

RESEARCH ARTICLE

Conditioned discrimination of magnetic inclination in a spatial-orientation arena task by homing pigeons (*Columba livia*)

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ABSTRACT

It has been well established that homing pigeons are able to use the Earth's magnetic field to obtain directional information when returning to their loft and that their magnetic compass is based, at least in part, on the perception of magnetic inclination. Magnetic inclination has also been hypothesized in pigeons and other long-distance navigators, such as sea turtles, to play a role providing positional information as part of a map. Here we developed a behavioral paradigm which allows us to condition homing pigeons to discriminate magnetic inclination cues in a spatial-orientation arena task. Six homing pigeons were required to discriminate in a circular arena between feeders located either in a zone with a close to 0 deg inclination cue or in a zone with a rapidly changing inclination cue (−3 deg to +85 deg when approaching the feeder and +85 deg to −3 deg when moving away from the feeder) to obtain a food reward. The pigeons consistently performed this task above chance level. Control experiments, during which the coils were turned off or the current was running anti-parallel through the double-wound coil system, confirmed that no alternative cues were used by the birds in the discrimination task. The results show that homing pigeons can be conditioned to discriminate differences in magnetic field inclination, enabling investigation into the peripheral and central neural processing of geomagnetic inclination under controlled laboratory conditions.

KEY WORDS: Homing pigeon, Magnetic inclination, Compass, Conditioning

INTRODUCTION

Homing pigeons (*Columba livia*), selectively bred for several thousand years for their ability to return to their loft from distant and unfamiliar sites, have been one of the main model species for studying the sensory mechanisms underpinning navigation behavior. It is generally accepted that true navigation requires the use of a map and a compass, as originally suggested by Kramer (Kramer, 1953; Kramer, 1961), to determine position relative to the goal and to set and maintain an appropriate direction of movement, respectively (for reviews, see Able, 2001; Wiltschko and Wiltschko, 2009; Wallraff, 2005). Over the past few decades, considerable evidence has accumulated that spatial information provided by the Earth's magnetic field plays an important role during pigeon navigation (for reviews, see Wiltschko and Wiltschko, 1995; Wiltschko and Wiltschko, 2009).

Firstly, homing pigeons possess a magnetic compass. Previous studies conducted with migratory birds had shown that the birds

adjusted their preferred direction for orientation during migratory restlessness whenever the horizontal component of the magnetic field vector experienced by the birds inside Emlen funnels was rotated by magnetic coils surrounding the funnels (Wiltschko, 1968). Analogous to this, pigeons carrying battery-operated magnetic coils atop their heads flew in the opposite direction from home under overcast conditions (i.e. without the availability of their sun compass) whenever the vertical component of the local magnetic field vector was inverted (Walcott and Green, 1974; Benvenuti et al., 1982). Thus, the avian magnetic compass, unlike a human-made magnetic compass, is an 'inclination' compass (for reviews, see Wiltschko and Wiltschko, 2007; Wiltschko and Wiltschko, 2009), which does not respond to polarity, but instead utilizes the direction of the magnetic field vector to determine the north–south axis and the inclination angle to distinguish between 'pole-ward' (downward inclination) and 'equator-ward' (upward inclination) direction.

Secondly, indirect evidence from field studies has suggested that pigeons may also, under some conditions, rely on spatial information provided by the Earth's magnetic field to determine their position with the vanishing directions of pigeons having been correlated to temporal and spatial variations in the local magnetic field under sunny conditions (Keeton et al., 1974; Frei and Wagner, 1976; Wagner, 1976; Walcott, 1978; Frei, 1982). Furthermore, magnetic pulse treatments under sunny conditions shifted the initial orientation direction of homing pigeons, thus providing further indication of the possible existence of a magnetic map (Beason et al., 1997). It should be noted though that such a pulse effect was not replicated in a recent study with GPS-tracked homing pigeons (Holland et al., 2013), which found no evidence of impairment of either initial orientation or navigation performance. Furthermore, there is also considerable evidence, which is not necessarily mutually exclusive to the possibility of the existence of a magnetic map, that homing pigeons use odours in the atmosphere at least at some locations on Earth to determine their position (for reviews, see Papi, 1992; Wallraff, 2004; Wallraff, 2006).

Relevant for the current study, it is noteworthy that although discussion of a hypothetical geomagnetic map generally focuses on spatial variation in intensity (Walker, 1998; Walker, 1999; Dennis et al., 2007; Mora and Walker, 2009; Wiltschko et al., 2009; Postlethwaite and Walker, 2011; Mora and Walker, 2012), magnetic inclination (the angle between the magnetic field vector and the Earth's surface) is also suitable for determining latitude (Gould, 1982) because it varies systematically, just like intensity, with latitude from the equator (0 deg) to the poles (+90 deg and −90 deg at the magnetic north and south poles, respectively). For example, it been suggested that newts, which generally move over relatively short distances of only a few kilometers during homing (e.g. Fischer et al., 2001; Phillips et al., 2002), use magnetic inclination to determine latitude. More impressive, several studies have also indicated that inclination has an important role in

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position determination for marine long-distance migrators. Loggerhead sea turtles (*Caretta caretta*) can detect the magnetic inclination angle (Lohmann and Lohmann, 1994) and it has been even suggested that sea turtles possess a magnetic map consisting of a grid of magnetic intensity and inclination values (e.g. Lohmann and Lohmann, 1996; Lohmann et al., 2007). Although this hypothesis may not be globally applicable (lines of equal intensity and inclination intersect at sufficiently large angles only in small geographic areas) nor evolutionary stable because of gradual shifts in the Earth's magnetic poles over time (Courtilot et al., 1997; Walker et al., 2002), magnetic inclination remains an intriguing possibility that may allow animals such as sea turtles and birds, if they are sensitive to it, to locate their position at least on one axis of a bi-coordinate map.

Conditioning experiments in the laboratory provide one useful avenue to investigate sensory mechanisms as well as perception limits. Previous attempts to condition homing pigeons or other birds to magnetic stimuli have focused either on magnetic anomalies, which varied both in magnetic intensity and inclination in an uncontrolled way (Mora et al., 2004; Thalau et al., 2007; Freire et al., 2012), or on changes in the horizontal component (Freire et al., 2005; Voss et al., 2007; Keary et al., 2009; Wilzeck et al., 2010). To investigate whether homing pigeons could be trained to discriminate differences in magnetic field inclination only, we developed a behavioral conditioning paradigm that required pigeons to discriminate changes in magnetic inclination to obtain a food reward in a spatial-orientation arena task. In addition to demonstrating that our experimental pigeons can discriminate inclination differences, the results of our study open up the possibility for future investigation into the peripheral and central neural processing of magnetic inclination, potentially offering insight into the neural organization of the avian magnetic compass, as well as provide a new approach to test the hypothesis of a geomagnetic 'inclination map' component under controlled laboratory conditions.

