (Beyaert et al., 2012) suggests that honeybees consolidate their navigation memory during sleep, so I always let there be a night of memory consolidation between training and tests.

Experiment 1

In the first experiment, I used a natural pollen mix as reward. The pollen was provided in a box at the end of one of the arms of the maze; the boxes in the other three arms were empty. The bumblebees accessed the maze through transparent plastic tubing connected directly to the entrances of their hive, which was placed on a wooden platform under the table holding the maze. For tests, the training boxes were replaced with identical boxes, which had never contained pollen. Preliminary video analysis of results of experiment 1 did not suggest any magnetically sensitive behaviour. The bumblebees showed only a very moderate interest in the pollen reward, and a much greater interest in walking around in small groups in a seemingly random fashion. Experiment 1 was replicated four times, each on a separate day.

Experiment 2

Due to the low interest in the pollen reward in experiment 1, we changed the protocol. The sugar feeding station was removed from the nest, and the pollen reward in the maze was substituted with sugar solution (1 M sucrose solution, which was also used in all subsequent experiments). We removed the sugar from the nest to make the bees more hungry, and thus more motivated to search for the reward. We used a saturated NaCl solution as a negative reward in all other arms, providing a slight punishment for bees trying to drink in the wrong box. We discovered that the white tinted Plexiglas we used for diffusing the light coming from our lamp blocked all UV light, meaning that none of the UV light from the LEDs reached the bumblebees in the maze. At that time we were convinced that bumblebees needed UV light for orientation. So, we moved the LEDs to unblocked locations in the periphery of the lamp, from where they provided unfiltered UV light for the arena. Experiment 2 was replicated six times, without improvement of the orientation abilities of the bees.

Experiment 3

For experiment 3, we switched to a protocol testing colour pattern cues instead of magnetic cues, since it has previously been shown that bees are able to learn colour. Patterns with blue and yellow were used, inspired by Blackawton et al. (2010). The coloured paper patches (size 2.5×3 cm) were attached to the sides of the entrance to the rewarded and non-rewarded boxes, two on each box, with blue-dominated patches marking the rewarded box and patches with more yellow on the non-rewarded boxes. The coloured paper was produced by colour laser printing (Ricoh Aficio MP C2800; Ricoh Company, Ltd., Japan) on ordinary white paper (Premium Label Paper, 80 g/m^2 ; Canon Inc., Japan). During training, the bees seemed to quickly identify and enter the box with the sugar solution, but during tests the overall orientation tendencies in the maze were not goal-oriented. Pollen was provided *ad libitum* in the center of the maze. Experiment 3 was replicated 5 times.

Experiment 4

Since preliminary analysis of experiment 3 did not suggest goal-oriented behaviour, I changed the size of the colour patches since I suspected that they were too small for the bees to discriminate from a distance. According to Spaethe and Chittka (2003), bumblebees need a visual angle of around $3.5\text{--}7^\circ$

for spatial resolution, depending on the size of the bee. The small patches used in the previous experiment occupied $\approx 4^\circ$ when viewed from the center of the maze, so it was not realistic to expect that the bee would be able to resolve the different colours in the patch from that distance. So, I replaced the small bi-coloured patches with visually simpler patches in one colour; blue representing a rewarded box and yellow a box with saline solution. The patches were also complimented with a larger paper piece (9.5×9.5 cm) of the same respective colour attached immediately above each feeding box, increasing visibility and giving a visual angle from the center of the maze of approximately 15° .

The experimental setup was also rebuilt. We removed the plastic tubing that had connected the bumblebee hive directly to the maze, and instead attached aluminium netting from the edge of the circular table holding the maze to a wooden plate on the floor of the coil. The hive was placed on the wooden plate, inside the net cage. In this way, we constructed a space where the bumblebees could fly freely at all times. To make individual testing of trained bees possible, bees who visited the rewarded box during training sessions were marked by gluing a small number tag (Joel Svenssons Vaxfabrik, Munka-Ljungby, Swe.) dorsally on their thorax. We also mounted a sliding transparent door in the central opening to the maze, making it possible to see if a marked (and thus trained) bee was approaching the hole from the flight cage below, and then letting it up into the maze to be tested if it was marked. In total, 17 tests were performed with 11 individual bees.

In experiments 4 and 5, several bees were tested two times or more, since it was impossible to control before the test which bee was going to come up through the entrance to the maze. However, this pseudoreplication is not a big problem, since the purpose of the experiments was to demonstrate a learning effect. Still, only the first test of each bee has been included in the main statistical analysis and the presentation of the results in this report. Data from experiment 5, including all tests, is available in appendix A.

Experiment 5

Preliminary analysis of the data from experiment 4 suggested that there was a learning effect, meaning that we finally had an experimental setup, where we could see that the bumblebees were learning to associate a colour with a reward, which they are known to be able to learn (Blackawton et al., 2010). The coloured papers were now removed, and training and testing with magnetic fields was resumed. The experimental protocol was otherwise similar to that of experiment 4. The same colony was used as in experiment 4, and several bees were tested in both experiments. In experiment 5, I made 33 tests of 15 individuals.

