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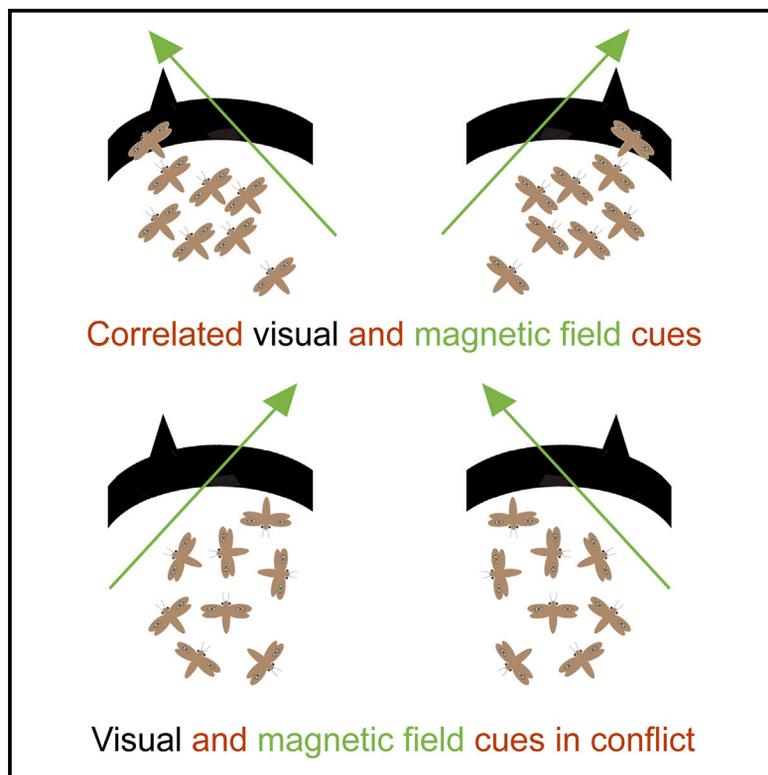
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Current Biology

The Earth's Magnetic Field and Visual Landmarks Steer Migratory Flight Behavior in the Nocturnal Australian Bogong Moth

Graphical Abstract



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In Brief

The nocturnal Bogong moth performs a highly directed long-distance migration to and from alpine caves in the Australian Alps. Dreyer et al. show that this moth senses the Earth's magnetic field and uses it together with visual landmarks to steer migratory flight behavior. The geomagnetic field might thus be used as a compass during migration.

Highlights

- The Australian Bogong moth, a nocturnal migrant, possesses a magnetic sense
- Bogong moths correlate visual and magnetic cues to steer migratory flight behavior
- When these cues are placed in conflict, moths become disoriented after a few minutes
- Bogong moths use the Earth's magnetic field and visual landmarks to steer flight



The Earth's Magnetic Field and Visual Landmarks Steer Migratory Flight Behavior in the Nocturnal Australian Bogong Moth

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SUMMARY

Like many birds [1], numerous species of nocturnal moths undertake spectacular long-distance migrations at night [2]. Each spring, billions of Bogong moths (*Agrotis infusa*) escape hot conditions in different regions of southeast Australia by making a highly directed migration of over 1,000 km to a limited number of cool caves in the Australian Alps, historically used for aestivating over the summer [3, 4]. How moths determine the direction of inherited migratory trajectories at night and locate their destination (i.e., navigate) is currently unknown [5–7]. Here we show that Bogong moths can sense the Earth's magnetic field and use it in conjunction with visual landmarks to steer migratory flight behavior. By tethering migrating moths in an outdoor flight simulator [8], we found that their flight direction turned predictably when dominant visual landmarks and a natural Earth-strength magnetic field were turned together, but that the moths became disoriented within a few minutes when these cues were set in conflict. We thus conclude that Bogong moths, like nocturnally migrating birds [9], can use a magnetic sense. Our results represent the first reliable demonstration of the use of the Earth's magnetic field to steer flight behavior in a nocturnal migratory insect.

RESULTS AND DISCUSSION

Many species of nocturnal moths undertake seasonal migrations of hundreds or thousands of kilometers to favorable new habitats, which are typically broad geographic regions, to take advantage of suitable temperatures and more abundant food and to reduce the risk of predators and infectious diseases [2]. When the season turns, one or more descendant generations of these original migrants then return. In contrast, newly eclosed

Bogong moths make a highly directed spring migration, from the mostly flat arid plains of their breeding areas in southeast Australia to a geographically restricted assemblage of high mountain caves in the Australian Alps, more than 1,000 km away (and thus well beyond their visual and olfactory range; Figure 1A). Once in the mountains, Bogong moths enter a period of 3–4 months of dormancy (aestivation) [3, 4]. At the beginning of autumn, the same individuals make a return migration to their breeding grounds to reproduce and die.

The Natural Migratory Directions of Bogong Moths

To study these forward and return migrations, we light-trapped Bogong moths during their southward spring migration near Narrabri in northern New South Wales, as well as during the return autumn migration in the Australian Alps near Adaminaby in southern New South Wales (see Figure 1B). Captured moths were tethered and flown outdoors for 10 min within a modified Mouritsen-Frost flight simulator [8], a cylindrical Perspex arena placed vertically on a table. Moths were tethered at the center of the arena to the end of a vertical shaft connected to an encoder that continuously measured the instantaneous orientations of steadily flying moths that were free to turn in any azimuthal direction under natural night skies (see Figures S1 and S2 for details of experimental sites and the simulator). Natural migratory directions of tethered moths in each location were as expected (Figure 1B): spring migrants on average flew geographic SSW (geographic south is 180° in the plot) toward the Australian Alps ($n = 18$, mean vector [MV] = 214°, 95% confidence interval 176°–238°, $R^* = 1.624$, $p < 0.001$), whereas autumn return migrants flew NNW ($n = 36$, MV = 337°, 95% confidence interval 290°–353°, $R^* = 1.668$, $p < 0.001$). Since the breeding grounds of Bogong moths stretch from western Victoria to southeast Queensland [10] (gray arrows in Figure 1B), we did not expect the autumn migratory direction to be the exact reverse of the Narrabri spring migratory direction.

The Earth's Magnetic Field and Visual Landmarks Steer Migratory Flight Behavior

Which sensory compass cues are used by Bogong moths to steer migration in the correct forward and return directions?