RESULTS

Discrimination of magnetic inclination zones

All experiments were conducted in a circular arena situated centrally atop cinder blocks inside a three-axis magnetic coil system (Fig. 1A) with pigeons being individually harnessed to a horizontal tracker arm (Fig. 1B). Four automated feeder-response units were situated against the wall of the circular arena aligned with the four cardinal directions in the testing room [geographic north (N), south (S), east (E) and west (W); Fig. 1C; see also Materials and methods). During the initial conditioning series, six pigeons were required to select one of two possible feeders associated with changing inclination out of a total of four feeders available (chance level 50%; Fig. 2). Responses to feeders in the 'zero inclination' zones were not rewarded. Our results clearly show that homing pigeons are able to discriminate changes in magnetic inclination because they were able to distinguish the two feeders associated with a rapid change in inclination (-3 deg to $+85$ deg) from the two feeders associated with a constant, low inclination value (-3 deg) (Fig. 3A).

Furthermore, we observed a statistically significant improvement in the performance of the pigeons over the course of the sessions (Linear mixed-model ANOVA, type III test of fixed effects: $F_{\text{Session}}=26.085$, $P<0.001$). However, it is important to note that reported data do not take the form of a traditional learning curve because most birds were already above chance level in the first session after having received prior discrimination training with the magnetic stimulus during the pre-training phase. Because pre-training was customized for each bird's acquisition of the task (see the Materials and methods), discrimination performances during pre-training were not comparable for individual birds and therefore are not included in the graph. A systematic difference between subjects was detected (Linear mixed-model ANOVA, type III test of fixed effects: $F_{\text{Subject}}=16.202$, $P<0.001$) due to birds 703 and 724 taking longer than the other birds to acquire a performance level that was consistently above chance (they did so toward the end of the initial

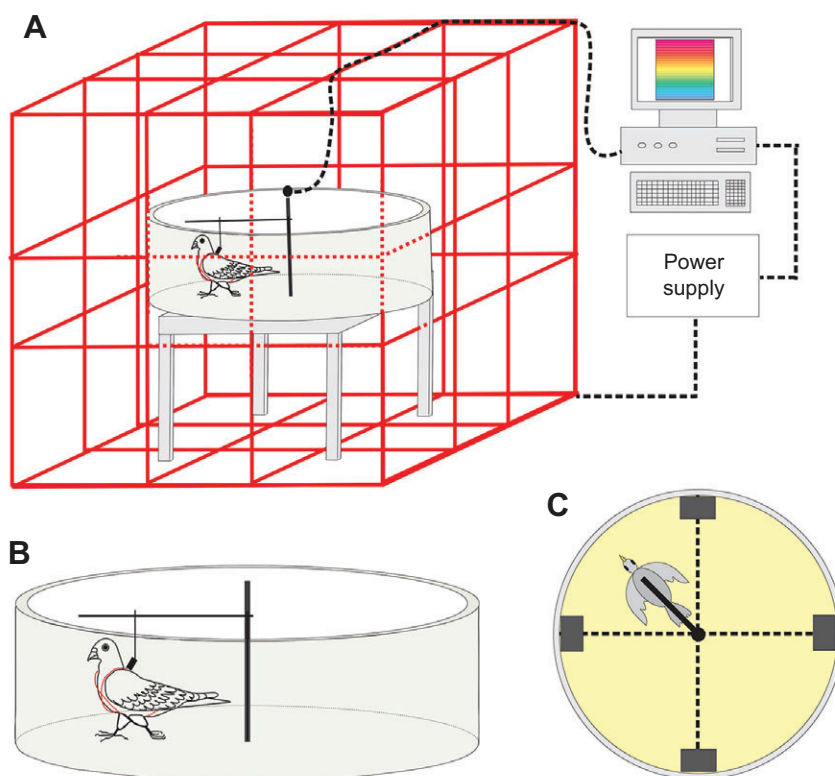


Fig. 1. Experimental setup for magnetic inclination conditioning paradigm. (A) Circular orientation arena (diameter 110 cm) surrounded by three-axis coil system (red lines) [figure adapted from Merritt (Merritt, 1983)], which generated a spatially uniform magnetic field inclination cue throughout the entire arena. This type of magnetic cue is in contrast to spatially variable magnetic anomalies used by past conditioning studies (e.g. Mora et al., 2004). Magnetic field intensity in the arena was controlled in real time by customized software based on the position of the horizontal tracker arm over time. Note that the four feeder-response units are not shown for clarity in A and B. (B) Pigeon walking in arena whilst attached by harness (red) to horizontal tracker arm [adapted from previous sea turtle studies (e.g. Lohmann, 1991)], with tracker arm orientation in the arena detected by angular decoder every 200 ms. (C) Top view of arena showing pigeon attached to horizontal tracker arm as well as position of four feeder-response units (gray rectangles), each with a pecking key above an automated food reservoir, located around periphery of the circular arena in the four cardinal directions (geographic north, south, east and west; dashed lines). Note that images are not drawn to scale.

