

RESEARCH ARTICLE

Spontaneous magnetic orientation in larval *Drosophila* shares properties with learned magnetic compass responses in adult flies and mice

Michael S. Painter^{1,*}, David H. Dommer², William W. Altizer¹, Rachel Muheim³ and John B. Phillips¹

¹Department of Biological Sciences, Virginia Polytechnic Institute and State University (Virginia Tech), 2119 Derring Hall, Blacksburg, VA 24061-0406, USA, ²Department of Science and Mathematics, Mount Olive College, 652 R.B. Butler Drive Mount Olive, NC 28365, USA and ³Department of Biology, Lund Vision Group, Lund University, Lund 223 62, Sweden

*Author for correspondence (mipainte@vt.edu)

SUMMARY

We provide evidence for spontaneous quadramodal magnetic orientation in a larval insect. Second instar Berlin, Canton-S and Oregon-R × Canton-S strains of *Drosophila melanogaster* exhibited quadramodal orientation with clusters of bearings along the four anti-cardinal compass directions (i.e. 45, 135, 225 and 315 deg). In double-blind experiments, Canton-S *Drosophila* larvae also exhibited quadramodal orientation in the presence of an earth-strength magnetic field, while this response was abolished when the horizontal component of the magnetic field was cancelled, indicating that the quadramodal behavior is dependent on magnetic cues, and that the spontaneous alignment response may reflect properties of the underlying magnetoreception mechanism. In addition, a re-analysis of data from studies of learned magnetic compass orientation by adult *Drosophila melanogaster* and C57BL/6 mice revealed patterns of response similar to those exhibited by larval flies, suggesting that a common magnetoreception mechanism may underlie these behaviors. Therefore, characterizing the mechanism(s) of magnetoreception in flies may hold the key to understanding the magnetic sense in a wide array of terrestrial organisms.

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INTRODUCTION

The role of geomagnetic field cues in behaviors such as navigation, migration, homing and passive alignment has been documented in a diverse range of taxa (Becker and Speck, 1964; Wiltschko and Wiltschko, 1972; Stabrowski and Nollen, 1985; Phillips, 1986; Chew and Brown, 1989; Wiltschko and Wiltschko, 1995; Wiltschko and Wiltschko, 2005; Begall et al., 2008; Dommer et al., 2008). While the adaptive significance of magnetoreception for large-scale spatial tasks such as migration and homing is intuitive, the significance of magnetic alignment behavior, defined as non-goal-directed alignment of the body axis with respect to the magnetic field, remains unclear (Begall et al., 2012). Furthermore, although theoretical and empirical evidence has provided insight into the molecular and biophysical properties underlying magnetoreception, the specific mechanism(s) mediating magnetic alignment behavior has not been identified.

Adult insects have provided a number of examples of alignment behaviors, first reported in termites that exhibited a tendency to align their body axes quadramodally along the cardinal compass directions (i.e. north, south, east or west) in their natural environment (Roonwal, 1958). In laboratory studies, two species of termites (Termitidae) showed a quadramodal distribution of body axis alignments along the cardinal compass directions that shifted when the magnetic field was deflected by strong magnets, providing the first evidence that quadramodal alignment was mediated by information derived from the external magnetic field (reviewed by Becker, 1964). Additional evidence for quadramodal magnetic

alignment in termites has come from studies of gallery-building behavior (Becker, 1976).

Quadramodal magnetic alignment along the cardinal compass directions was subsequently documented in adults of several Dipteran species, i.e. blowflies (*Calliphora erythrocephala*), houseflies (*Musca domestica*), fruit flies (*Drosophila melanogaster*) (Becker, 1963; Becker and Speck, 1964; Wehner and Labhart, 1970), as well as in honeybees (*Apis mellifera*) (Martin and Lindauer, 1977). A more recent study of resting American cockroaches (*Periplaneta americana*) also found a quadramodal magnetic preference coinciding with the cardinal compass axes (Vácha et al., 2010).

Although a seemingly widespread behavior among insects, the adaptive significance of quadramodal magnetic alignment remains unclear. In some cases, like in eusocial termites, quadramodal gallery building may represent an efficient mechanism for organizing the activities of a large and spatially distributed workforce in subterranean environments devoid of alternative cues. At the level of the individual, encoding changes in direction of movement with respect to a global reference system provided by the geomagnetic field could increase the accuracy of a path integration system (Cheung et al., 2008).

Alternatively (or in addition), the quadramodal response could reflect properties of the sensory mechanism(s) responsible for detecting the geomagnetic field. In terrestrial organisms there is evidence for two magnetoreception mechanisms sensitive to earth-strength magnetic fields (Phillips, 1986; Lohmann and Johnsen, 2000; Mouritsen and Ritz, 2005; Wiltschko and Wiltschko, 2005;

Phillips et al., 2010a). One mechanism relies on single-domain or superparamagnetic crystals of biogenic magnetite (Marhold et al., 1997; Munro et al., 1997; Burda et al., 1990; Kobayashi and Kirschvink, 1995; Wu and Dickman, 2011; Wu and Dickman, 2012). A mechanical force resulting from the rotation or torque experienced by single-domain magnetite particles in different relative alignments to the ambient magnetic field could directly or indirectly affect ion channel permeability. Alternatively, the local magnetic field generated by a magnetite particle that is free to rotate, tracking the alignment of the external field, could alter the rate of intracellular free-radical reactions that, in turn, affect the membrane potential (Binhi, 2006; Winklhofer and Kirschvink, 2010). In the case of superparamagnetic magnetite that lacks a stable magnetic moment, the magnetic field could interact with aggregations of crystals that produce attractive or repulsive forces, resulting in mechanical stress on the surrounding cell membrane generating nerve impulses (Davila et al., 2003).

The second magnetoreception mechanism is based on a light-dependent process presumed to involve a specialized class of photopigments that form long-lived radical pair intermediates (Ritz et al., 2000; Ritz et al., 2004; Ritz et al., 2009; Rodgers and Hore, 2009; Phillips et al., 2010b; Solov'ov et al., 2011). In the light-dependent mechanism, the alignment of the magnetic field is proposed to affect the quantum spin dynamics of a photo-induced radical pair, altering the response of specialized photoreceptors to light. As a consequence, the magnetic field may be perceived as a 'visual' pattern of light intensity or color superimposed on the animal's surroundings that is fixed in alignment relative to the magnetic field (Ritz et al., 2000; Ritz et al., 2010; Phillips et al., 2010b).

Previous studies have led to conflicting conclusions about the mechanism of magnetoreception in insects. For example, Kirschvink showed that the effects of changes in magnetic field intensity on the strength of quadramodal orientation of honeybees obeys the Langevin function, which relates the variability in the alignment of single-domain particles relative to an external magnetic field to the opposing effects of thermal agitation (Kirschvink, 1981). These findings are consistent with a magnetite-based mechanism involving single-domain crystals of magnetite (but see Wiltshko and Wiltshko, 1995).

In contrast, studies of the effects of light on learned magnetic compass orientation by adult male *D. melanogaster* and mealworm beetles (*Tenebrio molitor*) have shown a wavelength-dependent 90 deg shift in orientation that is consistent with a light-dependent (presumably radical pair-based) magnetic compass (Phillips and Sayeed, 1993; Vácha et al., 2008). A similar light-dependent 90 deg shift in orientation in amphibians has been shown to result from a direct effect of light on the underlying magnetoreception mechanism, consistent with the involvement of a radical pair-based mechanism. Interestingly, studies of adult *Drosophila* conditioned to a strong magnetic anomaly (10× the geomagnetic field strength) have also provided evidence for a light-dependent mechanism (Gegeer et al., 2008; Gegeer et al., 2010) (see Discussion). However, the possibility that light-dependent effects on compass orientation could result from an interaction between a non-light-dependent (e.g. magnetite-based) magnetic compass and non-magnetic, photoreceptor-based input cannot be excluded (Phillips et al., 2002; Winklhofer and Kirschvink, 2010; Jensen, 2010).