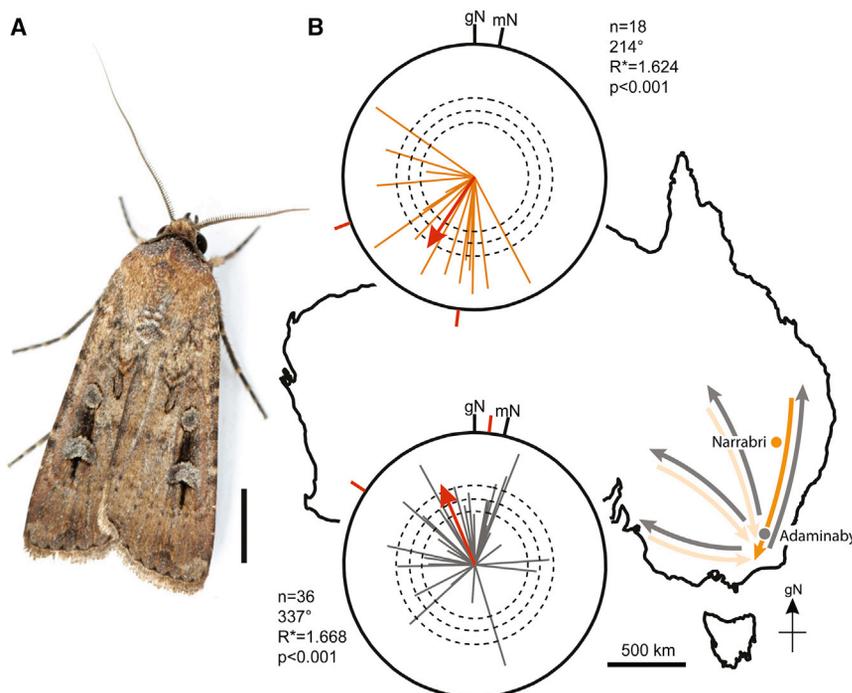


Figure 1. Natural Migratory Orientations of Bogong Moths

(A) A male Bogong moth (scale bar, 5 mm). Photo: Ajay Narendra.

(B) Moths caught during the spring migration near Narrabri ($n = 18$; orange vectors, upper plot) and the autumn return migration near Adaminaby ($n = 36$; gray vectors, lower plot) headed in their seasonally appropriate migratory directions (heavy orange and gray arrows on the map). The directedness (length) of each individual moth vector is given by its r value ($0 \leq r \leq 1$), where the outer radius of plots corresponds to $r = 1$. Each red mean vector (MV) results from weighting the mean directions and mean directedness (vector lengths) of all individual moths (Moore's modified Rayleigh test; see STAR Methods). Directedness of MV is given by R^* value. Dashed circles indicate required R^* value for statistical significance: $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, for increasing radius. In the outer radius of plots, $R^* = 2.5$. Red radial dashes indicate 95% confidence intervals. gN, geographic north; mN, magnetic north. A Mardia-Watson-Wheeler test confirmed that the spring and autumn migratory directions differ significantly ($W = 21.661$, $p = 1.98E-5$). See also Figures S1 and S2.

Radar studies reveal that migrating moths generally exploit favorably directed winds, adopting optimal flight headings that partially correct for crosswind drift to maximize distance traveled in their preferred inherited direction [5, 11], a feat requiring an internal directional compass sense [5, 6]. However, the nature of this compass remains enigmatic [5–7].

Visual cues, including celestial cues (such as the stars or the moon) and terrestrial landmarks, are obvious candidates, although these tend to be less reliable due to their changing positions (and/or prominence) over time. A more reliable compass cue is the Earth's magnetic field. Although the importance of a magnetic compass for long-distance migration in birds [9], sea turtles [12], and fish [13] has been long established, its role in long-distance migration in insects is unknown. Tentative evidence suggests that some migratory moths may sense magnetic fields [14–16], as may some butterflies [17, 18], such as the diurnal long-distance migratory monarch butterfly, *Danaus plexippus* [18], although in many of these studies very strong (unnatural) magnetic fields were used to show effects and the evidence is also conflicting [7, 19]. Hitherto studied migratory butterflies instead appear to rely primarily on a sun compass [7, 20–22], and it remains unclear how, or whether [19], they use the Earth's magnetic field for navigation in natural circumstances.

To test whether Bogong moths use a magnetic compass, we placed the flight simulator outdoors within a pair of single-axis Helmholtz coils that could turn the azimuthal direction of a natural Earth-strength magnetic field clockwise by about 120° without changing the field's strength or inclination angle (Figures S1B–S1F). In initial experiments over two experimental seasons, newly captured migratory moths, tethered in the simulator, were subjected to a 120° azimuthal field change to test whether they changed their flight direction accordingly (as occurs in birds hopping in an Emlen funnel during a similar stimulation paradigm

(e.g., [23]). Unlike birds, moths either failed to react or reacted in an unpredictable manner, suggesting that they do not rely solely on a magnetic compass. Since many nocturnal insects have exquisite dim-light vision [24], remaining weak visual orientation cues within the flight simulator (that we had attempted to minimize) may have confounded the experiment (see STAR Methods). We thus lined the interior of the simulator arena with featureless white felt and introduced two dominant moveable visual landmarks (Figure 2, see STAR Methods for justification): a triangular black “mountain” above a lower black “horizon” within the flight simulator arena, and a black stripe on a rotatable circular UV-transmissive diffuser above the moth (the diffuser's angular extent removed all remaining visual landmarks above the arena walls). These landmarks were chosen to be visible in most parts of the moth's visual field since we had no *a priori* knowledge of which visual field areas are used for analyzing landmarks.

To investigate whether Bogong moths use both magnetic and visual cues to navigate, we subjected tethered moths, captured during their autumn migration near Adaminaby, to two five-phase experiments (experiments 1 and 2) in which these cues were placed in congruent and conflicting configurations (Figures 2A and 2B, left columns). In each phase, moths flew for 5 min (i.e., a total continuous flight of 25 min). In phase A of experiment 1 (Figure 2A, upper panels), the magnetic field was aligned toward magnetic north (mN, close to the putative migratory direction; see Figure 1B), but the visual landmarks were aligned 60° further east to detect whether one cue was dominant over the other. In phase B, the field and the landmarks maintained their relative positions but were both moved clockwise by 120° (with landmarks now at 180° , mS). In phase C₁, the landmarks remained at mS, but the field was returned to mN (cues now in conflict). To study the effect of this conflict over time, we continued phase C₁ for a further