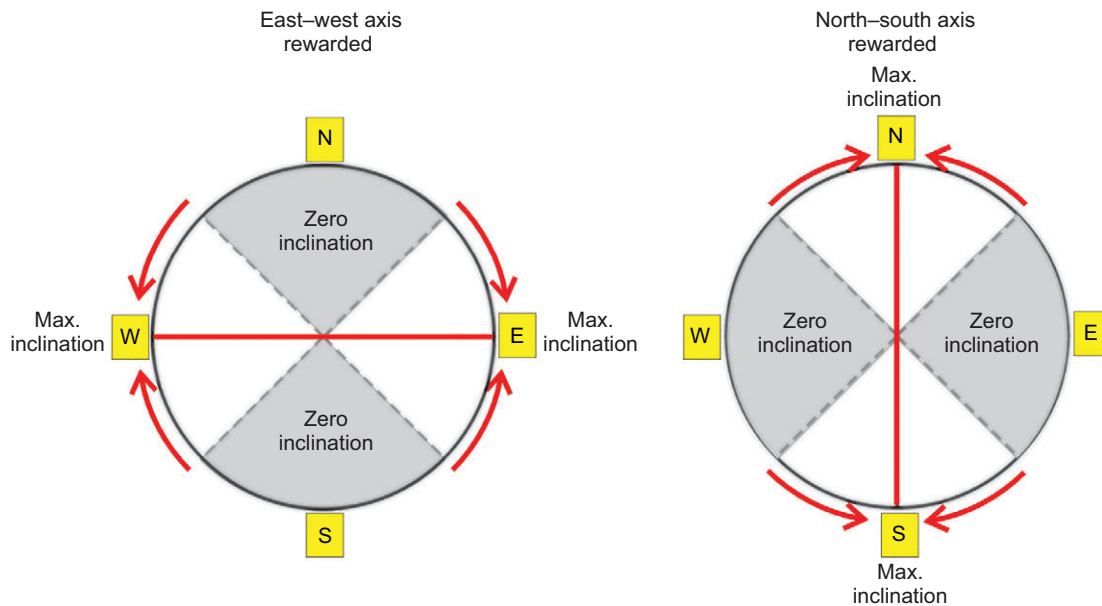


Fig. 2. Arrangement of reinforcement contingencies for north-south and east-west trials. During north-south trials the 90 deg zones with either the north and south feeders at their center were associated with magnetic inclination rapidly increasing (red arrow) from -3 deg to a maximum inclination value of $+85$ deg (-3 deg to $+71$ deg for truncated magnetic inclination range series) when the pigeon approached either of these two feeders. In contrast, the 90 deg zones with either the east or west feeders at their center were associated with a constant value of -3 deg ('zero inclination' zones). During these trials, a pecking response at either the north or south feeder was rewarded with access to the food reservoir for 10 s. During east-west trials the east and west feeders were associated with the rapid inclination change and responses at these feeders rewarded with food access. North-south and east-west trials were presented in pseudo-random order during each 32-trial session. Feeders indicated by yellow boxes labeled with cardinal direction and rewarded axis indicated by red line.

conditioning series and performed well during the parallel-anti-parallel control series). After the initial eight sessions, the standard error for the mean performance across birds for each session no longer included the chance level (50%) for almost all sessions (for sessions 14 and 16 the s.e. crossed the chance level, although it is not clear why the birds performed more poorly on these two days). We then calculated the mean performance over these last 17 sessions for each bird and then the mean discrimination performance across all birds ($n=6$; mean \pm s.e., $64.98 \pm 3.31\%$; 95% confidence interval 56.47% to 73.49%). This was significantly different from chance level (50%), both when comparing individual mean bird performances to chance level (unpaired t -test: $t=4.442$, $P<0.01$) as well as when looking at the mean performance of all birds being above chance level over the course of all sessions in this series (Wilcoxon signed ranks test: t -value=0, $P<0.001$). In summary, the pigeons were successfully able to perform the discrimination task and that performance was consistently above chance level over time.

Coil on-off control sessions

White noise was used to mask any humming sounds emanating from the coils. Furthermore, the axis of correct feeder choices (north-south or east-west) was disassociated from any visual cues in the experimental room by selecting from a pseudorandom schedule which of the two feeder axes in the arena was associated with the changing inclination values for a given trial. To test whether any other alternative cues may have been used by the pigeons to identify a correct feeder, we conducted a coils on-off series of control sessions with four of the original six pigeons. Four sets of two coils-off control sessions (no current sent to coils) were alternated with two consecutive standard sessions (same procedure as for the initial conditioning series) (Fig. 3B).

Mean discrimination performance averaged across all birds for all standard sessions of the coils on-off control series was $66.95 \pm 2.16\%$

($n=4$, 95% confidence interval 60.06% to 73.83%). This was significantly above chance level (50%; unpaired t -test: $t=7.550$, $P<0.001$) and slightly higher than the performance during the initial conditioning series. The birds' mean performance for each session was also consistently above 50% over time (Wilcoxon signed ranks test: t -value=0, $P<0.001$). The performance in standard sessions was in contrast to the performance for the coils-off sessions ($n=4$, $50.30 \pm 1.68\%$, 95% confidence interval 44.94% to 55.65%), during which performance fell to around chance level (50%; unpaired t -test: $t=0.176$, $P>0.05$; Wilcoxon signed ranks test: t -value=14, $P>0.05$). Furthermore, each bird's individual performance was significantly different from its performance during coils-off sessions (paired t -test: $t=7.2289$, $P<0.01$). Therefore, the pigeons were not able to perform the discrimination task when the coils were turned off.

Coils parallel or anti-parallel control sessions

Next, we conducted a parallel-anti-parallel control series using double-wrapped coils as suggested by Kirschvink et al. (Kirschvink et al., 2010) with seven pigeons (three from the initial conditioning series as well as four additional birds, which were pre-trained until a comparable average performance as in the initial conditioning series was achieved). The purpose of this was to eliminate the possibility of the white noise not having masked all sounds emanating from the experimental equipment and thus being used as alternative cues. This approach also eliminated the use of any other alternative cues (e.g. heat or vibration) potentially associated with the varying amounts of current passing through the coils during a trial.

Four sets of two consecutive sessions with the current running through the double-wound coils in the same direction (parallel sessions, i.e. same magnetic inclination cues as for the initial conditioning series) were alternated with three sets of two consecutive sessions with the current running in the opposite