Here, we report the first evidence for a quadramodal magnetic alignment response in a larval insect, second instar *D. melanogaster*. Furthermore, we provide a re-analysis of data from an earlier study of adult *D. melanogaster* (Phillips and Sayeed, 1993) and male C57BL/6 mice (Muheim et al., 2006) suggesting that a quadramodal

pattern of response may be a property of the magnetoreception mechanism that underlies not only the spontaneous quadramodal orientation of larval flies but also learned magnetic compass orientation by adult *Drosophila* and adult C57BL/6 mice.

MATERIALS AND METHODS

Stocks

Berlin and Canton-S wild-type strains of *D. melanogaster*, obtained from The Bloomington Stock Center, IN, USA, and Oregon-R × Canton-S wild-type crosses were maintained at the testing facility. Strains were selected based on previous studies of adult *Drosophila* magnetic compass orientation (see Phillips and Sayeed, 1993) and magneto-sensitivity responses (see Gegeer et al., 2008). All adult strains were reared in 200 ml plastic bottles containing 50 ml of Instant *Drosophila* Media (Formula 424 Blue, Carolina Biological Supply, Burlington, NC, USA) and 35 ml distilled water. Dry active granulated yeast was added to the surface of the media and a partially submerged laboratory tissue was inserted in the bottom of each bottle to provide adults with a dry substrate. Every 7 days, 100–150 adult flies were transferred to fresh bottles and once every 4 weeks adults were transferred to fresh bottles containing 1% penicillin-streptomycin solution to inhibit bacterial growth and maintain colony health. Flies were reared at 23±2°C, 35±10% relative humidity (RH), and maintained under a 12 h:12 h light/dark cycle produced by two 100 W incandescent bulbs.

Rearing vials

Larvae were reared in vials made of Pyrex glass (25 cm diameter, 95 cm long; Kimble Chase Opticlear, Vineland, NJ, USA) that transmit UV light down to ~320 nm. Fresh vials containing media consisting of distilled water, degerminated yellow cornmeal, unsulfured molasses and agar were prepared every 10 days and stored in a refrigerator. In preparation for use, vials were removed from the refrigerator, plugged with a cotton stopper and allowed to acclimate to room temperature (23±2°C) for a period of ~2 h, at which point the surface of the media was partially covered with dry granulated yeast and scored with a metal probe to facilitate egg deposition. Approximately 20–25 adult flies were passively transferred from a rearing bottle to each vial. The outside of each vial was cleaned with a laboratory tissue to remove fingerprints, and transferred into the rearing/testing enclosure.

Rearing/testing enclosure

Rearing and testing took place in the same light-tight, radio frequency-shielded enclosure (model no. 12W-2/2-I, ETS-Lindgren, Cedar Park, TX, USA). A 'floating' floor supported at the edges of the experimental chamber helped to uncouple vibrations caused by the observer inside the chamber from the experimental apparatus. Environmental conditions were maintained at 20.5±2.5°C and 30±25% RH regulated by a portable air-conditioner unit (MovinCool Office Pro 12, Denso, Long Beach, CA, USA) and dehumidifier (Freestar model, Ebac Industrial Products, Newport News, VA, USA) located outside the shielded enclosure. Power was supplied by a custom-built six-channel power supply (Design Solutions LLC, Dillsboro, IN, USA) located outside the enclosure. Wires entering the shielded enclosure passed through RF filters (model no. EC-2005-1C, ETS-Lindgren) mounted on the outside of the enclosure shield (100 dB attenuation from 150 kHz to 10 GHz).

Rearing

Vials containing adult egg layers were transported into the experimental enclosure and placed vertically in the center of three

horizontal Merritt coils, two aligned parallel and one perpendicular to geomagnetic north (north–south magnetic axis). This coil design allows for the cancellation of the horizontal component of the geomagnetic field, achieved by one of the two coils aligned on the north–south axis, while leaving the vertical component unchanged. The two remaining coils are then used to produce a magnetic field aligned in one of four cardinal compass directions (i.e. magnetic north at north, south, east or west) with inclinations and total intensities similar to those of the ambient magnetic field. In the current experiments, all rearing vials experienced the same alignment of magnetic north (i.e. topographic north) prior to testing (total intensity $48,100 \pm 900$ nT, inclination 59.5 ± 1 deg). Eggs laid in the vials were allowed to develop to the late second instar stage (130–160 h in total).

Rearing vials were exposed to monochromatic UV light (365 nm, 12 nm bandwidth) produced by six Nichia UV light-emitting diodes (LEDs) (NSHU no. 550A, Nichia, Tokushima, Japan) with constant (24 h) light from a circular light source centered above the rearing apparatus. Three layers of UV-transmitting frosted mylar diffusers located between the light source and the tops of the coil elements ensured that the UV light reaching each vial was non-directional. Light intensity reaching the top of each vial was set to 10.1 ± 0.05 log quanta $\text{cm}^{-2} \text{s}^{-1}$ using a Keithly RFA 486 picoammeter (Cleveland, OH, USA) with a calibrated United Detector Technology photodiode (Gamma Scientific, San Diego, CA, USA).

Testing

The spontaneous directional responses of individual second instar larvae were observed on 150 mm diameter plastic Petri plates filled with 60 ml of a distilled water, grape juice, agar and sugar solution. Plates were covered and allowed to cool to room temperature prior to testing. The agar concentration prevented larvae from burrowing into the testing plate, restricting them to movement along the plate's surface. Using an incandescent headlamp equipped with a 630 nm long-pass filter, the observer entered the testing enclosure and removed the appropriate rearing vial from the rearing apparatus. A non-magnetic stainless steel probe was used to remove the media from the rearing vial, which was then placed into a 40% sucrose solution to help separate larvae from the rearing media. Fine-bristled paintbrushes were used to collect second instar larvae floating on the top of the sucrose solution. The larvae were then cleaned of residual media and sucrose in a distilled water bath and placed together in the center of an acclimation plate (identical to the testing plates). Once 12–15 larvae had been collected, the acclimation plate was covered and placed inside a light-tight black felt bag to minimize light exposure prior to testing trials. Larvae remained on the acclimation plate until tested; however, no larvae were tested that had remained on the plate for longer than 60 min.

Using a paintbrush moistened with distilled water, an individual larva was transferred from the acclimation plate to the center of the testing plate and immediately covered with an opaque 3 cm high, 1.5 cm diameter plastic cap that served as a release device. The testing plate was then lowered into a circular plate holder centered inside the testing coil elements and aligned with a reference mark at geomagnetic north. The testing plate was illuminated with diffuse monochromatic 365 nm light from an overhead circular light source identical to that in the rearing apparatus. The light intensity was adjusted to 10.1 ± 0.05 log quanta $\text{cm}^{-2} \text{s}^{-1}$ at the surface of the testing plate, equivalent to the intensities experienced during rearing. After 30 s, the release device was removed from the testing plate and the larva was allowed to move freely on the surface of the testing plate. In all trials, larvae were observed from a fixed position approximately 0.5 m

from the center of the testing arena. Directional responses were recorded once the larvae crossed a 30 mm scoring radius, at which point the testing plate was immediately removed from the testing arena, and placed on a 360 deg circular template. The position of each larva was then transferred to the circular template to create a permanent record of the larva's directional response. Tests were terminated and no directional responses were recorded if a larva failed to reach the 30 mm scoring boundary within 8 min, was found to have crawled inside the opaque release cap when it was removed or had burrowed into the surface of the testing media, or if an auditory or vibratory disturbance (i.e. any noticeable disturbance from within or outside the testing enclosure) occurred during the trial. The alignment of magnetic north was changed between trials (i.e. magnetic north at geographic north, south, east or west). Testing coil configurations and conditions were identical to those described for the rearing coil design. Overall, approximately equal numbers of larvae were tested in each of the four magnetic field alignments, with magnetic north at topographic north, east, south or west (each larva tested only once). Bearings from larvae tested in the four magnetic field alignments were pooled to help identify any effects of non-magnetic biases on the distribution of bearings.