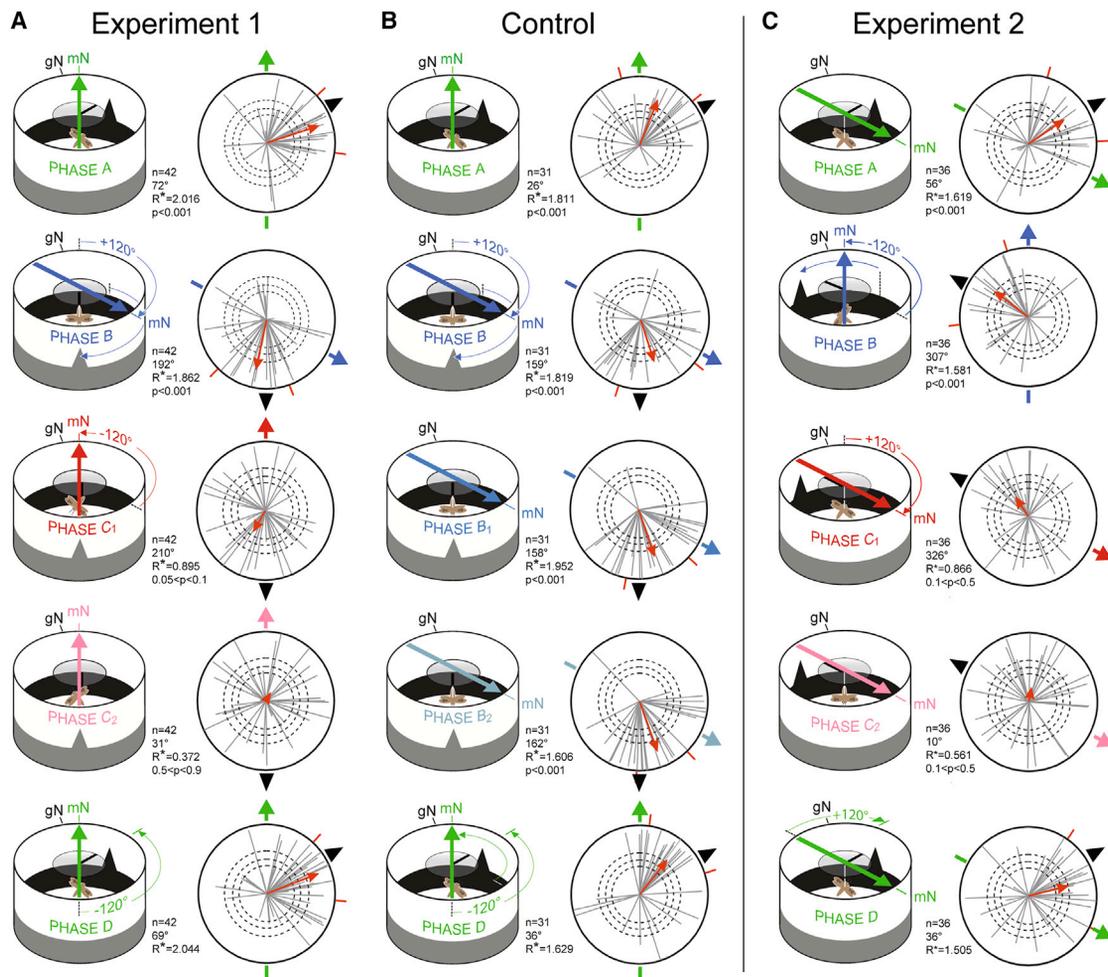


Figure 2. Magnetic and Visual Cues Steer Migratory Flight Behavior in Bogong Moths

(A) Experiment 1. When the positions of the magnetic field (colored arrows) and visual landmarks (black triangular “mountain” and dorsal stripe) are correlated (phases A, B, and D), moths ($n = 42$; gray vectors) are significantly oriented near the landmarks ($p < 0.001$). When the two cues are in conflict (phase C₁/C₂), moths become disoriented (C₁: $0.05 < p < 0.1$; C₂: $0.5 < p < 0.9$).

(B) In a control of experiment 1 for fatigue, where the magnetic field is not placed in conflict (phase B₁/B₂), moths remain oriented.

(C) Experiment 2. As for experiment 1 (A), with landmarks again initially at 60° , but with magnetic field direction at 120° . Moreover, an anti-clockwise (rather than clockwise) shift of cues occurs between phases A and B. The magnetic field and visual landmarks are again correlated in phases A, B, and D and moths ($n = 36$; gray vectors) are once more significantly oriented ($p < 0.001$). When both cues are in conflict (phase C₁/C₂), moths are again disoriented (C₁: $0.05 < p < 0.1$; C₂: $0.1 < p < 0.5$).

Each phase is 5 min of flight. Other plot conventions are as in Figure 1. Vector data for flight trajectories in selected individual moths from experiment 1 are shown in Figure S4B. Histograms showing the distributions of flight direction and flight trajectory directedness (given by the r value) for the 42 moths of experiment 1 are shown in Figure S4C. These indicate that most moths were quite directed in all five phases ($r \geq 0.5$), irrespective of the direction they flew, indicating that a loss of orientation in phases C₁ and C₂ was not necessarily associated with a simultaneous loss in directedness. See also Figures S1–S4.

5 min (phase C₂). In phase D, a quality control, the conditions of phase A were repeated—to be included in the analysis, a moth was required to return to a flight direction in phase D that was not significantly different to that in phase A (see STAR Methods). Experiment 2 (Figure 2B) repeated experiment 1 but with different visual and magnetic starting conditions: in phase A, landmarks were again aligned at 60° , but magnetic field direction was at 120° . Moreover, an anti-clockwise (rather than clockwise) 120° shift of both cues occurred between phases A and B.

In experiment 1, moths were significantly oriented in a direction close to the landmarks in phase A ($p < 0.001$; see the MV for each phase in Figure 2), indicating that in our experiment

moths preferred to align with visual rather than magnetic cues. That moths did not orient in their inherited migratory direction (as they did in Figure 1B) was most likely an artifact [25] of using an unnatural single set of aligned and disproportionately salient visual beacons in an otherwise visually impoverished arena (in the wild, moths would experience an entire panorama of naturally salient celestial and terrestrial visual cues). In phase B, moths turned on average by 120° in response to the 120° rotation of both cues ($p < 0.001$) and still remained oriented toward the landmarks. In phases C₁ and C₂, when magnetic and visual cues were in conflict, moths became disoriented (C₁: $0.05 < p < 0.1$; C₂: $0.5 < p < 0.9$), although initially they continued to