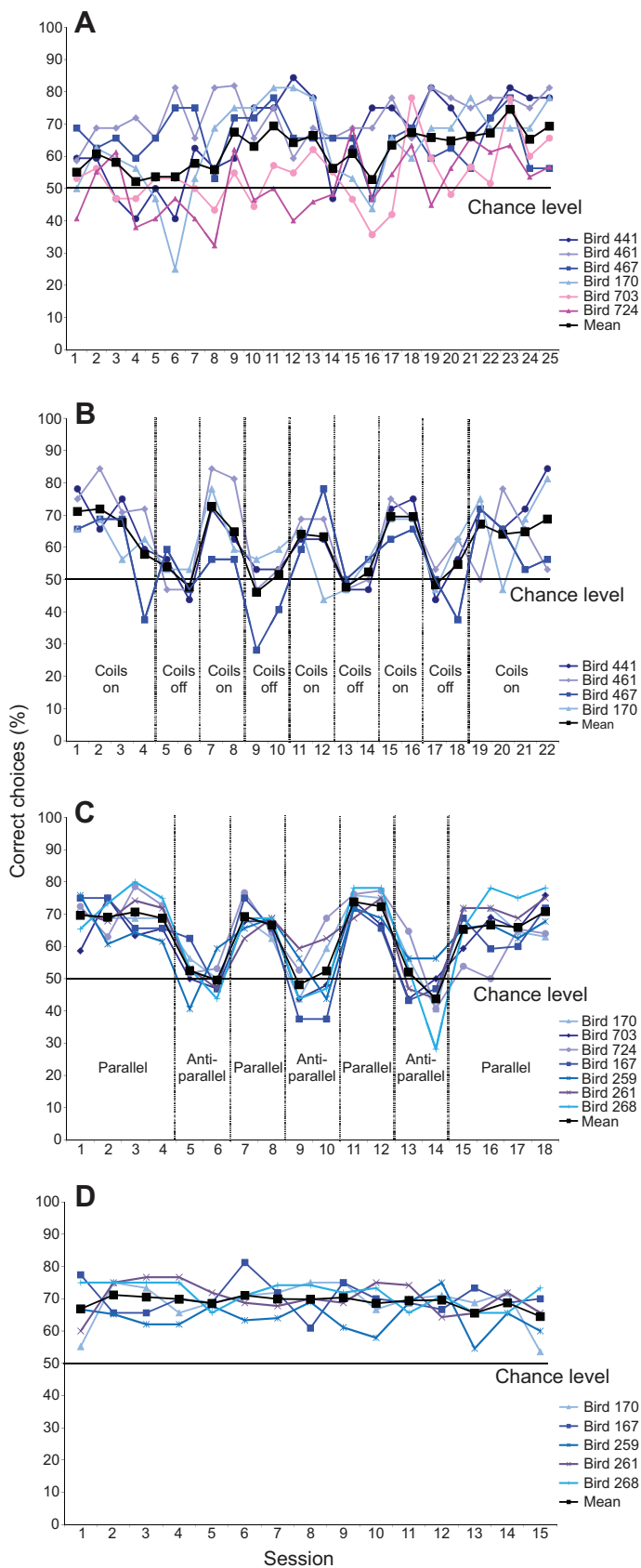


Fig. 3. Percentage of correct choices made by individual pigeons during each session. (A) Initial conditioning series (chance level 50%). (B) Coils on–off control series consisting of standard sessions as well as control sessions during the latter of which the coils system had no current input. (C) Parallel–anti-parallel control series for which sessions with current running parallel through a double-wound coil system were alternated with sessions with current running anti-parallel through the coil system. (D) Truncated magnetic inclination range series for which the range in magnetic inclination changes was reduced from -3 deg to $+85$ deg to a narrower range of -3 deg to $+71$ deg to eliminate simultaneous changes in declination being a possible alternative discrimination cue.

across all birds ($n=7$, $69.08\pm 0.91\%$, 95% confidence interval 66.86% to 71.30%) was significantly above chance level (50%; unpaired t -test: $t=19.763$, $P<0.001$; Wilcoxon signed ranks test: t -value=0, $P<0.001$). This was significantly different (paired t -test: $t=9.4691$, $P<0.001$) from the mean performance for each bird for the anti-parallel coils sessions ($n=7$, $49.73\pm 1.47\%$, 95% confidence interval 46.13% to 53.32%; chance level 50%; unpaired t -test: $t=0.186$, $P>0.05$; Wilcoxon signed ranks test: t -value=9, $P>0.05$). This shows that the pigeons were not able to perform the discrimination task when the current ran anti-parallel through the coils. One curious finding was that the relatively small standard error associated with the discrimination performances during the earlier parallel sessions was not maintained during the last four parallel sessions. We do not know why this occurred, but complex conditioning paradigms inherently yield a certain amount of variability in the discrimination performances.

Mean discrimination performance during both coils on–off and the parallel–anti-parallel series fell to around chance level with relatively little variance. The birds were still very motivated during coils-off trials as well as during anti-parallel sessions to move between feeders and peck the response keys when they were lit, i.e. they did not make their choices completely randomly nor did they just sit in front of a single feeder for the entire session pecking only that response key. Instead they sometimes adopted a combination of alternative choice behaviors, with the combination being unique to each bird.

We are confident for two reasons that such alternative strategies did not significantly contribute to the discrimination performance observed during standard sessions. Firstly, these strategies were not sufficient to raise performance above chance level during the two control series for which no magnetic discrimination cues were available to the birds. Secondly, an analysis of the discrimination performance for the last 10 standard sessions in Fig. 3A revealed that all six pigeons generally favored choosing a different feeder on a subsequent trial irrespective of whether they were rewarded (Win-shift: $70.24\pm 1.75\%$ of subsequent trials with choices to a different feeder) or not (Lose-shift: $73.51\pm 2.93\%$ of trials). This is as would be expected if the birds did not follow any other alternative behavioral strategy in making feeder choices. This is because when four feeders are available then an unbiased behavioral strategy would result in the bird choosing the same feeder in a quarter of the time as during the previous trial and three-quarters of the time choosing one of the other three feeders. We would like to point out here that shifting to another feeder even though the stimulus presented could be the same as in the previous trial can still result in a correct response, because for each trial both feeders on the axis associated with the changing magnetic inclination stimulus were rewarded. Therefore, we observed no behavioral-strategy bias in their feeder choices across trials that could have influenced the pigeons' discrimination performance on inclination-meaningful test trials.

direction (anti-parallel sessions, i.e. background magnetic inclination cues) (Fig. 3C). Similar to the coils on–off control experiment, for parallel coil sessions the mean discrimination performance averaged

As described above, retro-fitting our coil system for the anti-parallel sessions resulted in a weak residual magnetic intensity gradient being produced by the coils instead of complete cancellation of the coils' fields, yet the pigeons' discrimination performance fell to chance level during anti-parallel sessions. This is not surprising because such a weak stimulus would be considerably more difficult to discriminate and thus the birds were highly likely to switch for the same level of motivation (85% free-feeding weight and 10 s feeder access per correct choice) to alternative behavioral strategies (see above), which still yielded a reward for 50% of the trials. This is especially true given that the birds were only exposed to this weaker stimulus for two sessions at a time and for a total of only eight sessions. Therefore, no conclusions can be drawn from this control experiment about whether or not pigeons are able to perceive such small changes in magnetic inclination. To test such a possibility, a carefully designed threshold study will need to be performed in the future.