A Rayleigh test was used to test for a non-random clustering of bearings. To test for quadramodal orientation, each bearing was doubled twice (modulo 360 deg) (Batschelet, 1981), and the resulting distribution of quadrupled bearings was analyzed using the Rayleigh test.

Double-blind horizontal-cancelled field experiment

Horizontal-cancelled field experiments were carried out with Canton-S strain larvae to determine whether magnetic cues were mediating the quadramodal orientation. All protocols for rearing and testing remained identical to those described above. However, during this experiment, a second investigator located outside the testing enclosure controlled a switch that prevented power from reaching the stimulus coils. This eliminated the horizontal component of the magnetic field inside the testing apparatus to less than 1% of the natural geomagnetic field ($\sim 15 \pm 6$ nT), and prevented the animals from using any directional information provided by the magnetic field. Trials were conducted double-blind, with the observer unaware of the magnetic conditions (i.e. coils used to generate magnetic fields were powered or unpowered), while the second investigator was unaware of the directional responses of the larva. Communication between the observer and second investigator was restricted to indicating the beginning and end of each trial, so the magnetic field condition could be reset for the next trial. The presence or absence of the horizontal component of the magnetic field was determined by the second investigator, while the alignment of the horizontal component (if present) was set by the observer using a switchbox located inside the testing enclosure.

Re-analysis of adult *Drosophila* data

Training

Detailed training and testing methods can be found elsewhere (see Phillips and Sayeed, 1993). Groups of Oregon-R *D. melanogaster* adult flies ranging from 4 to 8 days old were transferred into Pyrex bottles and placed in a training enclosure equipped with a 365 nm overhead light source in a natural magnetic field. Bottles were placed such that light from above reflected off a central pyramid and into one of four arms corresponding to magnetic north, south, east and west. Each arm contained up to four bottles. The intensity of 365 nm (60 nm bandwidth) light reaching flies in the bottles was 10.0 log quanta $\text{cm}^{-2} \text{s}^{-1}$. Each group was trained for 6–10 days.

Testing

After training, groups of flies were tested in a radially symmetrical eight-arm maze made of opaque Plexiglas under diffuse 365 nm UV light at $11.3 \pm 0.1 \log \text{ quanta cm}^{-2} \text{ s}^{-1}$. A double-coil system was used to test flies in earth-strength fields aligned to magnetic north, south, east or west of equal intensity ($\pm 1\%$) and inclination ($\pm 0.5 \text{ deg}$). At the beginning of each test, flies were transferred to a release device centered underneath the eight-arm maze. After an acclimation period, flies were released and allowed to move freely into the maze and given 30 min to exit the maze. Flies exiting the maze passed through a funnel trap into a test tube attached to each arm of the maze. In the original experiments (Phillips and Sayeed, 1993), mean vectors were calculated by vector addition from the distribution of adult male bearings from each group of flies, and the distribution of mean vectors for the 16 conditions (four trained directions \times four magnetic field alignments in testing) plotted relative to the trained magnetic direction was analyzed using the Hotelling's one-sample test. The re-analysis of these data (Phillips and Sayeed, 1993) was carried out to examine the fine structure of the distribution of adult male flies. We compared the distribution of individual flies in each of the four trained directions (each pooling data from four groups of flies tested in the four magnetic field alignments; see above).

Re-analysis of C57BL/6 trained magnetic compass response

Training

Detailed training and testing methods are given elsewhere (Muheim et al., 2006). Male C57BL/6 mice, a common laboratory strain widely used as models of behavior and human disease, were trained in individual cages placed on a narrow wooden shelf with one end shaded (dark end) and the opposite end exposed to overhead light (light end). Four differently aligned shelves were used so that the dark end was aligned in one of four magnetic directions, i.e. 70, 160, 250 or 340 deg relative to geomagnetic north. A nestbox was located at the dark end of each cage where mice typically built a nest, and served to reinforce the training direction. Mice were held in training cages for a minimum of 5 days under a 15 h:9 h light/dark cycle illuminated by a diffuse overhead tungsten/halogen light source.

Testing

Directional responses were obtained from nest positions constructed overnight by mice tested individually in a radially symmetrical circular arena devoid of visual cues. Mice were tested in one of four magnetic field conditions corresponding to the cardinal compass axes (magnetic north 360, 90, 180 or 270 deg). We compared the distribution of individual mice in each of the four trained directions (each pooling data from mice tested in the four magnetic field alignments; see above).

RESULTS

Under 365 nm light, untrained second instar Berlin, Canton-S and Oregon-R \times Canton-S *D. melanogaster* larvae exhibited a spontaneous preference for one of four absolute or 'topographic' directions corresponding to magnetic NE, SE, SW and NW ('anti-cardinal') compass directions (Fig. 1, Table 1). In each strain, the overall topographic and magnetic distributions of bearings were indistinguishable from random, indicating that there was no consistent unimodal topographic or magnetic clustering of bearings (Fig. 1, left and center; see also supplementary material Figs S1, S2). When the directional responses of larvae from all three strains were combined, the distribution of topographic bearings showed a weak clustering at $\alpha = 126 \text{ deg}$, where α represents the distribution mean,

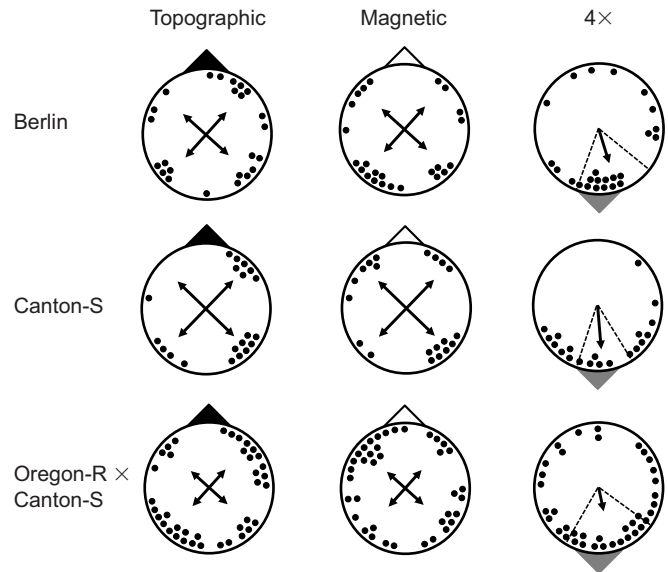


Fig. 1. Spontaneous orientation of second instar *Drosophila melanogaster* larvae. Distributions of bearings are shown for Berlin, Canton-S and Oregon-R \times Canton-S strains. The distributions are plotted as absolute or 'topographic' bearings (left), bearings relative to magnetic north (center) and quadrupled bearings (4x; right). None of the distributions of topographic or magnetic bearings differed significantly from a random distribution ($P > 0.05$, Rayleigh test). The distribution of quadrupled bearings was significant for all strains, i.e. Berlin ($N=24$, $\alpha=164 \text{ deg}$, $r=0.44$, $P < 0.009$), Canton-S ($N=20$, $\alpha=175 \text{ deg}$, $r=0.63$, $P < 0.001$) and Oregon-R \times Canton-S ($N=38$, $\alpha=168 \text{ deg}$, $r=0.29$, $P < 0.05$). The mean quadrangular directions of orientation for Berlin larvae were 41–131–221–311 deg, those for Canton-S larvae were 44–134–224–314 deg and those for Oregon-R \times Canton-S larvae were 42–132–222–312 deg. For non-random distributions ($P < 0.05$, Rayleigh test), the black arrow represents the mean angle of the distribution with the length of the arrow indicating the mean vector length ' r ' (radius of each circle corresponds to $r=1$). Dashed lines show the 95% confidence interval. Black dots represent individual larval bearings tested in one of four magnetic field alignments. Black triangles indicate the direction of geographic north, open triangles indicate the direction of magnetic north and gray triangles indicate the direction of quadrupled bearings corresponding to the anti-cardinal directions.