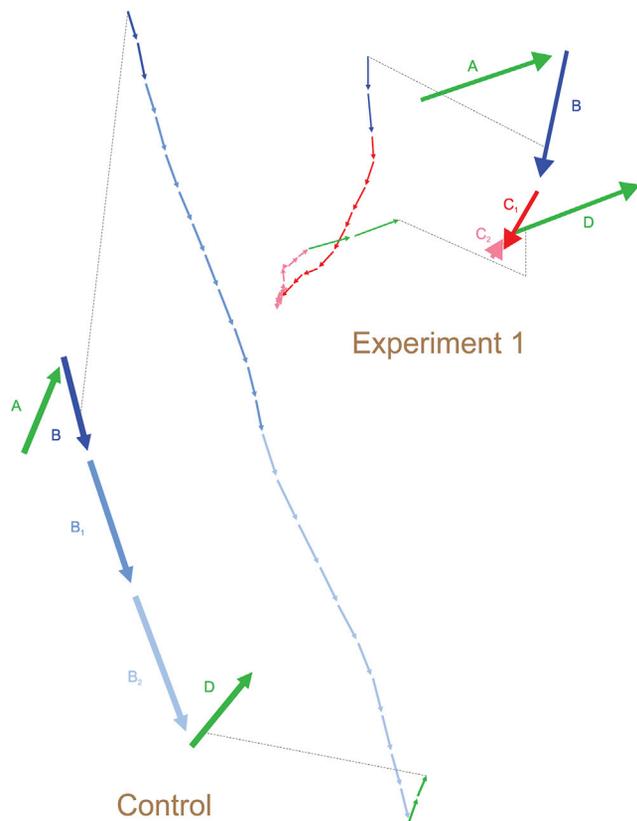


Figure 3. Vector Data for Bogong Moth Flight Trajectories in Experiment 1 and the Control, from Data Given in Figure 2

Plots show average vectors (of correct length and direction) throughout each phase, as well as an expanded view of these vectors where each is dissected into ten 30 s vector segments (i.e., 5 min total). When moths entered phase C₁ in experiment 1, moths initially continued to orient toward the landmarks but vector segments eventually began to shorten (indicating a declining directedness) and change direction. By phase C₂, vector segments were very short and oriented in random directions (i.e., moths were totally disoriented). In the control, in contrast, vector segments in phases B₁ and B₂ remained long (i.e., moths were highly oriented), and vector direction was maintained toward the landmarks (as in phase B). The color code for phases is as in Figure 2. See also Figures S2 and S4.

fly toward the visual landmarks (as seen by the longer MV in C₁; Figure 3). In phase D, when cues were again returned to their initial conditions, moths returned to the landmark-directed orientation of phase A. To control for the possibility that fatigue led to the disorientation seen in phases C₁ and C₂, we performed a 25-min control experiment that twice repeated phase B (phases B₁ and B₂; Figure 2B). The only difference between phases C₁/C₂ and B₁/B₂ is the direction of the magnetic field. Moths remained significantly oriented toward the landmarks in phases B₁ and B₂ (Figures 2B and 3), indicating that the disorientation seen in phases C₁ and C₂ was due to the altered direction of the magnetic field, and not fatigue. Despite different cue starting conditions, the same results and conclusions were obtained from experiment 2 (Figure 2C) and in one further experiment in which visual and magnetic cues were initially both aligned toward mN (Figure S3). The few individual highly directed moths that flew in directions well outside the population average in phase A of experiments 1 and 2 also usually reacted to changes in visual

and magnetic stimuli, but not in ways typical of the rest of the tested population (Figure S4A).

Landmark Fidelity Is Rechecked with the Magnetic Sense Every Few Minutes

In the absence of other cues, Bogong moths thus appear to require correlated visual and magnetic cues for orientation, becoming disoriented when these cues are placed in conflict. Even though their preferred visual landmarks remain unchanged and clearly visible upon entering phase C₁, moths nonetheless become incapable of orienting toward them (Figure 2). Interestingly, this incapacity is not instantaneous—moths continued flying for some time toward the visual landmarks before detecting the conflicting magnetic field and becoming disoriented. The time course of disorientation was revealed by analyzing the directedness (via the R^* value; Figures 4A and 4B) and direction (Figures 4D and 4E) of moth flight tracks in experiments 1 and 2 as a function of time during the transition from phase B to phases C₁ and C₂. Moths were significantly directed toward the landmarks in phase B in both experiments (significance = $R^* \geq 1$; horizontal dashed lines in Figures 4A–4C) and initially remained so even in phase C₁. Eventually, however, R^* fell permanently below 1 (at a “time to confusion,” t_c), after which moths became disoriented until the onset of phase D ($t_c \approx 3.5$ min and 1.5 min in experiments 1 and 2, respectively; Figures 4A and 4B). Moths also began to deviate significantly from their phase B mean direction ($t_c \approx 3.5$ min and 2.0 min; Figures 4D and 4E). In the control, R^* remained greater than 1 in all phases (Figure 4C), and flight direction was maintained (Figure 4F).

These results suggest that moths used visual landmarks as an orientation beacon but calibrated landmark fidelity using a magnetic sense. The fact that it took around 2–3 min for moths to detect a conflict between visual and magnetic cues suggests that either the magnetic sense is reliable and relatively noise-free and checks the directional fidelity of a landmark once every few minutes or it is relatively noisy and unreliable and must continuously accumulate data every few minutes for reliable calibrations. Either way, the calibration mechanism appears to be periodic.

Is the Earth’s Magnetic Field Used as a Compass in Conjunction with Visual Landmarks to Steer Migration?

Our results show that the steering directions of Bogong moths during flight is the result of an interaction between visual landmarks and the Earth’s magnetic field, providing the first indication that insects could use magnetic and visual cues in combination to navigate during long-distance nocturnal migration. However, since in some of our experiments (Figures 2 and S3) the moths did not fly in their natural migratory direction (Figure 1)—due to the dominating effect of our visual landmarks—we are unable to say at this stage with certainty whether Bogong moths integrate magnetic and visual information to follow an inherited migratory trajectory.

Nonetheless, the Bogong moth’s magnetic sense could potentially allow the Earth’s magnetic field to be used as a compass to select and hold an inherited migratory direction, as found in nocturnally migrating birds [26]. Since our results indicate that geomagnetic and visual cues are used in conjunction to steer flight behavior, a compass-selected migratory direction could be then aligned with a visual landmark in a nearby direction—a dark feature on the nocturnal horizon, one or more stars, or the moon.