In summary, because discrimination performance fell to chance level not only when current to the coils was disconnected, but also when current ran through the coil system in an anti-parallel fashion, the two control experiments demonstrated that neither the current itself nor any other alternate non-magnetic cues could have been used by the birds to discriminate the magnetic intensity cues in this experimental setup. This result is consistent with the fact that the coils felt barely warm to touch during sessions, the arena's support base rested on a concrete floor without contact to the coils, and auditory as well as visual cues were controlled for.

Truncated inclination range sessions

To eliminate the possibility that the pigeons used differences in declination between north-south and east-west trials to solve the discrimination task, we conducted with five of the seven birds from the parallel-anti-parallel series 15 sessions for which the upper magnetic range was truncated (see above) (Fig. 3D).

Our results show that homing pigeons are able to discriminate magnetic inclination changes from -3 deg to $+71$ deg from inclination values held constant at -3 deg with the mean performance of birds ranging between 64% and 71%. We next calculated the mean performance over all sessions for each bird and then the mean discrimination performance across all birds ($n=5$, $68.97 \pm 1.26\%$, 95% confidence interval 65.72% to 72.22%). The latter was slightly higher than the mean performance during the initial conditioning series and the coils-on sessions of the first control series, but comparable to the performance level during the parallel sessions of the second control series. Furthermore, the discrimination performance for the truncated inclination range was significantly different from chance level (50%). This was true when comparing individual mean bird performances with chance level (unpaired t -test: $t=14.432$, $P<0.001$) as well as when looking at the mean performance of all birds being above chance level over the course of all sessions in this series (Wilcoxon signed ranks test: t -value=0, $P<0.001$). Therefore the pigeons were able to perform the discrimination task and that performance was consistently above chance level over time.

We did not observe a statistically significant change in the pigeons' performance over the course of the sessions of the truncated inclination range series (Linear mixed model ANOVA, type III test of fixed effects: $F_{\text{Session}}=2.520$, $P=0.117$). That is, there was no traditional acquisition curve for the conditioned response to this inclination stimulus, which was not surprising given the considerable experience the birds already had with the overall conditioning paradigm. A systematic difference between subjects

was detected (Linear mixed model ANOVA, type III test of fixed effects: $F_{\text{Subject}}=4.556$, $P=0.003$) because bird 259 had a slightly poorer and more variable performance level.

DISCUSSION

The results presented here provide evidence that homing pigeons are able to discriminate differences in the properties of magnetic inclination during a conditioning task. During the initial conditioning series, homing pigeons were required to select within a circular arena one of two possible feeders associated with a rapid change in inclination value (-3 deg to $+85$ deg) to obtain a food reward whilst the other two available feeders were associated with an unchanging inclination value of close to 0 deg. All pigeons performed consistently above chance level (50%).

During two series of control experiments, the possibility that alternative cues (e.g. sound, vibration or pseudorandom order of cue presentation) were used by the pigeons to select correct feeders was tested with discrimination performances dropping to chance level whenever the magnetic coils were switched off or current through the coils was run in an anti-parallel fashion. During the latter series, the residual changes in magnetic intensity of up to 4300 nT, as experienced by the pigeons during anti-parallel conditions, could not be used by the pigeons to identify the correct feeders. These intensity changes were greater than the maximally 3500 nT changes occurring during parallel sessions, thus indicating that the birds were not able to use intensity changes in this conditioning paradigm to locate rewarded feeders.

During the truncated inclination range series, the rewarded inclination stimulus was reduced in range (-3 deg to $+71$ deg) to test the possibility of simultaneous residual changes in magnetic declination serving as a discrimination cue on their own. The pigeons continued to perform the discrimination task during the truncated series consistently above chance level whilst changes in both intensity and declination were below the levels that the birds had previously failed to discriminate during the anti-parallel control series.

This series therefore confirmed that the pigeons were able to detect and use magnetic inclination on its own as a discrimination cue to solve the discrimination task. Previous successful magnetic conditioning studies with pigeons had trained the animals to discriminate magnetic anomalies, which consisted of both changes in magnetic intensity and inclination, and were generated either by magnetic coils or a group of bar magnets (Mora et al., 2004; Thalau et al., 2007; Freire et al., 2012). Studies with homing pigeons (Wilzeck et al., 2010) as well as two other bird species, the domestic chicken (*Gallus gallus*) (Freire et al., 2005) and zebra finch (*Taeniopygia guttata*) (Voss et al., 2007; Keary et al., 2009), conditioned the birds to a shift in the horizontal component of the magnetic field.

Overall, the discrimination performance during the initial conditioning series was very similar to a previous conditioning study that required homing pigeons to discriminate the presence and absence of a magnetic anomaly varying in both intensity and inclination [mean discrimination performance of around 65% with a chance level of 50% (Mora et al., 2004)]. The mean discrimination performances presented here increased slightly over the course of the study to almost 70% during the parallel sessions of the second control series, probably as a result of increased experience with the experimental task. A similar level of performance was also achieved during the truncated inclination range series. Another recent study, which required homing pigeons to discriminate magnetic intensity cues within a virtual magnetic map paradigm had achieved relatively

better performance [45–55% with 25% chance level (Mora and Bingman, 2013)], but this was a substantially different type of discrimination task, which makes comparisons in performance levels difficult.

It is well established in the animal psychobiology literature that discrimination tasks, which require the animal to move between different manipulanda (feeder-response units) consequently cause a separation of the stimulus (magnetic inclination cues), response (key pecking) and reinforcement (food reward) in space and time (for a review, see Mora et al., 2009). This then results in a considerably lower discrimination performance, as observed here, compared with a discrimination task in a traditional Skinner box, which requires a stationary pigeon to discriminate sensory cues (typically 90–100% performance in a simple visual discrimination task). Consequently, how high above chance level the discrimination performance resides does not necessarily reflect the birds' sensitivity to magnetic inclination in the field in the behavioral context of free-flying navigation. To determine how sensitive pigeons are to inclination cues, a threshold study will have to be conducted, which progressively reduces the size of the stimulus to be discriminated until discrimination performance consistently resides around chance levels.