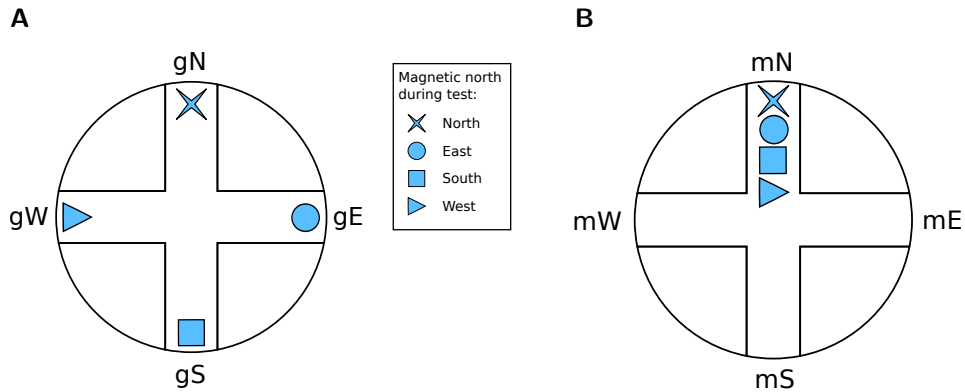


Figure 2: Illustration of the analysis model. (A) Geographical distribution of hypothetical bumblebees in four different tests. The tests had magnetic north shifted to different directions as indicated. (B) The same data as in A, but rotated so that magnetic north in each test is now 0° . It is now visible that the bees in all tests oriented towards magnetic north. Geographic north is abbreviated to gN, magnetic north to mN.

Analysis

For statistical analysis, the data was pooled in two different ways. In the first analysis, the directional choices of the bumblebees were plotted relative to the geographical direction (Fig. 2A). When this data is pooled, it should preferably show no significant preference for geographical direction, as that would otherwise mean that there is a problem with the experimental setup, i.e., that the bumblebees preferred to go towards a light source, sound source, following some temperature gradient or some other bias. In the second analysis, the data was transformed by rotation, so that the geographical direction chosen by the bumblebee was rotated relative to the magnetic direction, so that magnetic north was at 0° (Fig. 2B). Thus, if the bumblebees oriented using the magnetic field, there would be a significant orientation towards the trained magnetic direction.

On the other hand, if they did not, or could not, use the magnetic field information, or we could not detect that they used it, there would be no significant preference in this second analysis. All angles reported are in degrees, with 0° corresponding to north, either geographic or magnetic, and angles increasing clock-wise. The mean vector length (r) is a measure of scatter and ranges from 0 to 1.

All experiments were video recorded. The goal was to analyse videos in Matlab (R2012A; The MathWorks Inc., Natick, MA, USA) with respect to location and movement of bees in the maze over time, but time limitations hindered development of the analysing program. Instead, the videos were manually analysed. Experiments 1 to 3 were set up in a way that allowed

many bees to access the maze simultaneously, meaning that many bees were tested at the same time. These bees were not individually marked. For analysis the video was paused and the number of bees in each arm counted at specific times during the 30 minute test (0, 1, 3, 5, 7, 10, 15, 20, 25 and 30 minutes). Bees were counted as being in an arm of the maze when they had crossed an imaginary line at the beginning of each arm. The number of bees in each arm was then divided by the total number of bees in the maze at each time to get proportions of bees per arm at each separate moment. These proportions were then averaged across the entire 30 minute test, and compared against each other with a non-parametric Friedman's two-way rank variance analysis test, as described in Siegel (1985).

The videos of experiments 4 and 5 were analysed in several ways. For each bee, I recorded the first direction it chose to walk or fly, and also the second direction it chose after walking back to the center, after not finding a reward in the first arm. I also noted how much of the first 60 seconds the bee spent in each arm. Visits shorter than one second were not counted, since these could not be distinguished accurately. The time, counted in seconds, that the bee spent in each arm was considered a vector, and the vectors for the four arms of each test were summed together to a mean vector for each test using vector addition. This means that a theoretical bee that spent 30 seconds in the northern arm (0°), and 30 seconds in the eastern arm (90°), would get a mean vector with an angle of 45° and, using the Pythagorean theorem for right-angled triangles ($c^2 = a^2 + b^2$), a length of $\sqrt{30^2 + 30^2}$ which is ≈ 42.4 . This was then scaled to a vector length (r) between 0 and 1 using the maximal theoretical length of 60, resulting in $r=0.7$ in this example. Vector data was tested using circular statistics in Oriana 4 (Kovach Computing Services, Wales, U.K.), except 95% confidence ellipses for mean vectors, which were calculated and plotted in Matlab according to Batschelet (1981). When the confidence ellipse does not include the origin, the angular confidence interval can be constructed by measuring the angles of tangents to the ellipse from the origin. Data from experiment 5 were also analysed axially, but this analysis is not presented, since it did not give notably different results.

The Moore-Rayleigh test was used to calculate the significance of mean vectors (Moore, 1980). Fisher's tests for choices in experiments 4 and 5 were made in R (v. 2.15.3; The R Foundation for Statistical Computing, Vienna, Austria).