relative to geomagnetic north ($N=82$, $\alpha=126 \text{ deg}$, $r=0.2$, $P=0.04$) (Fig. 2, left), while the distribution of magnetic bearings (Fig. 2, center) was indistinguishable from random ($P > 0.1$), indicating that the clustering of bearings was due to a weak topographic bias in the testing apparatus and not to a consistent unimodal direction of orientation relative to the magnetic field (supplementary material Figs S1, S2).

The distributions of bearings were tested for quadrangularity by quadrupling the bearings (Batschelet, 1981), which resulted in significant orientation in all strains of larvae tested (Fig. 1, right). Because the alignments of the four magnetic fields differed by 90 deg, quadrupling the topographic bearings and quadrupling the magnetic bearings resulted in identical distributions. The pooled distribution of quadrupled bearings from all three strains combined was strongly oriented ($N=82$, $\alpha=170 \text{ deg}$, $r=0.42$, $P < 0.001$) (Fig. 2, right), corresponding to four clusters of bearings in the original distributions coinciding with the anti-cardinal axes (42–132–222–312 deg) (Fig. 2, left and center diagrams). For all strains, the latency to cross the 30 mm scoring radius ranged from 160 to 345 s, with a mean time to score of 218 s. Only one trial was discarded as a result of disturbances.

Table 1. Spontaneous alignment responses of Berlin, Canton-S and Oregon-R × Canton-S strains of *Drosophila melanogaster* second instar larvae

Strain	Date of test (month/day/year)	Direction of magnetic north (deg)	Topographic bearing (deg)	Magnetic bearing (deg)	Quadramodal bearing (deg)	Strain	Date of test (month/day/year)	Direction of magnetic north (deg)	Topographic bearing (deg)	Magnetic bearing (deg)	Quadramodal bearing (deg)
Berlin	1/28/2010	0	228	228	192	Oregon-R × Canton-S	2/4/2010	0	90	90	360
	2/4/2010	0	74	74	296		2/4/2010	0	135	135	180
	2/11/2010	0	44	44	176		2/12/2010	0	127	127	148
	2/18/2010	0	39	39	156		2/19/2010	0	240	240	240
	2/18/2010	0	84	84	336		2/19/2010	0	323	323	212
	1/28/2010	90	236	146	224		2/26/2010	0	212	212	128
	1/28/2010	90	288	198	72		2/26/2010	0	193	193	52
	2/4/2010	90	294	204	96		3/26/2010	0	50	50	200
	2/4/2010	90	231	141	204		3/26/2010	0	305	305	140
	2/11/2010	90	226	136	184		3/26/2010	0	213	213	132
	2/18/2010	90	40	310	160		2/4/2010	90	51	321	204
	2/18/2010	90	221	131	164		2/4/2010	90	192	102	48
	2/18/2010	90	50	320	200		2/12/2010	90	90	0	360
	1/28/2010	180	115	295	100		2/12/2010	90	230	140	200
	2/4/2010	180	41	221	164		2/19/2010	90	31	301	124
	2/4/2010	180	315	135	180		2/19/2010	90	85	355	340
	2/11/2010	180	135	315	180		2/26/2010	90	241	151	244
	2/18/2010	180	4	184	16		2/26/2010	90	221	131	164
	2/18/2010	180	13	193	52		2/26/2010	90	25	295	100
	1/28/2010	270	179	269	356		2/26/2010	90	124	34	136
	2/4/2010	270	115	205	100		3/26/2010	90	206	116	104
	2/11/2010	270	148	238	232		3/26/2010	90	50	320	200
	2/18/2010	270	129	219	156		3/26/2010	90	56	326	224
	2/18/2010	270	135	225	180		3/26/2010	90	38	308	152
Canton-S	4/8/2010	0	147	147	228	2/4/2010	180	79	259	316	
	4/9/2010	0	216	216	144	2/12/2010	180	18	198	72	
	4/9/2010	0	148	148	232	2/26/2010	180	159	339	276	
	4/15/2010	0	281	281	44	2/26/2010	180	209	29	116	
	4/15/2010	0	120	120	120	3/26/2010	180	291	111	84	
	4/15/2010	0	143	143	212	3/26/2010	180	144	324	216	
	4/8/2010	90	235	145	220	2/4/2010	270	169	259	316	
	4/9/2010	90	42	312	168	2/12/2010	270	254	344	296	
	4/9/2010	90	27	297	108	2/19/2010	270	195	285	60	
	4/15/2010	90	135	45	180	2/19/2010	270	135	225	180	
	4/15/2010	90	48	318	192	2/26/2010	270	307	37	148	
	4/15/2010	90	51	321	204	2/26/2010	270	71	161	284	
	4/15/2010	90	120	30	120	3/26/2010	270	315	45	180	
	4/9/2010	180	202	22	88	3/26/2010	270	240	330	240	
	4/15/2010	180	151	331	244						
	4/15/2010	180	215	35	140						
	4/8/2010	270	142	232	208						
	4/9/2010	270	33	123	132						
	4/15/2010	270	45	135	180						
	4/15/2010	270	58	148	232						

Data are 'topographic', 'magnetic' and 'quadramodal' bearings obtained from Berlin, Canton-S and Oregon-R × Canton-S wild-type second instar *D. melanogaster* larvae tested in one of four earth-strength magnetic field alignments; each larva was tested only once.

Canton-S larvae were tested in a double-blind series in which magnetic field conditions (i.e. horizontal component of the magnetic field present or absent) were intermixed in each test. Larvae exhibited anti-cardinal quadramodal orientation when magnetic compass cues were present (35–125–215–305 deg; $N=25$, $r=0.39$, $P=0.02$) (Fig. 3A, Table 2). There was a weak unimodal distribution of topographic bearings ($\alpha=73$, $r=0.35$, $P=0.046$) (Fig. 3A, left), although again the distribution of magnetic bearings was indistinguishable from random ($P>0.1$; Fig. 3A, center), indicating that the clustering of topographic bearings was due to a weak topographic bias in the testing apparatus and not to a consistent unimodal direction of orientation relative to the magnetic field. In contrast, when the horizontal component of the magnetic field was cancelled, both topographic ($N=18$, $P>0.1$) and magnetic ($P>0.1$)

distributions were indistinguishable from random, when analyzed either unimodally or with the bearings quadrupled (Fig. 3B, Table 2). During the double-blind series, the latency to cross the 30 mm scoring radius when the magnetic field was present ranged from 120 to 300 s, with a mean time to score of 190 s. Latency times to score in trials when the magnetic field was cancelled ranged from 120 to 360 s, and averaged 185 s. No trials were discarded as a result of disturbances.