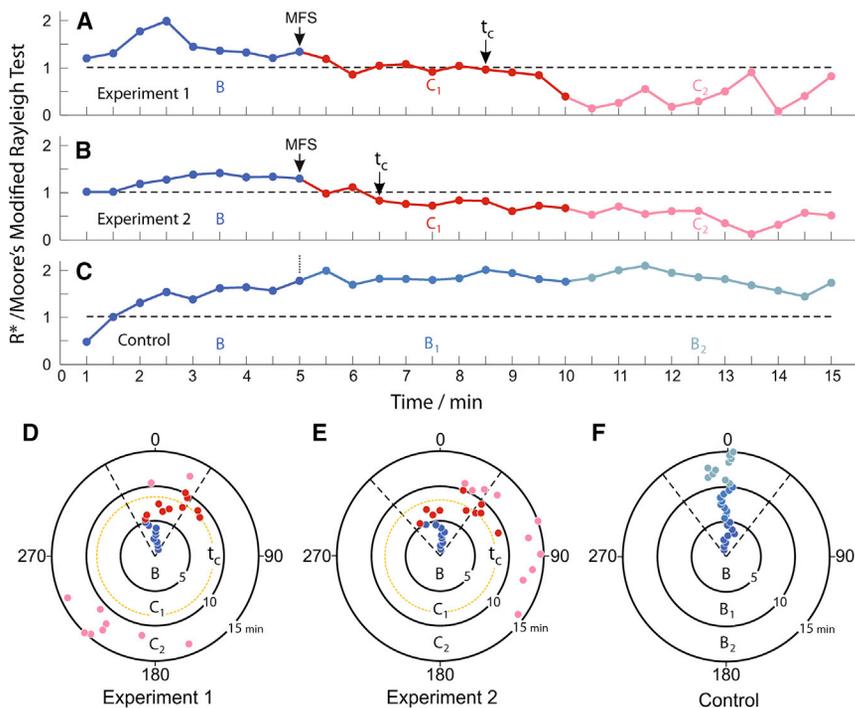


Figure 4. The Time Course of Navigational Disorientation during Cue Conflict

The directedness (R^* value; A–C) and mean directions (D–F; 0° = mean direction in phase B) of moths in phases B (blue dots), C₁ (red dots), and C₂ (pink dots) of experiments 1 (A and D) and 2 (B and E), as well as in phases B and B₁ (light blue dots) and B₂ (pale blue dots) of the control (B and F), as a function of time ($t = 0$; beginning of phase B). Dashed lines in (A)–(C) indicate the significance level for directedness ($p < 0.05$: $R^* \geq 1.011$, A; $R^* \geq 1.015$, B; $R^* \geq 1.016$, C). Dashed lines in (D)–(F) indicate 95% confidence intervals (as in Figure 2 but with $MV = 0^\circ$ to aid comparison). t_c indicates “time to confusion”: the time after the phase C₁ magnetic field shift (MFS) when R^* permanently falls below significance (A–C) or the time after MFS when MV first deviates significantly from MV in phase B (D–F). Data are from Figure 2. See also Figure S2.

Such a landmark could be then used as a temporary orientation cue, with the directional fidelity of the landmark regularly affirmed by the magnetic compass. When the landmark becomes obscured (by the terrain or clouds) or moves over time (such as the moon), the entire process could then be repeated with a new landmark. Such a strategy—which could even involve generating a sequence of temporary multisensory navigational “snapshots” [27] of the visual and magnetic “scene”—would have the advantage that it does not require time compensation to hold an accurate bearing using the variable and comparatively unreliable disk of the moon as a celestial cue. Such a strategy would of course require that the Bogong moth uses the Earth’s magnetic field as a magnetic compass, and further experiments using more naturalistic visual stimuli (terrestrial panoramas and projected starry skies) are needed to confirm whether this is indeed the case.

The Earth’s magnetic field is arguably the most stable cue for nocturnal long-distance migration, and our results indicate that Bogong moths, like birds [9, 26], have a magnetic sense. Moreover, our results also provide the first reliable demonstration of the use of the Earth’s magnetic field to steer flight behavior in a nocturnal migratory insect. It is possible that other nocturnal moths also rely on a magnetic sense, although this—and the hitherto unknown sensory mechanisms responsible for sensing and analyzing the Earth’s magnetic field [28–31]—remain enticing topics for future research.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at <https://doi.org/10.1016/j.cub.2018.05.030>.

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AUTHOR CONTRIBUTIONS

E.W. and B.F. conceived the project. K.G. contributed detailed knowledge of Bogong moth ecology and aestivation sites and provided critical logistical support in the Australian Alps. M.W. provided crucial logistical and experimental support in Narrabri. E.W., B.F., D.D., H.M., A.G., and S.J. designed the experiments, manufactured the experimental apparatus, and executed the experiments. D.D. calibrated the magnetic stimulation apparatus and wrote the MATLAB scripts to control it. D.D., E.W., and B.F. analyzed the results. D.D. and E.W. made the figures. S.H. made intellectual contributions to the interpretation of the results. E.W. and D.D. wrote the initial version of the manuscript, and all authors made significant contributions to the final version.

DECLARATION OF INTERESTS

The authors declare that they have no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited Data		
Raw and analyzed data files	This paper	Figures 1, 2, 3, 4, S1, S3, and S4
Experimental Models: Organisms/Strains		
Bogong moths (<i>Agrotis infusa</i>)	Wild caught	N/A
Software and Algorithms		
USB1, USB4	US Digital, Vancouver, WA, USA	https://www.usdigital.com/support/software/usb4-software
Custom software for analyzing data files generated in USB1 and USB4 (MATLAB)	This paper; MathWorks, Natick, MA, USA	RRID:SCR_001622
Custom software for generating optic flow	This paper; Martin York, Queens University, Canada	N/A
Oriana	KCS, Pentraeth, UK	http://www.kovcomp.co.uk/oriana/oribroc.html

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Eric Warrant (eric.warrant@biol.lu.se).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Male and female Bogong moths (*Agrotis infusa*, Lepidoptera: Noctuidae) were caught in the wild during their migration using a powerful (1000 W) vertical beam search light (model GT175, Ammon Luminaire Company, Shanghai, China), and a suspended white sheet at ground level, during their natural southerly spring migration in October 2012 on Mt. Kaputar (near Narrabri, northern NSW, Australia (elevation 1489 m): Google Maps coordinates: 30.281°S, 150.151°E) and during their return autumn migration in March 2015, 2016 and 2017 on a mountain pass in the Australian Alps close to Thredbo (Dead Horse Gap (elevation 1580 m), southeast NSW, Australia: Google Maps coordinates: 36.524°S, 148.260°E). Each captured moth was transferred to its own small plastic container to isolate it from the influences of other moths, fed with 10% honey solution (in water) and stored in a cool and sheltered place (but with exposure to the natural light cycle) to recover from stress induced by capture. Following capture, the moths were transported 74 km to the testing site near Adaminaby, NSW (Google Maps coordinates: 36.038°S, 148.863°E).