Results

In experiment 1, where we used pollen as a reward and tested many bees at the same time, the data was analysed both according to geographic and

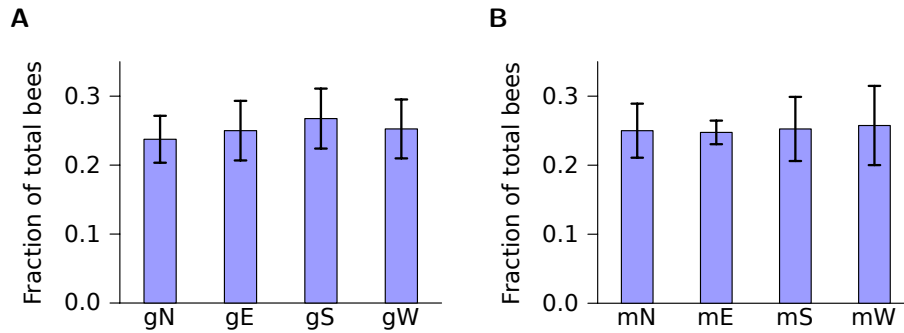


Figure 3: Results from experiment 1, in which bees were trained to magnetic north, and rewarded with pollen during training sessions. Magnetic north was shifted between tests. (A) Average geographic distribution of bees in the maze during tests. (B) The same data as in A, but rotated so that the arms with the same magnetic direction are averaged together. Error bars are ± 1 standard deviation. Geographic north is abbreviated to gN, magnetic north to mN. Friedman’s two-way rank analysis $p \geq 0.9$ in both A and B.

magnetic directional preference (Fig. 3). Tests for geographic orientation (Friedman two-way rank analysis, $\chi_r^2=0.525$, $k=4$, $p=0.9$) and magnetic orientation (Friedman, $\chi_r^2=0.225$, $k=4$, $p>0.9$) were both non-significant, meaning that there was no statistically significant preference for either geographic or magnetic direction. Both categories gave mean distributions of bumblebees for each direction that averaged close to 0.25, also suggesting a random distribution.

Experiment 2, where bees were rewarded with sugar and tested many at the same time, was replicated six times, on separate days (Fig. 4, A-B). Tests for preference for geographic direction (Friedman, $\chi_r^2=3.95$, $df=3$, $0.3>p>0.2$) and magnetic direction (Friedman, $\chi_r^2=1.25$, $df=3$, $0.8>p>0.7$) were not significant. If anything, there might have been a slight tendency to prefer the geographic north direction judging from the graphs.

Experiment 3 was the first experiment where we used colour instead of magnetic cues (Fig. 4, C-D). The first impressions of a lack of orientated behaviour from watching the bees on the video screen was confirmed by statistical analysis of the results. There was no preference for either geographic direction (Friedman, $\chi_r^2=2.7$, $df=3$, $0.5>p>0.3$) or the direction towards the box marked with the previously rewarded colour combination (Friedman, $\chi_r^2=4.14$, $df=3$, $0.3>p>0.2$).

In experiment 4, where bees were trained to big coloured papers and tested individually, the bees appeared to know what they were doing during experiments. A preliminary Fisher’s test was performed on the directional choices of all bees, including the multiply tested ones. There were 10 correct choices and 7 incorrect ones, which I tested against an expected random

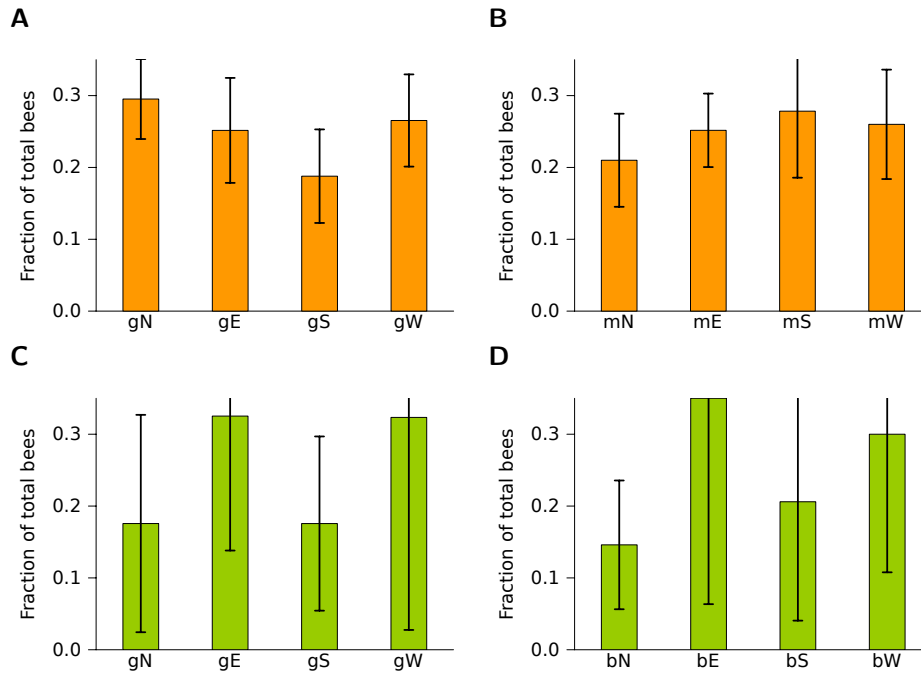


Figure 4: Results from experiment 2 (A-B), where bees were trained to magnetic fields, and experiment 3 (C-D), where bees were trained to colour patches. (A) The average geographic distribution of bees in the maze during experiment 2. (B) The same data as A, but rearranged so that arms with the same magnetic direction are analyzed together. (C) The average geographic distribution of bees during experiment 3. (D) The same data as C, but rotated according to the direction of the blue colour patch, which was rewarded during training. Error bars are ± 1 standard deviation. Geographic north is gN, magnetic north is mN, blue colour patch is bN.