DISCUSSION

The findings in Figs 1 and 2 show quadramodal alignment responses in second instar *Drosophila* larvae. Although the larval response requires movement from the center of the testing plate, we refer to the behavior as an alignment response rather than compass

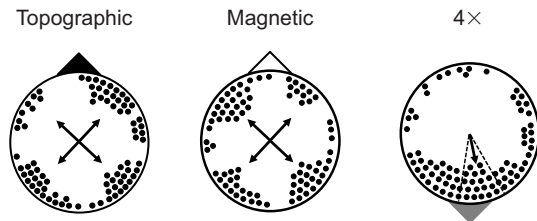


Fig. 2. Pooled distribution of directional responses from all three strains of *D. melanogaster* larvae. The combined distribution of quadrupled bearings (4x) was significantly oriented ($N=82$, $\alpha=170$ deg, $r=0.42$, $P<0.001$; Rayleigh test). This indicates there was significant quadramodal clustering of bearings along the anti-cardinal axes at 42–132–222–312 deg. The topographic distribution also exhibited a weak unimodal clustering ($\alpha=126$ deg, $r=0.198$, $P=0.04$; mean vector not shown). Symbols are the same as in Fig. 1.

orientation because it appears to be a spontaneous movement that is not goal oriented. A double-blind experiment in which larvae were tested in the presence and absence of directional earth-strength magnetic cues confirmed that the quadramodal alignment is dependent on the magnetic field, and that no alternative, non-magnetic cue was present in the testing environment (Fig. 3).

Although quadramodal compass responses appear to be widespread in insects (Roonwal, 1958; Becker and Speck, 1964; Becker, 1976; Martin and Lindauer, 1977; Vácha et al., 2010), the sensory mechanism(s) mediating this behavior has yet to be identified. As discussed earlier, there are two candidate mechanisms proposed to mediate magnetoreception in terrestrial organisms: (1) a magnetite-based mechanism (MBM), based on single-domain or interacting superparamagnetic particles of magnetite believed to produce mechanical deformation of, or torque on, cell membrane structures that activate a coupled transduction mechanism (Winklhofer and Kirschvink, 2010) or, in the case of freely rotating single-domain particles, to secondarily affect the rate of intracellular free-radical reactions that influence the opening or closing of membrane channels; and (2) a photoreceptor-based mechanism involving a specialized class of photopigments (cryptochromes) that form photo-excited radical pair intermediates sensitive to magnetic fields (radical pair mechanism or RPM) that in some animals may cause the magnetic field to be perceived as a pattern of light intensity superimposed on the animal's surroundings (Ritz et al., 2000; Phillips et al., 2010b).

In insects, evidence has been obtained for both MBM and RPM in different species. For example, honeybees dancing on a horizontal comb, which prevents the use of a gravity reference to align the waggle run, exhibit a strong quadramodal preference for the cardinal compass directions and a secondary preference for the anti-cardinal compass directions (Martin and Lindauer, 1977). A re-analysis of these data by Kirschvink found that the relationship between the strength of quadramodal orientation and the intensity of the magnetic field plateaus at higher intensities, and conforms to the Langevin function that predicts the ratio of magnetic to thermal energies of single-domain particles of magnetite (Kirschvink, 1981). These findings are consistent with a MBM that involves single-domain particles of magnetite in which variation in the alignment of the particles, and therefore the scatter in the alignment of the waggle run, decreases with increasing magnetic field intensity. Unlike the MBM, the effect of the magnetic field on a RPM involves a resonance process that functions within a relatively narrow range of magnetic field intensities. Therefore, the strength of quadramodal orientation mediated by a RPM should peak at an intermediate

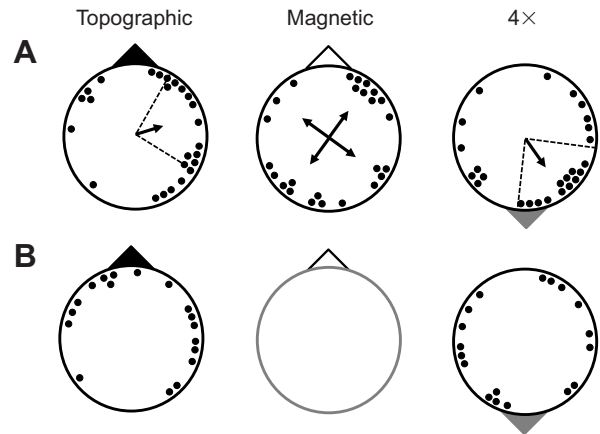


Fig. 3. Distributions of directional responses from second instar Canton-S *D. melanogaster* larvae tested with the ambient horizontal component in all four magnetic field alignments (A) and with the horizontal components cancelled (B). (A) Larvae tested with magnetic cues present showed a non-random distribution of topographic bearings ($N=25$, $\alpha=73$ deg, $r=0.35$, $P=0.05$; left). However, the distribution of magnetic bearings (center) was indistinguishable from random ($P>0.1$), indicating that the 'topographic bias' in the left diagram was not a consistent unimodal response to the magnetic field. The quadrupled bearings (4x) were non-randomly distributed ($\alpha=145$ deg, $r=0.40$, $P=0.02$), indicating that the distributions of bearings were clustered quadramodally at 36–126–216–306 deg. (B) Larvae tested with the horizontal component of the magnetic field cancelled (no magnetic compass cues) showed topographic bearings (left; $N=18$, $P>0.10$) and quadrupled bearings (right; $P>0.10$) that were indistinguishable from random. Symbols are the same as in Fig. 1.

intensity and decrease at both high and low magnetic field intensities (Timmel et al., 1998), and is not consistent with the behavioral responses exhibited in honeybees.

In contrast to evidence for the involvement of a MBM in honeybees, learned magnetic compass responses in both adult fruit flies and adult mealworm beetles appear to be mediated by a light-dependent mechanism (reviewed by Phillips et al., 2010a). Adult male *Drosophila* exhibit robust unimodal magnetic orientation when trained and tested under short-wavelength light (365 nm). However, when trained under short-wavelength light (365 nm) and tested under long-wavelength light (500 nm), males show a 90 deg clockwise shift in orientation relative to the trained magnetic direction, suggesting that the magnetic compass of adult *Drosophila* is affected by the wavelength of light (Phillips and Sayeed, 1993). Similar light-dependent responses were reported in mealworm beetles. When trained and tested under short-wavelength light (390 nm), beetles oriented in the trained magnetic directions. When trained under the same short-wavelength light and tested under blue-green light (500 nm), beetles shifted their orientation 90 deg clockwise relative to the trained magnetic direction (Vácha et al., 2008). While the wavelength-dependent effects of light on magnetic compass orientation in flies and beetles could result from a change in the behavioral response rather than a change in the directional input used to guide behavior, similar wavelength-dependent 90 deg shifts in magnetic compass orientation in both anuran and urodele amphibians have been shown to result from a direct effect of light on the underlying magnetoreception mechanism (Phillips and Borland, 1992; Freake and Phillips, 2005; Diego-Rasilla et al., 2010). Furthermore, preliminary evidence for photoreceptors sensitive to the alignment of an earth-strength magnetic field has been obtained in neurophysiological recordings from the frontal organ (an

Table 2. Spontaneous alignment responses of second instar *Drosophila melanogaster* tested with or without an earth-strength magnetic field