METHOD DETAILS

Objectives of the study

The objectives of the study were (1) to determine whether nocturnal Bogong moths (*Agrotis infusa*) possess a magnetic sense, and if so (2) to determine how information from the Earth's magnetic field might be used with other sensory information (in particular visual information) to steer long-distance migration in the inherited migratory direction at night. To achieve these objectives, wild Bogong moths were captured during their autumn migration, tethered within a flight simulator and subjected to controlled azimuthal changes of a natural Earth-strength magnetic field and correlated visual landmarks (placed in congruent and conflicting configurations).

Attachment of tethering stalks on moths

Before attachment of tethering stalks, moths were chilled in a freezer for 5–10 min in order to immobilize them. The scales on the moth's dorsal thorax were removed by suction using a micro-vacuum pump (custom built by B.F.). Afterward a thin vertical tungsten stalk (which is ferromagnetic free), fashioned at its end to create a small circular footplate, was glued to their dorsal thorax using contact cement while being restrained by a weighted-down plastic mesh (Figure S2C). Each moth was transferred to its own clear UV-transmissive Perspex container (not airtight), given a drop of 10% honey solution on cotton wool and stored in a cool outdoor location with a natural light cycle. Moths were tested on the day of stalk attachment. Shortly before sunset, containers holding moths were placed on an elevated location (such as a rock) and provided with a clear view of the western sky and the setting sun (and the skylight polarization pattern), in case these cues were important for calibrating the magnetic compass (as found in birds [32–34]). After sunset

moths could also see the stars (and the celestial rotation). On cold nights ($< 10^{\circ}\text{C}$), the containers holding moths were placed in a warmed plastic box to maintain ambient temperature at $\approx 20^{\circ}\text{C}$ and prevent them from cooling.

Behavioral apparatus

Two ferromagnetic-free modified Mouritsen-Frost flight simulators [8] (built from aluminum, plastic and UV-transmissive Perspex) were used to continuously record the heading directions of tethered migratory Bogong moths. Briefly, each flight simulator consisted of a cylindrical Perspex arena (diameter 50 cm, height 35 cm), placed vertically on an aluminum table (Figure S2A). The table top was made of clear Perspex. The two flight simulators (and their tables) were placed around 8 m apart. For tethering, the tungsten stalk of the moth was attached to the bottom end of a second long, fine vertical tungsten rod (the encoder shaft) using a short length of thin rubber intravenous medical tubing (see below). The encoder shaft was connected to an optical encoder suspended at the center of the open top end of the arena by a thin horizontal Perspex arm. The encoder instantaneously measured the moth's heading and allowed us to reconstruct its virtual flight path, thus tracking the heading of the flying moth relative to magnetic north (mN) in the presence or absence of induced sensory cues.

The modification to the simulator consisted of incorporating an optic-flow system below the moths to produce sustained stable flight and to provide them with the appearance of forward progress over terrain. For each flight simulator, a projector (Philips PicoPix PPX3610 or BENQ GP2/3), a mirror placed at 45° and neutral density filters (optical density between 4 and 5 log units), were used to produce a very dim bitmap image of the Earth's surface near Narrabri (at approximately 800 m altitude) that was back-projected onto a tracing paper screen placed below the arena (Figure S2A). Custom written software (Martin York, Queens University, Canada), that controlled the direction of movement of the image (but not its orientation), was coupled to the encoder system (USB1, USB4: US Digital, Vancouver, WA, USA) via a feedback loop. This feedback ensured that the resultant ventral flow-field image always moved 180° relative to the moth's heading (i.e., backward from nose to tail), creating a visual sensation of forward movement irrespective of which direction the moth flew, instantaneously turning with the moth as it changed flight direction. The average light intensities of the optic flow at the moth were $1.31 \times 10^{-3} \text{ cd m}^{-2}$ (Flight Simulator 1) and $6.70 \times 10^{-4} \text{ cd m}^{-2}$ (Flight Simulator 2) and in both simulators it moved continuously at 10 mm s^{-1} . From their position in the center of an arena, the moths could see a 2D 120° sector of sky (equivalent to a 3D solid angle of 3.5 steradians). Great care was taken to ensure that landmarks external to the arena, such as trees or other structures, could not be seen by moths while they were performing navigational flight behavior in the simulator.

All experiments were conducted outdoors, after sunset, during the migratory periods in the Australian spring (test sites 1 and 2 (October): Australian Cotton Research Institute, Narrabri, NSW (Google Maps coordinates: 30.200°S , 149.612°E ; magnetic declination $+10^{\circ}53'$) and a helipad in Mount Kaputar National Park, Narrabri, NSW (Google Maps coordinates: 30.279°S , 150.174°E) and autumn (test site 3 (March): "Glenhare," Adaminaby, NSW (Google Maps coordinates 36.042°S , 148.862°E ; magnetic declination $+12^{\circ}29'$). Background levels of radio-frequency disturbances were measured at the experimental sites using a Rohde and Schwarz FSV7 Signal and Spectrum Analyzer and a calibrated passive loop antenna (ETS Lindgren, Model 6511). The experimental sites were in very rural locations, and measured noise levels were extraordinarily low and similar to the screened conditions under which the magnetic compass of night-migratory songbirds is not disturbed [35, 36] (Figure S1A).

All moths that were tested to determine their general migratory direction (see Figure 1) at testing sites 1, 2 and 3, were tested under ambient local magnetic field conditions. To prevent unnoticed stray light generated by the electronic equipment (such as battery indicators) entering the experimental arenas, the interior walls of the testing arenas were initially covered with black cardboard. Even though all efforts were made to remove visual landmark cues from the interior of the arena, defects in the cardboard (such as slight buckles and seams) may have provided confounding visual cues during later initial experiments using magnetic field manipulation alone. For the main experiments reported in this paper, the cardboard lining was replaced with a very even felt (see below).