distribution of 4 correct and 13 incorrect choices. This gave $p=0.079$, which was good enough to move on to experiment 5. For further analysis, only the first test of each bee was counted ($n=11$) to avoid pseudoreplication (Fig. 5). The mean vector for the first 60 seconds (334° , $r=0.441$) gave significant preference for the direction to the blue box (Moore-Rayleigh test, $R^*=1.409$, $p<0.005$; 95% CI for mean vector: $316^\circ-6^\circ$; Fig. 5B), which was the trained rewarded direction, whereas the mean vector for geographic direction (308° , $r=0.152$) was not significant (Moore-Rayleigh test, $R^*=0.401$, $0.9>p>0.5$; 95% CI could not be calculated; Fig. 5A).

Experiment 5 initially had 33 replicates, but after keeping only the data of the first test of each bee to avoid pseudoreplication, only 16 tests remained. Counting the choices of these bees revealed an interesting pattern – their first choice of direction was correct in 3 cases of 16 (19%), which is similar to chance (25%). However, for their second choice of arm, they went in the right direction in 7 cases of 16 (44%). This is not significant in a Fisher's

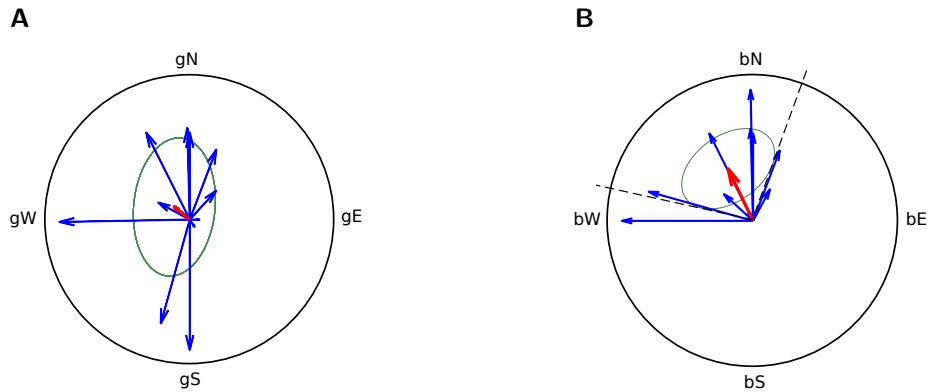


Figure 5: Results from experiment 4, where the bees were trained to rewarded colour. (A) Orientation relative to geographic north. ($n=11$, mean vector angle 308° , Moore-Rayleigh test $0.9 > p > 0.5$) (B) The same data as in A, but rotated according to the placement of the blue rewarded box, so that the rewarded direction is at 0° , irrespective of the geographic direction to the reward. ($n=11$, mean vector angle 334° , $r=0.441$, Moore-Rayleigh test $p < 0.005$) Geographic north is indicated by gN, direction to the blue box by bN. Thinner blue arrows are directional vectors constructed from the time the bee spent in each arm of the maze. The thick red arrow is the mean vector; the green circle is the 95% confidence interval for the mean vector. Dashed lines indicate the angular confidence interval.

test ($p=0.45$) because of small sample size, but could indicate a tendency of learning.

The mean orientation relative to geographic north (117° , $r=0.08$) was not significant (Moore-Rayleigh test, $R^*=0.326$, $0.9 > p > 0.5$) (Fig. 6A). The same was true when the data was rotated relative to the trained magnetic direction (310° , $r=0.26$), (Moore-Rayleigh test, $R^*=0.962$, $0.1 > p > 0.05$; Fig. 6B). However, in the latter case, the magnetic mean vector is more than three times the length of the geographic mean vector (0.26 compared to 0.08).

A subgroup analysis of the magnetic vector data from experiment 5, including all the 33 original tests and splitting the data by tests done in magnetic north-south or east-west directions, showed that the bees were well able to orientate in the north and south fields (Fig. A1, C-F), but were disoriented in the east and west fields. The mean vector for magnetic north-south tests, rotated to have magnetic north at 0° , was 325° with $r=0.46$ (Moore-Rayleigh test, $R^*=1.407$, $p < 0.005$). For the east-west tests the mean vector was 257° with $r=0.189$ ($R^*=0.756$, $0.5 > p > 0.1$). However, because of small sample size ($n=15$ for north-south tests, $n=18$ for east-west tests), this should be taken with reservations.