Date of test (month/day/year)	Field	Direction of magnetic north (deg)	Topographic bearing (deg)	Magnetic bearing (deg)	Quadrmodal bearing (deg)
4/23/2010	On	0	119	119	116
4/23/2010	On	0	305	305	140
4/29/2010	On	0	45	45	180
5/6/2010	On	0	124	124	136
5/11/2010	On	0	221	221	164
5/11/2010	On	0	135	135	180
5/12/2010	On	0	32	32	128
5/12/2010	On	0	31	31	124
4/23/2010	On	90	275	185	20
4/23/2010	On	90	58	328	232
4/29/2010	On	90	307	217	148
5/6/2010	On	90	310	220	160
5/11/2010	On	90	123	33	132
5/11/2010	On	90	161	71	284
5/12/2010	On	90	113	23	92
5/12/2010	On	90	148	58	232
4/23/2010	On	180	20	20	80
5/6/2010	On	180	109	289	76
5/11/2010	On	180	15	195	60
5/12/2010	On	180	57	237	228
4/23/2010	On	270	30	120	120
4/29/2010	On	270	329	59	236
5/11/2010	On	270	155	245	260
5/12/2010	On	270	102	192	48
5/12/2010	On	270	79	169	316
4/29/2010	Cancelled	n/a	34	n/a	136
4/29/2010	Cancelled	n/a	143	n/a	212
5/6/2010	Cancelled	n/a	335	n/a	260
5/6/2010	Cancelled	n/a	139	n/a	196
5/6/2010	Cancelled	n/a	71	n/a	284
5/6/2010	Cancelled	n/a	294	n/a	96
5/6/2010	Cancelled	n/a	323	n/a	212
5/6/2010	Cancelled	n/a	98	n/a	32
5/6/2010	Cancelled	n/a	303	n/a	132
5/11/2010	Cancelled	n/a	5	n/a	20
5/11/2010	Cancelled	n/a	111	n/a	84
5/11/2010	Cancelled	n/a	79	n/a	316
5/11/2010	Cancelled	n/a	345	n/a	300
5/11/2010	Cancelled	n/a	232	n/a	208
5/12/2010	Cancelled	n/a	63	n/a	252
5/12/2010	Cancelled	n/a	94	n/a	16
5/12/2010	Cancelled	n/a	284	n/a	56
5/12/2010	Cancelled	n/a	336	n/a	26

Data are 'topographic', 'magnetic' and 'quadrmodal' bearings of Canton-S *D. melanogaster* larvae tested in one of four alignments of an earth-strength magnetic field (On) or tested when the horizontal component of the magnetic field was cancelled.

outgrowth of the pineal organ) of bullfrogs *Lithobates catesbeianus*, showing wavelength-dependent properties consistent with the behavioral effects observed in both amphibians and insects (J.B.P. and C. Borland, in preparation). These findings suggest that similar light-dependent magnetic compass mechanisms may be present in multiple orders of insects and amphibians. In addition to adult insects, learned compass orientation in second instar *D. melanogaster* larvae trained and tested in four different magnetic directions under short wavelength light (365 nm) have also been reported (Dommer et al., 2008). However, these findings did not address the spectral dependence of the magnetic compass response.

Additional evidence for a light-dependent magnetoreception mechanism has come from experiments investigating naive and conditioned responses of adult *D. melanogaster* to a magnetic anomaly 10 times the intensity of the geomagnetic field presented in one arm of a T-maze (Gegear et al., 2008; Gegear et al., 2010). Under full spectrum light, naive flies avoided magnetic stimuli, while

flies exhibited a conditioned response towards the magnetic stimulus when paired with a sucrose reward. In transgenic flies lacking a functional cryptochrome photopigment, as well as in wild-type flies tested under lighting conditions that excluded short-wavelength light (<420 nm), both naive and conditioned responses were abolished. Although Gegear and colleagues' findings are consistent with a RPM-based magnetoreception mechanism in *Drosophila*, the relevance of these findings to quadrmodal alignment and learned magnetic compass responses remains unclear as the strong magnetic stimuli used in these experiments could affect other physiological processes that are not directly involved in the detection of earth-strength magnetic fields (e.g. cryptochrome-based photo-entrainment of circadian rhythms) (Yoshii et al., 2009).

A re-analysis of the earlier study (Phillips and Sayeed, 1993) of light-dependent magnetic compass orientation by adult *Drosophila* found evidence that adult flies trained and tested under UV light to magnetic north or south showed a unimodal distribution of bearings

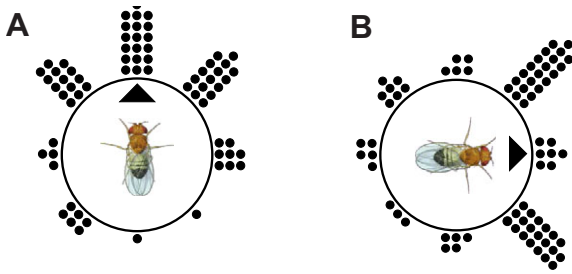


Fig. 4. Magnetic compass response of adult *D. melanogaster* depends on whether flies were trained along the magnetic north–south (A) or east–west (B) axis (data from Phillips and Sayeed, 1993). (A) Flies trained to the north or south (plotted relative to the trained magnetic direction at the top of the diagram) exhibited a unimodal distribution of bearings coinciding with the trained magnetic direction. (B) Flies trained to the east or west (plotted relative to the trained magnetic direction on the right of the diagram) exhibited a distribution of magnetic bearings that was split by ± 45 deg on either side of the trained magnetic direction. Black symbols are the magnetic bearings of individual flies tested in groups in one of four magnetic field alignments (magnetic north at north, east, south or west). Each diagram includes directional bearings from flies tested in eight different conditions (2 trained directions \times 4 magnetic field alignments) (see Phillips and Sayeed, 1993). Black triangles indicate the trained magnetic direction.

centered on the trained direction (Fig. 4A), while flies trained to magnetic east or west under the same lighting conditions exhibited a 90 deg (i.e. ± 45 deg) split around the trained direction (Fig. 4B) (data from Phillips and Sayeed, 1993). These responses suggest that when flies are orienting along the north–south axis, the directional information obtained from the magnetic field is qualitatively different from that obtained when they are orienting along the east–west axis. This difference in behavior along the north–south and east–west axes is consistent with flies receiving magnetic input that exhibits a complex, axially symmetrical pattern of response (i.e. identical components, 180 deg apart) (see discussion of mouse findings below).

The splitting on either side of the east–west-trained axis (i.e. clusters of bearings at 45 and 135 deg for east-trained flies, and at 225 and 315 deg for west-trained flies) is consistent with the anti-cardinal quadramodal alignment response exhibited by *Drosophila* larvae (Figs 1, 2). This raises the possibility that the same magnetoreception mechanism may be involved in the light-dependent magnetic compass orientation of adult *Drosophila* and the quadramodal magnetic alignment response of *Drosophila* larvae.

Although the pattern of response generated by a RPM-based magnetic compass is unknown, theoretical models of magnetically sensitive, radical pair systems are compatible with a complex, radially symmetrical pattern that intersects the horizontal plane in multiple (e.g. two or four) directions and could produce quadramodal patterns of response (Cintolesi et al., 2003; Rodgers and Hore, 2009; Lau et al., 2012). Modeling of mechanisms involving single-domain or super-paramagnetic magnetite indicates that in some configurations a MBM can also exhibit axial symmetry (Winklhofer and Kirschvink, 2010). Given evidence that the intensity dependence of the quadramodal dance of honeybees fits the predictions of a MBM (Kirschvink, 1981), but not a RPM (Ritz et al., 2000), a magnetite-based receptor could be involved in the responses of both larval and adult flies. However, it remains to be determined whether a quadramodal pattern of response is likely to be generated by a MBM.