The pigeons in this study could have (1) used the actual inclination values at the individual feeders to solve the discrimination task, (2) compared the changing inclination with the steady value that differentiated the areas on either side of the two feeder types, or (3) utilized a combination of these two strategies. Further experiments are needed to differentiate these possibilities, but it appears likely that the pigeons are at least capable of the first option because birds inexperienced with the task spent a considerable amount of the sampling period walking in circles in the arena from feeder to feeder. In contrast to this, during the truncated inclination range sessions, birds typically either remained during the sampling period at the feeder chosen during the previous trial or moved clockwise (or counterclockwise) by 90 deg to the neighboring feeder before making their choice. That is, they either only minimally moved around the arena or not at all depending on whether they chose the same feeder as during the previous trial or one of the neighboring feeders.

The question, however, also arises as to whether absolute inclination values and/or the switch from positive to negative inclination were detected. The latter refers to the fact that because the inclination changed from –3 deg at the unrewarded feeders to +85 deg (+71 deg for the truncated series) at the rewarded feeders, there was a moment where inclination switched from negative to positive. In all the species studied so far, the avian magnetic compass has been shown to be an inclination compass, i.e. birds tend to disregard the polarity of the magnetic field vector, but only attend to the downward- versus upward-pointing aspect of the vector to discern pole-ward versus equator-ward directions along the north–south axis (for a review, see Wiltschko and Wiltschko, 2007; Wiltschko and Wiltschko, 2009). Therefore, the fact that the inclination with our magnetic coil setup decreased from +85 deg (later +71 deg) to –3 deg, and thus changed polarity, should not have been relevant to the discrimination task. This is because the birds would have experienced with their inclination compass only an absolute change from 85 deg (later 71 deg) to 0 deg and then a slight increase again to 3 deg when moving from a rewarded feeder toward an unrewarded feeder on either side of the rewarded one.

In terms of sensitivity, it had been previously shown that pigeons are able to discriminate the presence and absence of a magnetic anomaly varying mostly in intensity (peak intensity of 189 μ T compared with 44 μ T background intensity), but nevertheless

varying also significantly in inclination (peak inclination of –80 deg compared with –64 deg) (Mora et al., 2004). The results presented here show that pigeons are able to detect differences in magnetic inclination independent of any meaningful variation in intensity. To facilitate learning of the discrimination task, the size of the inclination change was initially set to be as close as possible to the maximum change of 90 deg whilst minimizing any simultaneous changes in magnetic inclination and declination. The truncated inclination range series indicates that the birds are at least able to discriminate a minimum inclination change of 70 deg. The tracker arm was, however, typically not exactly lined up with the feeder position as the pigeon was sitting in front of a feeder. Furthermore, inclination changed by 1.6 deg per 1 deg rotation of the tracker arm (70 deg over 45 deg arena zone). Therefore, a tracker arm positioned 15 deg to either side of a feeder, which was frequently observed, would reduce the inclination change experienced by the bird to ~50 deg. Given that birds use inclination cues as part of their magnetic compass during homing, sensitivity to inclination cues considerably greater than the one indirectly inferred by our results is almost certain.

Irrespective of what property of the inclination stimulus the pigeons utilized, the fact that the pigeons were able to discriminate differences in magnetic inclination provides evidence that they must possess the ability to detect, differentiate and process magnetic inclination information. Very little is yet known about how magnetic inclination is perceived, or where and how such information is processed in the brain. Researchers have been looking for a candidate magnetoreceptor in homing pigeons and migratory birds for several decades. Some progress has been made in recent years in relation to a putative receptor system for the magnetic compass in the retina of migratory birds (for reviews, see Mouritsen, 2012; Mouritsen and Hore, 2012). A candidate molecule (cryptochrome) is thought to alternate between a singlet and a triplet state in a light-mediated radical-pair process with the ratio of the two states depending on the orientation of the magnetic field vector axis (Ritz et al., 2000). Such a system would, however, not be suitable to determine the vector's inclination angle. It is this angle that is thought to help distinguish pole-ward from equator-ward directionality during the compass step of navigation.

A second theoretical mechanism, based on behavioral, electrophysiological and anatomical work in rainbow trout (*Oncorhynchus mykiss*) (Walker et al., 1997), proposes an iron-mineral-based receptor in the olfactory epithelium, which responds to magnetic field intensity changes and potentially also to changes in inclination angle (Walker et al., 2002). However, it should be noted that the existence of a magnetite and maghemite-containing receptor structure on the underside of the pigeon's upper beak as proposed by Fleissner et al. (Fleissner et al., 2007) has been recently called into question because macrophages may have been interpreted as iron-mineral particles (Treiber et al., 2012). Most recently, the lagena otolith organ in the pigeon's inner ear has also been raised as a possible location for a third type of magnetoreceptor (Wu and Dickman, 2011). Future impairment studies with the conditioning paradigm presented here should provide the opportunity to distinguish between the various alternative mechanisms of inclination perception described above as well identify the regions in the brain where such information is processed.

In terms of the nerve carrying magnetic inclination information to the brain, the ophthalmic branch of the trigeminal nerve was identified early on as a likely candidate. Beason and Semm (Beason and Semm, 1996) were first to demonstrate in a bird species, the

bobolink (*Dolichonyx oryzivorus*), that the ophthalmic branch of the trigeminal nerve carries magnetic information, although it was not clear which component of the magnetic field (magnetic intensity, magnetic inclination or possibly both) was transmitted. Also, the ability to discriminate the presence and absence of a magnetic anomaly with changes in intensity and inclination was abolished following the sectioning of this nerve in homing pigeons (Mora et al., 2004). Whilst a possible role of the trigeminal nerve during homing by pigeons in Italy at distances of up to 105 km has been dismissed (Gagliardo et al., 2006; Gagliardo et al., 2009), several recent studies have investigated the role of the ophthalmic branch of the trigeminal nerve in transmitting magnetic information to the brain in migratory and non-migratory birds such as European robins (*Erithacus rubecula*) (Heyers et al., 2010) and Pekin duck (*Anas platyrhynchos domestica*) (Freire et al., 2012) and its role in correcting for displacement during migration in, for example, reed warblers (*Acrocephalus scirpaceus*) (Kishkinev et al., 2013). Two recent studies by Wu and Dickman (Wu and Dickman, 2011; Wu and Dickman, 2012) have also shown involvement of pigeon trigeminal neurons in magnetoreception, as well as recorded neuronal responses in the pigeon's brainstem in response to changes in direction, intensity and polarity of the magnetic field.