For the cue conflict experiments (performed at Adaminaby on migratory moths returning from the Australian Alps: Figure 2), the outside of each arena was covered with black cardboard, while the inside of the arena was covered with a white and very even felt glued to the arena wall. At the bottom of the arena wall, a strip (12 cm high) of black felt was glued over the white felt in order to create the visual sensation of a "dark horizon" against a "less dark" sky. In order to check for a putative role of landmarks during compass orientation, a triangular-shaped (isosceles) piece of cardboard-supported black felt (height 12 cm, base width 10.5 cm) was connected to the top edge of the arena wall by a thin strip of diffusing paper, superimposed on the horizon to provide a dominant ("mountain-like") landmark. To ensure that the moths were not able to use celestial cues (or the optical encoder arm) for compass orientation, the sky was occluded by a rotatable 15 cm diameter disc made (for rigidity) from a layer of translucent UV-transmissive diffusing filter paper (Lee Filters 251 1/4 white diffuser) centered above the moths such that its angular subtense at the moth diffused all visual details above the top of the arena walls. A thin (1 cm) strip of black cardboard extending from the center of the disc to its edge served as a secondary landmark. As we had no *a priori* knowledge of which visual field areas moths used for analyzing landmarks, the two landmarks were chosen so that they were visible in most parts of the dorsal and frontal visual fields (or even in the lateral or posterior visual fields if tethered moths turned away from the mountain-like landmark). During some experimental nights in which the moon was visible in the sky, large garden parasols made from thick fabric were positioned to shade the arenas, thus preventing direct moonlight from interfering with the experiment. All light-generating components of the equipment (such as laptops and magnetic coil power supplies) were positioned at a distance behind a tarpaulin wall in a tent that served as a visual "hide" (Figure S2B).

Magnetic field shifts

During cue conflict experiments, the azimuthal direction of mN in a natural Earth-strength magnetic field was turned within each flight simulator arena by a computer controlled, double-wrapped [37] Helmholtz two-coil system (custom built by the workshops of the University of Oldenburg) placed around each arena with its long axes 60° relative to mN (Figure S2A). The azimuthal direction was turned clockwise by $\sim 120^\circ$ (120.4°) without significantly altering the field's strength or inclination (Figures S1B–S1F). A switchbox (also custom built by the workshops of the University of Oldenburg) placed between the power-supplies and the coils, enabled the current to be directed in the same (or parallel) direction through the double wound coils, or in opposite (or antiparallel) directions. In the antiparallel configuration the magnetic field produced by the coils cancels out so that only the Earth's natural magnetic field remains (Natural Magnetic Field, NMF). In the parallel mode, the current sent through the coils was carefully adjusted to create a resultant magnetic field vector similar to the natural local magnetic field vector but with mN deflected to a clockwise azimuth of $\sim 120^\circ$ (30° south of the actual (real) magnetic East: Changed Magnetic Field, CMF). Control of the magnetic field shifts in CMN was enabled by a High-Speed USB Carrier (USB-9162, National Instruments) connected between a laptop computer (see below) and the constant-current power supplies feeding the coil-systems (Kepco, BOP 50-2M). Fine adjustment of the magnetic fields was controlled by a custom-written code in MATLAB (Mathworks, Natick, MA). Before each experimental session, the local magnetic field parameters at the experimental site and at the centers of the two flight simulator arenas were measured using a Meda FVM-400 magnetometer.

At test sites 1 and 2 (Narrabri), the electrical power required to run the coil systems (and all other devices) was provided by a portable petrol-powered generator (Honda, EU2000i) displaced from the experimental area by at least 30 m using extension cables. Access to the local power grid was provided at test site 3 (Adaminaby).

Experimental procedures

An individual moth was taken out of its container by using a pair of forceps (a haemostat) to grasp the stalk attached to its dorsal thorax. The stalk was then inserted into a small section of intravenous tubing attached to the encoder shaft, thus enabling the moth to rotate freely around its yaw axis and choose a direction that its conspecifics would choose in the wild.

Each moth was first aligned by hand to mN and the encoder reset to zero to calibrate the system. The instantaneous heading directions (relative to mN) for each individual moth were recorded as angles by the encoder system at a sampling rate of 1 kHz (with a horizontal resolution of 3°) and saved as text files on the hard drive of a notebook (Dell Latitude E6430ATG). Using the recording software (USB1, USB4: US Digital), we sampled 5 heading directions per second. The encoder software featured a graphical rotary dial interface, enabling the experimenter to continuously monitor the current heading direction of the moth in the arena.

Moths chosen for analysis were required to fulfil three *ante hoc* and, in the case of the cue conflict experiments (Figure 2), one *post hoc* criterion (see below). The three *ante hoc* criteria were: (1) the tethering stalk was perfectly vertical, (2) wing flapping was vigorous and its amplitude was large and equal for both wings (indicating that the contact cement had not interfered with the wings), and (3) that the moth flew continuously for 10 min (Figure 1) or 25 min (Figure 2). In the case of the third *ante hoc* criterion, an individual tethered moth would generally start vigorously flapping its wings (creating a faint noise), “flying” for many minutes. If a moth stopped flying, the arena was gently tapped in order to stimulate the moth to continue flight behavior. A moth that stopped flying 4 times was rejected and the recording aborted. Likewise, moths that flew in continuous spirals (usually an indication of a non-vertical stalk) were also rejected. If a moth was suspected of having stopped flying, the arena was approached from below to check acoustically if the animals were truly flying – the sound of powered flight is easily distinguished from gliding. Headlamps with dim red LEDs were used while the moths were handled to keep them in a dark-adapted state.

In cue-conflict experiments, moths were subjected to two five-phase experiments, each phase requiring the moth to fly for 5 min (25 min total). In experiment 1 (Figure 2A), the two landmarks in phase A were displaced from the magnetic field vector by 60° toward the East (with the magnetic field vector at natural mN (NMN) = 0°). At the transition to phase B, the triangle at 60° was manually flipped from the inside to the outside of the arena wall, while as quickly as possible a second triangle (located at 180° at mS) was flipped from the outside to the inside. The stripe on the diffusing landmark disc was likewise rotated 120° toward mS. At the same time, the magnetic coil system was remotely switched from anti-parallel to parallel, which caused a shift of the magnetic field vector from NMN (0°) to CMN (120°). Thus, in phase B the magnetic field azimuth and the two landmarks remained correlated in position (and unchanged from the moth's perspective). In phase C₁ and C₂, the magnetic field direction was shifted back to NMN (0°), while the landmarks remained at 180° , misaligning visual and magnetic cues and creating a cue conflict. Phases C₁ and C₂ were together 10 min long in order to see the effect of the cue conflict in detail over time. In phase D, partly to ensure that moths performed robustly, the magnetic field was left at NMN (0°), while the landmarks were manually returned to 60° . Thus, phase D is a repeat of phase A.

Experiment 2 (Figure 2C) was the same as experiment 1, apart from the initial starting conditions for correlated visual and magnetic cues in phase A. Here landmarks were again at 60° , but the magnetic coil system was switched to parallel, meaning that the magnetic field direction started at CMN (120°). At the transition to phase B, the magnetic coil system was switched to anti-parallel, causing an anti-clockwise shift of the field by 120° to mN (NMN), and landmarks were removed from the 60° position, and shifted anti-clockwise by 120° by insertion at the 320° (-60°) position – from the moth's point of view, the relative positions of the magnetic field and visual landmarks remained correlated. The rest of experiment 2 proceeded according to the same logic as experiment 1.