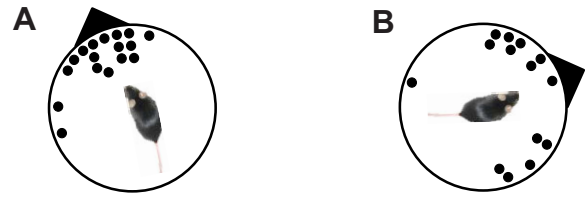


Fig. 5. Learned magnetic compass response of adult male C57BL/6 mice trained along the north–south or east–west magnetic axis (from Muheim et al., 2006). (A) Mice trained in either direction along the north–south axis (340 and 160 deg) exhibited a robust unimodal response coinciding with the trained magnetic direction (responses of north- and south-trained mice combined and plotted relative to the expected magnetic direction, shown by the black triangle). (B) In contrast, the magnetic bearings of mice trained along the east–west axis (70 and 250 deg) were split into two clusters, ± 45 deg on either side of the trained magnetic direction (responses of east- and west-trained mice combined and plotted relative to the expected magnetic direction, shown by the black triangle). Black circles represent individual nest positions; each mouse was tested only once.

Surprisingly, dependence of the magnetic compass response on the axis of training similar to that observed in adult flies (Fig. 4) was also found in a re-analysis of data from an earlier study of magnetic compass orientation by male C57/BL6 mice. A ~ 90 deg split was observed in mice trained in directions close to magnetic east or west (70 and 250 deg) (Fig. 5B), but not to magnetic north or south (340 and 160 deg) (Fig. 5A) (data from Muheim et al., 2006). It is noteworthy that the split in the response of east- and west-trained mice is centered on the cardinal compass directions (magnetic east and west), rather than the magnetic trained directions that were 20 deg counter-clockwise of the cardinal compass directions. Although nothing is known yet about spectral dependence of the mouse magnetic compass response, these findings suggest that both adult flies and mice are responding to different directional information along the north–south and east–west axes, i.e. that the magnetic compass produces a pattern of response with multiple components, rather than a simple reference direction. Both responses exhibit axial symmetry that is compatible with the involvement of either a RPM or a MBM.

While quadramodal magnetic responses have been demonstrated in adult insects belonging to a variety of taxonomic groups, it is interesting that *Drosophila* larvae aligned themselves along the 45 deg, or ‘anti-cardinal’, axes in contrast to the cardinal compass alignments reported from previous studies of adult insects. This discrepancy does not appear to be species specific as previous studies have reported quadramodal alignment behaviors in adult *D. melanogaster* along the cardinal compass axes (Wehner and Labhart, 1970) in contrast to the anti-cardinal orientation exhibited by second instar larvae. However, it should be noted that these alignment experiments were performed in complete darkness, suggesting these responses, like the quadramodal waggle run of honeybees, are mediated by a MBM. This suggests that a MBM could mediate quadramodal magnetic orientation at least under certain conditions. Alternatively, if the quadramodal response exhibited by larval *Drosophila* is mediated by a light-dependent mechanism, the cardinal and anti-cardinal quadramodal responses could reflect more general differences in the response to light intensity by adult and larval flies. For example, wild-type adult *Drosophila* exhibit positive phototaxis, whereas second instar larvae exhibit negative phototaxis (Grossfield, 1978; Sawin-McCormack et al., 1995; Hassan et al., 2000). If flies using a RPM-based magnetic compass perceive the magnetic field as a symmetrical

quadramodal pattern of increased light intensity, separated by contrasting regions of decreased light intensity (Ritz et al., 2000; Phillips et al., 2010b), the 'complementary' responses of larval and adult flies could reflect attraction towards, or away from, contrasting components of the pattern (i.e. perceived brightness) that parallels developmental changes in phototactic behavior.

If quadramodal magnetic orientation in both honeybees (Kirschvink, 1981) and adult flies is mediated by a MBM, the light dependence of these responses could result from a separate non-magnetic input. Jensen proposed that the properties of the light-dependent magnetic compass in birds and possibly other organisms can be explained by an interaction between a non-light-dependent magnetic compass (presumably magnetite based) and a vision-based celestial compass (e.g. a spectral gradient or polarized light compass) (Jensen, 2010). It is noteworthy that Jensen's model suggests that changes in the wavelength and intensity of light could alter the directional response of a hybrid magnetite-based magnetic and light-dependent polarized light (or spectral gradient) compass in a manner similar to that shown in amphibians (Phillips and Borland, 1992).

In summary, our findings in Figs 1–3 provide the first evidence for quadramodal magnetic alignment by a larval insect. Re-analysis of magnetic compass orientation by adult *Drosophila* and adult C57BL/6 mice suggests that a common underlying mechanism may mediate behaviors that are dependent on magnetic cues in larval and adult flies (Figs 2–4), and in some vertebrates (Fig. 5) (and see earlier discussion of similar light-dependent magnetic compass responses in flies and amphibians). Remarkably, magnetic responses in larval and adult flies exhibit properties that are consistent with the involvement of both MBM and RPM detectors. Therefore, the fly system is well suited to investigate whether these responses are mediated by a MBM, a RPM or both, and whether the wavelength-dependent effects of light on magnetic compass orientation are due to the involvement of a light-dependent, RPM-based detector, or to secondary effects of light on a MBM-based detector (Jensen, 2010). Most importantly, evidence that magnetic responses in adult and larval flies share properties with those of honeybees, cockroaches, amphibians and mice suggest that characterizing the mechanism of magnetoreception in flies will have broad implications for the magnetic sense in a wide variety of terrestrial organisms.

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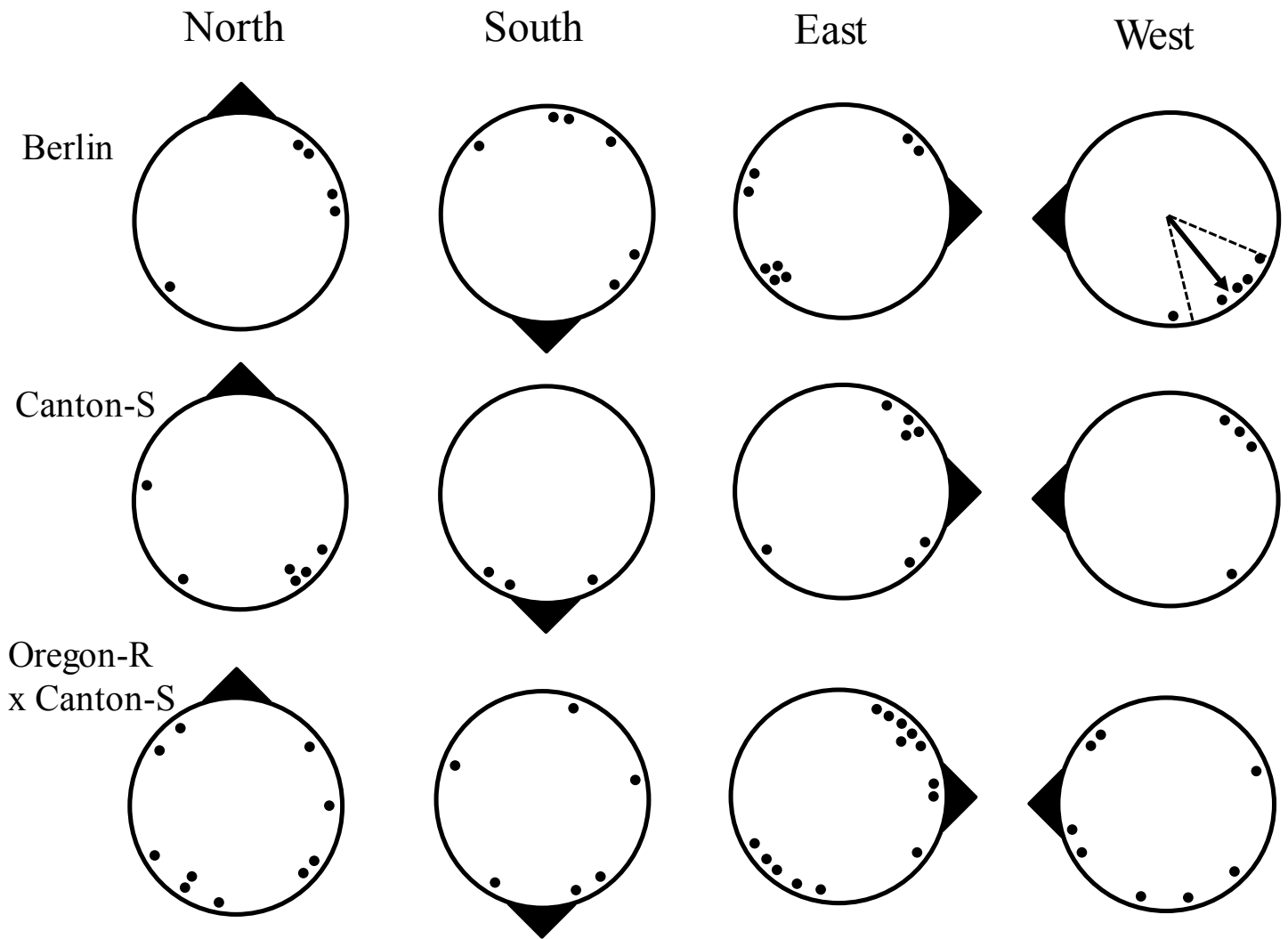