When reviewing the data for experiment 5, it looked as if there was a learning effect over time. In the first tests, individual bumblebees usually went in the wrong direction for their first choice, but then surprisingly often

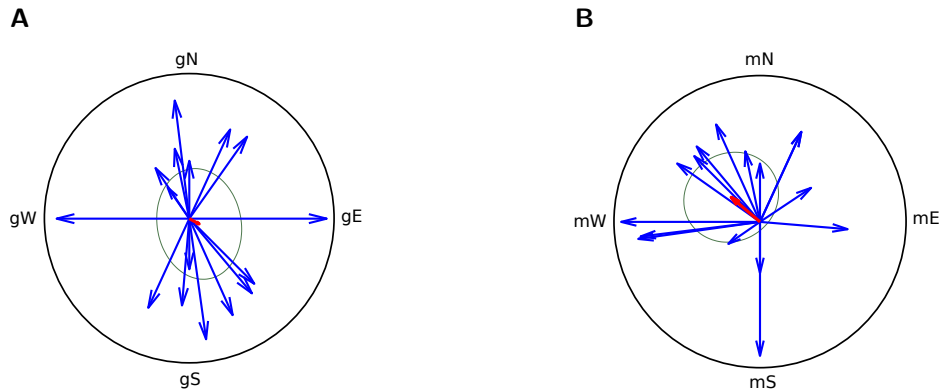


Figure 6: Results from experiment 5, where bees were trained to find a reward at magnetic north. (A) Orientation relative to geographic north. ($n=16$) (B) The same data as in A, but rotated relative to the direction of the magnetic field in each test. Magnetic north is indicated by mN. For details on interpreting the graphs, see figure 5.

made their second choice in the right direction, being correct in 9 of 16 tests (56%). During the second half of experiments, when they had already been tested and trained to magnetic cues for over a week, they were more often correct on their first choice, being right in 9 of 17 tests (53%).

Discussion

The aim of this study was to develop a behavioural assay to study magnetic orientation in bumblebees, something that I accomplished in experiment 5. I also present some assays that do not seem generally suitable. The results of the final tests indicate that there is probably a learning effect related to magnetic fields, since the mean vector length for the magnetic distribution was more than three times that of the mean vector of the non-rotated geographic data, and orientated more towards magnetic north. However, since the mean vectors were not statistically significant in themselves, more data is needed to firmly establish the presence of a magnetic sense in *B. terrestris* bumblebees. The tendency of the bees to become better at making the right choice over time probably reflects that they need longer time to learn to use magnetic cues than learning to use vision. A similar phenomenon is seen in honeybees, where it has been more difficult to condition them to magnetic fields than to traditional stimuli (Walker et al., 1989a). The reason for this is still unknown, but fits well with observed tendencies in birds to prefer other senses such as vision over magnetoreception, when given a choice (R. Muheim, pers. com.).

The results of the sub-grouping analysis of the data from experiment 5 suggested that the bees did not orientate well in the east and west fields. We

then suspected that the east-west coil might be malfunctioning, either magnetically or by introduction of RF fields. According to the radical-pair theory of magnetoreception, a RF field of frequency 1.4 MHz (locally, frequency varies with magnetic field strength) can make magnetoreception impossible (Henbest et al., 2004, Ritz et al., 2004). Measurement of the fields produced by our coils did not indicate significant RF interference, and the magnetic fields were equivalent to when calibrated. However, when measuring directly on the outputs of the electronics controlling the coils, we found some noise in the MHz range on the north output to the north-south coil, but the significance of this is unclear. A possible counter-argument to magnetosensitive orientation in our experiments could be that the bees would have learned to orientate relative to this RF noise output in the north-south coil, whereas this might have been absent from the east-west coil, and the tests therefore failed there. However, the subgroup analysis suggested just as good orientation in the south as in the north field, and a RF field in the MHz range is just as likely to disturb magnetoreceptive orientation as to help it.

There are several reasons why we had to try several setups before arriving at something that worked. Neither me nor my supervisor had much experience in working with bumblebees or insects before. Since bumblebees use pollen as the protein source when feeding the larvae, we chose to reward them with pollen in the first experiment. This proved to be very inefficient – the bees did not show more interest in the pollen than maybe exploring it for a bit of time, and we could not see any bees carrying pollen back to the nest. In experiment 2, we rewarded the bees with sugar solution, and also took away their unlimited sugar supply in the nest, which made them a lot more motivated to seek out the reward. However, in experiment 1-3, we tested many bees simultaneously, which turned out to be a bad strategy, since the bees followed and thereby influenced each other. In experiment 3, we did what we should have done already – established a control method, where we tested something that we knew the bumblebees would be able to learn, which is associating a colour with a reward. Since we were testing many bees at the same time, this still did not work. So, for experiment 4, we built a cage separate from the maze, where all bees could fly freely, thus enabling testing of one bee at a time. We tested for colour learning. Once this worked, we finally moved on to experiment 5, where we again tested for learning with magnetic fields.