MATERIALS AND METHODS

Experimental subjects

Ten adult homing pigeons (*Columba livia livia f. domestica* Gmelin 1789), between 2 and 3 years old and with previous homing experience, were housed individually at Bowling Green State University in Bowling Green, OH, USA. They were provided with water *ad libitum* and maintained between 85 and 87% free-feeding body weight to ensure motivation during the conditioning task. Because the experiments described below took place over the course of almost 1 year because of logistical constraints, not all birds participated in the same experiments.

Experimental setup

All experiments were conducted in a circular arena (diameter, 110 cm; wall height, 38 cm) situated centrally atop cinder blocks inside a three-axis magnetic coil system (Fig. 1A). Pigeons were individually harnessed to a horizontal tracker arm (Fig. 1B). The harness consisted of two 1.5-cm-wide strips of fabric cat collars sewn together in the shape of an 'X' with a clip attached at the joint and resting between the wings on the pigeon's back for attachment to the tracker arm.

The horizontal tracker arm was attached to a central, vertical shaft in the arena (Fig. 1B). The pigeon was able to walk freely around the periphery of the arena in either direction at a distance of 35 cm (point of attachment of harness on pigeon's back to tracker arm) from the center of the arena. An angular decoder located at the base of the shaft recorded the position of the pigeon to the nearest degree once every 200 ms. Four automated feeder-response units were situated against the wall of the circular arena aligned with the four cardinal directions in the testing room [geographic north (N), south (S), east (E) and west (W); Fig. 1C]. Each feeder-response unit contained an illuminated food magazine with food pellets (Purina® Check Pigeon Chow pellets) that could be made accessible to the pigeon and a response pecking key above the food magazine that could be illuminated. Each feeder's food magazine was raised and lowered by compressed air to avoid any localized distortions to the magnetic field typically associated with motor-driven feeders. An incandescent white light was mounted centrally above the circular arena as the trial light. The behavior of a pigeon in the arena was monitored via a centrally-mounted close-circuit video camera viewing the arena from above.

Pre-training procedure

The pigeons were familiarized with the harness initially by being fed in their home cage whilst wearing the harness. During pre-training sessions, they were next attached via the harness to the tracker arm in the experimental

arena with food placed on the floor in the locations where the four feeders would be later situated. Once the pigeons had acclimated to being attached to the tracker arm and ate freely in the arena, the four feeders were added to the arena as described above. Pigeons were then familiarized with the food magazine being raised and lowered via custom-written software in a pseudo-random order on each of the four feeders to allow food access for 10 s. Finally, the pigeons were required to peck a feeder's illuminated pecking key before the feeder's food magazine was raised, with pecks being detected by a micro-switch situated behind the key. Illuminated pecking keys were made available in a pseudorandom order during each pre-training session's 16 trials to avoid any response biases.

Next, the pigeons were exposed for three to five sessions to the reinforcement contingencies associated with the two types of inclination patterns described above. Trials were performed as described below, but if an incorrect choice was made, the same stimulus presentation was repeated until a correct choice was made (correction trials). After a correct choice, the next stimulus (changing magnetic inclination at either the north-south feeders or the east-west feeder) would be selected from the pseudorandom order of stimulus presentation. The order consisted of two consecutive eight-trial blocks, with each block being comprised of a random order of four north-south and four east-west trials. Therefore each session consisted of at least 16 trials with 16 food rewards being delivered.

During pre-training, the pigeons were not only exposed to the reinforcement contingencies, but on some trials birds were lured to the correct feeder by repeatedly illuminating the pecking key and/or raising the feeder magazine of the correct feeder closest to the bird's position in the arena. This strategy was used whenever birds appeared to develop alternative choice behaviors (e.g. remaining stationary in front of the same feeder over the course of several trials or going from one feeder to the next from trial to trial independently of stimulus presentation) to ensure the birds experienced the association of the correct feeder for a given stimulus in the presence of a food reward. Over the course of the five sessions, the number of trials with assistance was gradually reduced at a pace individually tailored to each bird's progress in learning the task, and the number of trials during which the pigeon was allowed to make its own choices was increased. Therefore, by the end of pre-training, the pigeons were already very familiar with the basic discrimination task, which is why no learning curve was observed during the first experimental conditioning series. Only when the bird made a correct choice upon first presentation of a given stimulus from the pseudorandom order was this choice counted toward the percentage of correct choices for a given session.

Magnetic inclination stimulus

The three-axis magnetic coil system [four 240×240 cm square coils per axis with a coil spacing of 89/62/89 cm; coil-winding ratio of 26:11:11:26; 14 AWG (2.08 mm²), PVC-insulated copper coil wire, aluminium frame, adapted from Merritt (Merritt, 1983)] (Fig. 1A) was powered by three power supplies (BK Precision, Model 9123A, 0–30 V/0–5 A single-output programmable DC power supply with constant current output), one assigned to each axis (*x*, *y* and *z*) of the coil system. This coil system was able to generate a sphere-shaped area in the center of the coils, approximately the size of the diameter of the experimental arena, within which the generated magnetic field was very uniform, albeit not perfectly uniform, as is typically the case with this type of coil system. That is, the magnetic field vector was very similar in terms of spatial orientation (inclination and declination) and length (intensity) for all spatial points inside this 'bubble'. By changing independently the current output to each of the three coil axes, we were able to either increase or decrease the magnetic field inclination in real time and relatively uniformly throughout the entire experimental arena. A white noise generator positioned next to the coil system masked any potential humming noise emanating from the coil system. The power supplies and associated relays were fully automated and located in a control room adjacent to the room containing the coil system. The coil wiring remained cool to the touch throughout the conditioning sessions.

The amount of current supplied to each coil axis was fully automated using custom-written software. The arena was divided into four 90 deg zones with each zone extending 45 deg on either side of one of the four feeders (Fig. 2). As the pigeon rotated the horizontal tracker arm while walking

around the periphery of the arena, the position of the tracker arm was determined by the angular decoder. Based on this position, the software simultaneously generated a magnetic field vector, whose inclination was adjusted to match that required for the tracker arm's position for one of two possible patterns as follows. For north–south trials (see magnetic conditioning procedure below), inclination was held constant at close to 0 deg in the zones with the east and west feeders at their center. Whenever the pigeon moved from the border of the east or west zone toward the north or south feeders, inclination rapidly increased from –3 deg at the border to +85 deg at the feeder and then decreased again from +85 deg at the north or south feeders to –3 deg at the border of the north and south zone with the east and west zones. For east–west trials, this pattern of inclination change was rotated by 90 deg. That is, inclination was held constant at close to 0 deg around the north and south feeders, but changed rapidly around the east and west feeders.