Fig. S1. Spontaneous orientation of second instar *Drosophila melanogaster* larvae relative to the alignment of magnetic north (black triangles). Distributions of topographic bearings for Berlin, Canton-S and Oregon-R \times Canton-S larvae showing bearings of each larva in the four testing fields. In only one condition was there a non-uniform distribution (Berlin strain, west magnetic field alignment; 231 deg, $r=0.93$, $P=0.005$) indicating that the overall quadramodal distributions were not dependent on the alignment of magnetic north. Furthermore, there is no consistent unimodal bias in the distributions of topographic bearings (Fig. 1, left) or magnetic bearings (Fig. 1, center) for any of the strains, or in the pooled distributions of topographic bearings (Fig. 2, left) or magnetic bearings (Fig. 2, center), that could account for the significant clustering of quadrupled bearings (Figs 1 and 2, right). Black dots indicate individual larval bearings. For non-random distributions ($P<0.05$, Rayleigh test), the black arrow represents the mean angle of the distribution with the length of the arrow indicating the mean vector length ' r ' (radius of each circle corresponds to $r=1$). Dashed line indicates the 95% confidence interval for the mean vector bearing.

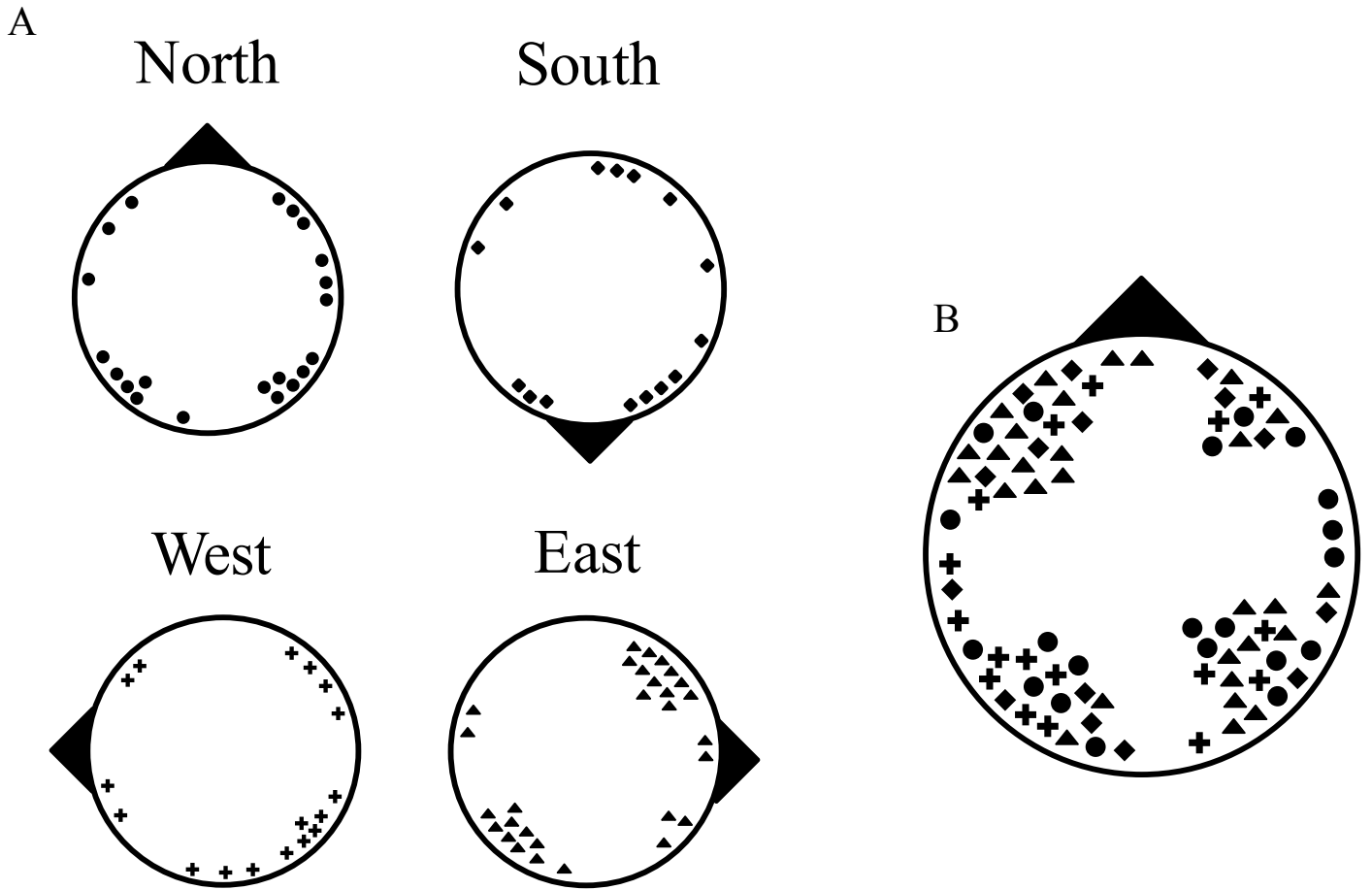


Fig. S2. Pooled distributions of bearings from three stains of second instar *Drosophila melanogaster* larvae. (A) Distributions of topographic bearings obtained in each of the four alignments of magnetic north (magnetic north aligned to north, south, west or east). (B) Pooled distribution of bearings from all four alignments of the magnetic field plotted relative to magnetic north. Symbols indicate individual larval bearings and correspond to those shown in A. Black triangles at the edge of each circle indicate the direction of magnetic north. The unimodal distributions of bearings pooled from all three strains were indistinguishable from random in all four alignments of magnetic north, as well as when the data were pooled with respect to magnetic north ($P > 0.10$, Rayleigh test) indicating that there was no consistent unimodal orientation relative to the magnetic field.