In the current setup, it is not possible to identify the bee before it is tested, which led to multiple testing of bees in experiments 4 and 5, and using some individuals in both experiments, since the same colony was used. This might have biased experiment 5, since some of these bees already had a learning experience from experiment 4. Walker et al. (1990) showed that *transfer along a continuum* exists in honeybees, meaning that learning of

a difficult discrimination task is facilitated, if one already has experience in an easier task in the same modality. This might not be really relevant here, since colour discrimination and magnetic discrimination are probably different modalities, but future experiments should still seek to use experimentally naive animals. Also, the results in experiment 5 seemed to improve over time, showing that the bees tested first, with previous experience from experiment 4, did not perform better than the inexperienced bees trained and tested later. The aim of these experiments was to establish a method, but for later experiments, a more strict protocol should be used, where either bees are tested only once and then sacrificed, or where a limited number of individuals are tested repeatedly in only one experiment, making it possible to more reliably evaluate individual performance.

An alternative experimental setup that could prove interesting, would be to use local magnetic anomalies, which honeybees are known to be able to perceive (Walker and Bitterman, 1989b). One could e.g. place small bar magnets under the arms of the maze, out of sight for the bees, with brass staffs used as non-magnetic controls. If this worked, it would on the other hand only prove that bumblebees can differentiate between magnetic field anomalies, but not that they navigate using ambient magnetic fields.

If some kind of interesting results come out of further experiments in the current setup, a negative control would be needed, where magnetic field information can not be used by the insects. This could be done, for example, by training the insects to use a magnetic field as cue for finding the reward, but in the test situation only give them a vertical field without directional information.

The implications of the finding that bumblebees probably have a magnetic sense are potentially large – since it is known from before that RF fields can disrupt magnetoreception, this could mean that modern society with its mobile masts, radio transmitters and WiFi-networks may make it very difficult for bumblebees, as well as other insects and animals, to use magnetoreception – one of their fundamental senses. Possibly, this could also partly explain the decline in many pollinator species that is presently seen, both bumblebees and honeybees, as reviewed in Potts et al. (2010). I even speculate that there could be a connection between a disturbance of magnetoreception and the disoriented behaviour, that is seen in honeybees exposed to neonicotinoid pesticides (Bortolotti et al., 2003), and that is probably involved in Colony collapse disorder (CCD) in honeybees.

Also, from a more general biological point of view, I think that the probable existence of a previously undescribed sense in bumblebees is really exciting. It is fascinating that a sense of a totally separate modality such as magnetism has gone unnoticed for such a long time in animals, probably

only because we humans are not consciously aware of having it – if we have it (Baker, 1987). I also think that magnetoreception in animals is an area that should get a lot more attention since it is an area where human activity, in the form of electrosmog, could cause disturbance.

Acknowledgements

A big thank you to my supervisor Rachel Muheim, for all your patient support and help! You are a star in many ways. Thanks also to Marie Dacke, Emily Baird, Aravin Chakravarthi and Therese Reber for valuable help and advice concerning experiments and handling of the bees. And thank you to all the rest of the vision group for valuable input and moral support during difficult experimental times. Thanks also to family and friends for fostering my curiosity, and thanks to myself for making the choice to go into biology, and those around me who have supported that choice. Gratitude also goes out to the more than 70 drivers who have picked me up while hitchhiking to university for thesis work.

References

- Baker, R. R.** (1987) Human navigation and magnetoreception: the Manchester experiments do replicate. *Anim. Behav.* **35**, 691-704.
- Dyer, A. G. and Chittka, L.** (2004). Bumblebee search time without ultraviolet light. *J. Exp. Biol.* **207**, 1683-1688.
- Batschelet, E.** (1981) *Circular statistics in biology*. London: Academic Press Inc.
- Blackawton P. S., Airzee S., Allen A. et al** (2010). Blackawton bees. *Biol. Lett.* **7**, 168-172.
- Beyaert, L., Greggers, U. and Menzel, R.** (2012). Honeybees consolidate their navigation memory during sleep. *J. Exp. Biol.* **215**, 3981-3988.
- Bortolotti, L., Montanari, R., Marcelino, J., Medrzycki, P., Maini, S. and Porrini, C.** (2003) *Bulletin of Insectology* **56**, 63-67.
- Çamlitepe, Y., Aksoy, V., Neslihan, U., Ayse, Y. and Becenen, I.** (2005) An experimental analysis on the magnetic field sensitivity of the black-meadow ant *Formica pratensis* Retzius (Hymenoptera: Formicidae). *Acta Biol. Hung.* **56**, 215-224.
- Chittka, L., Williams, N. M., Rasmussen, H. and Thomson, J. D.** (1999). Navigation without vision: Bumblebee orientation in complete darkness. *Proc. Biol. Soc.* **266**, 45-50.
- De Jong, D.** (1982). Orientation of comb building in bumblebees. *J. comp. phys.* **147**, 495-501.
- Gegear, R. J., Foley L. E., Casselman, A. and Reppert, S. M.** (2010) Animal cryptochromes mediate magnetoreception by an unconventional photochemical mechanism. *Nature* **463**, 804-807.
- Henbest, K. B., Kukura, P., Rodgers, C. T., Hore, P. J. and Timmel, C. R.** (2004) Radio frequency magnetic field effects on a radical recombination