The background field and the magnetic field parameters generated by the coil system were characterized with a FVM handheld three-axis vector fluxgate magnetometer (Meda Inc.) at the head height of a walking pigeon and at a distance of 30 cm from the center of the arena. Because of structural steel and electrical circuits in the walls of the experimental room, the background inclination and declination varied between +61.9 deg and +68.2 deg ($+65.0 \pm 0.4$ deg, mean \pm s.e.) and +4.8 deg and +24.5 deg ($+17.6 \pm 1.2$ deg), respectively (see Mora and Bingman, 2013). Background magnetic intensity varied around the periphery of the arena along a SW to NE gradient ($47,300 \pm 330$ nT with values ranging from 45,350 to 49,100 nT).

In contrast to the variations in the background field, the coil system itself generated a magnetic field vector whose inclination varied, as expected, strongly during the trial from –3 deg to +85 deg when the birds moved 360 deg around the periphery of the arena from feeder to feeder with the pattern of change being 90 deg out of phase for the north–south and east–west trials (supplementary material Fig. S1A). Magnetic intensity and declination changes experienced by the bird whilst walking from feeder to feeder around the periphery of the arena ranged for intensity from 45,500 to 49,000 nT (supplementary material Fig. S1B) and for declination from +9 deg to +26 deg (supplementary material Fig. S1C) for both north–south and east–west trials as a result of the variations in the background field described above. During control sessions with anti-parallel current (see below), there were very small changes in inclination (62 deg to 66 deg) as well as some changes in intensity (42,000 to 49,000 nT) and declination (15 deg to 26 deg). Therefore the magnetic parameters experienced during anti-parallel sessions were generally comparable to those experienced during sessions with parallel current, i.e. during training and during sessions of the initial conditioning series as well as the coils-on sessions of the first control series and the parallel sessions of the second control series (see below; supplementary material Fig. S2).

When we measured the differences between the two types of trials, i.e. between the north–south and east–west trials, in terms of inclination, intensity and declination specifically for each position around the arena, the maximum differences were 87 deg, 3500 nT and 13 deg for normal (parallel) sessions and 1 deg, 4300 nT and 4 deg for anti-parallel sessions. Whilst the difference in intensity between the two types of trial was greater for the anti-parallel than the parallel sessions, discrimination performance nevertheless fell to chance level for anti-parallel sessions so that differences in intensity were not used as a discrimination cue by the birds during parallel sessions (see also Results and Discussion sections). There was, however, a greater difference in declination between the two types of trials in parallel sessions compared with anti-parallel sessions such that the theoretical possibility remained that the observed discrimination behavior was due to differences in declination between north–south and east–west trials. We therefore conducted an additional experimental series for which we truncated the upper magnetic range used. For truncated sessions, maximum differences between the north–south and east–west trials in terms of inclination, intensity and declination were 73 deg, 3500 nT and 3 deg for normal (parallel) sessions (supplementary material Fig. S3).

Magnetic conditioning procedure

Each session consisted of 32 discrete trials. The start of each trial was indicated by the trial light being switched on. During a sampling period (15 s),

measured with a stop watch, the pigeon was able to move freely around the periphery of the arena. During this time, the custom-written software tracked the pigeon's position around the periphery of the arena in real time. The current output to the three coil axes was simultaneously adjusted to generate uniformly throughout the entire arena the magnetic inclination value appropriate for the pigeon's current position around the periphery of the arena for either a north–south or east–west trial. The pigeon was thus experiencing a dynamic magnetic inclination environment with the inclination presented to the pigeon changing based on the position of the pigeon's tracker arm and whether a current trial was a north–south or east–west trial.

Whether the north and south feeders or the east and west feeders were associated with the rapid increase in inclination to +85 deg (and correspondingly the east and west or north and south feeders with the steady inclination close to 0 deg), was determined for each trial based on a pseudorandom schedule to avoid the pigeons using any visual cues to solve the spatial conditioning task. At the end of the 15 s sampling period, pecking lights on all four feeders were illuminated. The pigeons were trained to choose one of the two feeders associated with the rapidly increasing magnetic inclination. A correct choice was rewarded with a 10 s access to the food magazine, whereas incorrect choices resulted in a time penalty of 10 s being added to the 5 s inter-trial interval (ITI), during which the arena was dark and only the background magnetic field was present. The magnetic stimulus for the next trial depended on a pseudo-random sequence rather than the correctness of the choice made during the preceding trial (see above).

Coil control procedures

For the coils on–off controls series, coils-on sessions were alternated with coils-off sessions. During a coils-on session the procedures described above were followed. By contrast, during coils-off sessions the custom-written software did not supply any current output from the three power supplies to the three-axis magnetic coil system. Therefore, the relays in the control room were still producing audible clicks as if the direction of current coming from one or more of the power supplies were switched from clockwise to counterclockwise for a coil axis, but no magnetic field was produced by the coil system.

For the parallel–anti-parallel control series, the number of each coil's wire loops was halved and a switch added that allowed the current in both halves of the coil to run either parallel (in the same direction) or anti-parallel (in opposite directions). Whilst the 26 loops of the outer coil were halved into two sets of 13 loops, for the 11 loops of the inner coils, we added an additional loop of wire that was only supplied with current during the anti-parallel setting so that current ran through 6 loops in one direction and through 5+1 loops in the other direction. When running parallel, the same magnetic field inclination was produced as for standard sessions, but when running anti-parallel the two coil halves effectively cancelled each other out (supplementary material Fig. S2). A mean residual magnetic field inclination change of 0.5 ± 0.08 deg (mean residual intensity and declination were 1366 ± 98 nT and 1.0 ± 0.2 deg, respectively) remained that was probably due to the retro-fitting of the double coils system having not exactly halved the coils.

Statistical analysis

For each session performed by each bird, the percentage of correct choices out of 32 trials was calculated. We also calculated for each session the mean discrimination performance across all birds, which was plotted against the individual bird's percentage of correct choices for each session (Fig. 3). For details of all statistical tests, see Zar (Zar, 1999).

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of Bowling Green State University (Permit Numbers: 09-001 and 12-002).

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Competing interests

The authors declare no competing financial interests.

Author contributions

C.V.M. conceived and designed the experiments, interpreted the findings, and drafted and revised the article; M.L.A. executed the experiments and revised the article; V.P.B. interpreted the findings and revised the article.

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Supplementary material

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