During the course of an evening, experiments 1 and 2 were alternated randomly on the two flight simulators (i.e., any systematic bias on either experiment due to the apparatus itself could thus be excluded).

A control experiment (Figure 2B) was performed to check whether the disorientation seen for the cue conflict during phases C₁ and C₂ in the two experiments was simply due to fatigue. This control was identical to experiment 1 except that the cue conflict of phases C₁ and C₂ was not included in the control. Instead, in the control, phase B was repeated a further two times (phases B₁ and B₂). The only difference between the control and experiment 1 was the magnetic field direction in phases C₁ and C₂, which was turned by 120° (from 0° (mN) in phase B) to break its correlation with the visual landmarks – in phases B₁ and B₂ the field direction remained unchanged at 0°.

In experiments 1 and 2 and the control, one further *post hoc* performance criterion was applied: to be included in the analysis, moths were required to return to the same direction in phase D as they had in phase A, that is, to a direction within the natural spread of directions (i.e., within 99% confidence limits) experienced by moths that continuously flew in phase A conditions for 25 min (i.e., were not subject to changes in the direction of the magnetic field or the position of landmarks – see Figure S3B). We also excluded a small number of moths that entirely failed to react to changes in the visual and magnetic stimuli (2, 15 and 5 moths tested in experiment 1, experiment 2 and the control, respectively). However, even if these moths are included, they make little difference to the significance levels of the results and no difference to the conclusions (in phase B of experiment 2, and in phases B, B₁ and B₂ of the control, the *p* value changes from *p* < 0.001 to *p* < 0.005). Of 84, 80 and 42 moths tested in experiment 1, experiment 2 and the control, respectively, 42, 36 and 31 moths fulfilled all three *ante hoc* criteria mentioned above as well as the *post hoc* criterion.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data analysis

Since the manual change of landmarks at the beginning of phases B and D caused a temporary disturbance to the moth, the first 30 s of each track in each of the five phases was removed from the analysis (except for the analysis in Figure 2). Oriana (KCS, Pentraeth, UK) and custom written code (MATLAB, MathWorks, Natick, MA, including the Circular Statistics Toolbox version 1.21 by P. Berens, and Excel (Microsoft, Redmond, WA)) was used to analyze the recorded tracks and to calculate a mean vector for the flight trajectory of each moth.

Each gray vector (or orange – Figure 1B) in the circular plots (Figures 1 and 2) encodes the mean orientation direction θ of a moth's individual recorded flight path as well as its *r* value (i.e., length, or directedness, of the flight path vector). To take advantage of the extra information in our data arising from the fact that the flight trajectories of moths not only had a mean direction (as used for a classic Rayleigh test [38]) but also a mean directedness (vector length), we applied Moore's modified Rayleigh test [39, 40] (Oriana, KCS, Pentraeth, UK). To paraphrase Moore [39], this tests the null hypothesis that a set of *N* independent vectors (r_n, θ_n) has been drawn from a uniform circular distribution. To keep the test non-parametric, vectors are ranked according to their length, r_n , with the vector of lowest rank given a length $r = 1$, that of second rank $r = 2, \dots$, and that of highest rank $r = N$. The resulting set of transformed numbered vectors, for notational simplicity, will then have polar coordinates (n, θ_n), with $n = 1, 2, \dots, N$. These transformed vectors are summed by vector addition to create a resultant Mean Vector (MV) that encodes the mean direction of the group. Thus:

$$X = \sum_{n=1}^N n \cos \theta_n, Y = \sum_{n=1}^N n \sin \theta_n, R^2 = X^2 + Y^2, R^* = R/N^{3/2}$$

where *X* and *Y* are the *X*- and *Y*-components of the MV and *R* is its length. The *R** value encodes the directedness of a population of *N* tested moths and reveals the likelihood that the combined flight direction of these moths – each with its own direction and directedness – differs significantly from random. For *N* = 42 (experiment 1), 36 (experiment 2) and 31 (control), the *R** value for 95% likelihood (*p* = 0.05) is 1.011, 1.015 and 1.016, respectively [39]. For 99% likelihood (*p* = 0.01), the corresponding values are 1.243, 1.244 and 1.245, and for 99.9% likelihood (*p* = 0.001), 1.504, 1.503 and 1.502 [39]. These values are shown as the radii of the dashed circles in Figures 1 and 2, and the 95% likelihood values are shown as dashed lines in Figure 4.

Since the highest recorded *R** value was 2.044 (experiment 1, phase D), for ease of comparison we normalized the lengths of all MVs and their significance levels (dashed circles) within circular plots of radius *R** = 2.5.

For moths tested to determine their general migratory directions during spring in Narrabri and during autumn in Adaminaby (see Figure 1), a Mardia-Watson-Wheeler test [38] (Oriana, KCS, Pentraeth, UK) was used to confirm that the two distributions of moth flight directions were significantly different.

Time course of cue conflict disorientation

Resultant vectors for tethered moths were calculated over 30 s bins throughout phases B, C₁ and C₂ (experiments 1 and 2) and phases B, B₁ and B₂ (control). The directedness (*R** value, Figures 4A–4C) and direction (Figures 4D and 4E) of these 30 s resultant vectors were calculated. *R** values were plotted as a function of experimental time for experiments 1 and 2 (phases B, C₁ and C₂) and the control (B, B₁ and B₂). *R** values below the level required for significant orientation of the moth population at the 95% level (dashed lines in Figures 4A–4C, see above) indicated disorientation.

Deviations in heading direction were calculated for each 30 s resultant vector, in both experiments and the control, relative to the MV direction in the corresponding phase B (this direction was set at 0° for easy comparison). Deviations were plotted outward on a circular diagram representing time *t*, with the center of the circle defined as *t* = 0 (start of phase B). Deviations were plotted as a function of experimental time for experiments 1 and 2 (phases B, C₁ and C₂) and the control (B, B₁ and B₂).

DATA AND SOFTWARE AVAILABILITY

Data availability

The authors declare that the data supporting the findings of this study are available within the paper and its [Supplemental Information](#). Individual data files used to generate the figures in the paper are available from the corresponding authors upon reasonable request (eric.warrant@biol.lu.se, david.dreyer@biol.lu.se).

Code availability

Custom-written MATLAB software (code) used in the analysis of data files generated using the recording software USB1 and USB4 (US Digital) is available from the corresponding authors upon reasonable request (eric.warrant@biol.lu.se, david.dreyer@biol.lu.se).