- reaction: diagnostic test for the radical pair mechanism. *J. Am. Chem. Soc.* **126**, 8102-8103.
- Holland, R. A., Thorup, K., Vonhof, M. J., Cochran, W. W. and Wikelski, M.** (2006) Navigation: Bat orientation using Earth's magnetic field. *Nature* **444**, 702.
- Intermagnet** (2013) *INTERMAGNET International real-time magnetic observatory network*. [Online] Available: www.intermagnet.org [April, 2013]
- Keeton, W. T.** (1971) Magnets interfere with pigeon homing. *Proc. Natl. Acad. Sci.* **68**, 102-106.
- Kirschvink, J. L. and Kirschvink, A. K.** (1991) Is geomagnetic sensitivity real? Replication of the Walker-Bitterman magnetic conditioning experiment in honey bees. *American Zoologist* **31**, 169-185.
- Kirschvink, J. L., Walker, M. M. and Diebel, C. E.** (2001) Magnetite-based magnetoreception. *Curr. Opin. Neurobiol.* **11**, 462-467.
- Koppert B.V.** (No date) *NATUPOL Beehive*. [Online] Available: www.koppert.com/products/pollination/products-pollination/detail/natupol-beehive [February 13, 2013].
- Martin, H. and Lindauer, M.** (1977). Der Einfluß des Erdmagnetfeldes auf die Schwereorientierung der Honigbiene (*Apis mellifica*). *J. Comp. Physiol. A* **122**, 145-187.
- Merritt, R., Purcell, C. and Stroink, G.** (1983). Uniform magnetic field produced by three, four and five square coils. *Rev. Sci. Instrum.* **54**, 879-882.
- Moore, B. R.** (1980) A modification of the Rayleigh test for vector data. *Biometrika* **67**, 175-180.
- Muheim, R., Edgar, N. M., Sloan, K. A. and Phillips, J. B.** (2006). Magnetic orientation in C57BL/6J mice. *Learning & Behavior* **34**, 366-373.
- Phillips, J. B.** (1986). Two magnetoreception pathways in a migratory salamander. *Science* **233**, 765-767.
- Phillips, J. B., Jorge, P. E. and Muheim, R.** (2010) Light-dependent magnetic compass orientation in amphibians and insects: candidate receptors and candidate molecular mechanisms. *J. R. Soc. Interface* **7**, Suppl. 2, S241-S256.
- Phillips, J. B. and Sayeed, O.** (1993) Wavelength-dependent effects of light on magnetic compass orientation in *Drosophila melanogaster*. *J. Comp. Phys.* **172**, 303-308.
- Potts, S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O. and Kunin W. E.** (2010) Global pollinator declines: trends, impacts and drivers. *Trends in Ecology and Evolution* **25**, 345-353.
- Ritz, T., Dommer, D. H. and Phillips, J. B.** (2002) Shedding light on vertebrate magnetoreception. *Neuron* **34**, 503-506.
- Ritz, T., Thalau, P., Phillips, J. B., Wiltschko, R. and Wiltschko, W.** (2004). Resonance effects indicate a radical-pair mechanism for avian magnetic compass. *Nature* **429**, 177-180.
- Riveros, A. J. and Srygley, R. B.** (2008) Do leafcutter ants, *Atta colombica*, orient their path-integrated home vector with a magnetic compass? *Anim. Behav.* **75**, 1273-1281.
- Siegel, S.** (1985) *Nichtparametrische statistische methoden*. (2nd ed) Eschborn bei Frankfurt am Main, Germany: Fachbuchhandlung für Psychologie GmbH
- Spaethe, J. and Chittka, L.** (2003). Interindividual variation of eye optics and single object resolution in bumblebees. *J. Exp. Biol.* **206**, 3447-3453.

- Vácha, M., Puzová, T. and Kvicalová, M.** (2009). Radio frequency fields disrupt magnetoreception in American cockroach. *J. Exp. Biol.* **212**, 3473-3477.
- Wajnberg, E., Acosta-Avalos, D., Cambraia Alves, O., Ferreira de Oliveira, J., Srygley, R. B. and Esquivel, D. M. S.** (2010) Magnetoreception in eusocial insects: an update. *J. R. Soc. Interface* **7**, Suppl 2, S207-S225.
- Walker, M. M., Baird, D. L. and Bitterman, M. E.** (1989a) Failure of stationary but not of flying honeybees (*Apis mellifera*) to respond to magnetic field stimuli. *J. comp. psych.* **103**, 62-69.
- Walker, M. M. and Bitterman, M. E.** (1985). Conditioned responding to magnetic fields by honeybees. *J. Comp. Physiol. A* **157**, 67-71.
- Walker, M. M. and Bitterman, M. E.** (1989b). Short communication: Honeybees can be trained to respond to very small changes in geomagnetic field intensity. *J. Exp. Biol.* **145**, 489-494.
- Walker, M. M., Lee Y. and Bitterman, M. E.** (1990). Transfer along a continuum in the discriminative learning of honeybees (*Apis mellifera*). *J. Comp. Psych.* **104**, 66-70.
- Wiltschko, W. and Wiltschko, R.** (1972). Magnetic compass of European Robins. *Science* **176**, 62-64.

Appendix A

Table 1: Choices of geographical directions in experiment 5 in chronological order. Correct choice of direction is indicated with a bold letter. (Total number of tests 33)

Date	Bee	mN	First ch.	Sec. ch.
130125	52	S	S	E
130125	42	W	N	W
130125	40	E	W	N
130125	44	N	N	S
130125	49	W	E	S
130125	41	E	N	E
130125	38	S	E	S
130125	32	N	E	N
130129	40	W	E	W
130129	56	E	W	E
130129	45	S	W	S
130129	58	N	W	E
130129	46	E	S	W
130130	52	W	E	W
130130	46	N	W	S
130130	50	S	E	S
130130	56	E	W	N
130130	41	N	W	N
130130	45	W	W	S
130130	57	E	W	N
130130	58	S	S	E
130131	56	W	W	N
130131	42	E	E	W
130131	55	N	W	E
130131	50	S	S	-
130131	57	E	E	W
130131	59	N	S	N
130131	58	W	S	W
130201	56	W	S	E
130201	41	E	N	-
130201	50	S	S	N
130206	55	S	S	S
130206	50	E	E	S

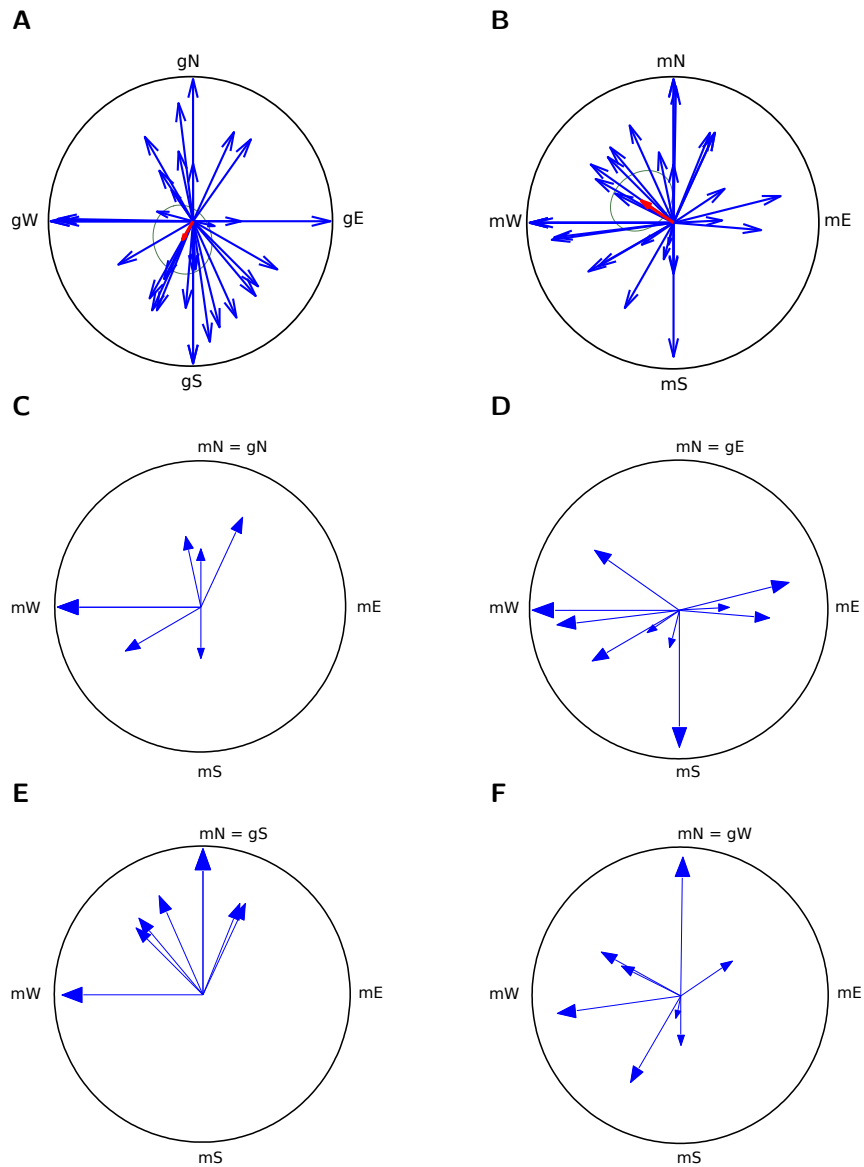


Figure 1: Graphs depicting all data for experiment 5, including all tests. (A) Geographic distribution of bees during experiment 5. (Mean vector 209° , $r=0.146$, Moore-Rayleigh $R^*=0.682$, $0.5 > p > 0.1$) (B) Same data as in A, but rotated according to the magnetic direction in each test, so that mN is 0° . The mean vector is significant when data is rotated according to magnetic direction. (Mean vector 304° , $r=0.266$, Moore-Rayleigh $R^*=1.356$, $p < 0.005$) (C-F) Data from experiment 5 subgrouped according to the magnetic direction for each test, and rotated so that mN is at 0° . C is bees tested with mN in geographic north, D with mN in east, E with mN in south and F with mN in west.