**Behavioral Investigation of the Light-Dependent Magnetoreception Mechanism of *Drosophila melanogaster***

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**By**

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Abstract

Use of a magnetic compass has been demonstrated in all major classes of vertebrates as well as several classes of invertebrates, and is proposed to involve a photo-induced radical pair mechanism (RPM). My dissertation research consisted of characterizing a magnetic compass in a model species, *Drosophila melanogaster*. Preliminary experiments were carried out with adult flies, however, due to the behavioral complexity of adult responses a new behavioral assay of magnetic compass orientation was developed using larval *Drosophila* that elicits a robust magnetic compass response in a trained magnetic direction. This manuscript describes experiments that were conducted showing that larval magnetic compass orientation: 1) demonstrates a complex 3-dimensional pattern of response consistent with a RPM; 2) is consistent with a receptor mechanism that utilizes short- and long-wavelength antagonistic photopigments, proposed to explain wavelength dependent effects in vertebrates (e.g. amphibians and birds); and 3) produces axially symmetrical patterns of response with respect to the geomagnetic field. Additionally, tests of adult *Drosophila* under low and high intensities of monochromatic long wavelength light revealed a similar behavioral response to varying intensities of monochromatic light as previously reported in migratory birds (*E. rubecula*).

These findings indicate that the magnetic compass of larval *Drosophila* shares a common functional architecture and similar biophysical mechanism with that of at least some vertebrates (e.g. amphibians and possibly birds), suggesting that the magnetic compass of modern vertebrates may have evolved once in a common ancestor of these three lineages over 450 million years ago. Furthermore, findings indicating a spontaneous preference for magnetic directions in *D. melanogaster* larvae suggest that a light-dependent magnetoreception mechanism is more widespread in insects than was previously suspected. The development of a behavioral assay to study the light-dependent magnetic compass in an organism with a simple nervous system, a limited behavioral repertoire, and with the possibility of using the full power of modern molecular and genetic techniques holds considerable promise to increase our understanding of the biophysical mechanism(s) and neurophysiological structures underlying magnetic orientation in terrestrial animals.

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## Introduction

Although use of the geomagnetic field in spatial orientation has been documented in a wide range of taxa, the underlying morphological structure(s) and physiological mechanism(s) remain enigmatic. Evidence for magnetoreception has been obtained in groups as phylogenetically diverse as all of the major classes of vertebrates (fish, amphibians, birds, reptiles and mammals; Phillips, 1986a; Burda et al., 1990; Wiltschko, W. and Wiltschko, R., 1995; Diebel et al., 2000; Lohmann et al., 2001) and many classes of invertebrates (insects, mollusks, crustaceans; Phillips and Sayeed, 1993; Arendse, 1978; Lohmann et al., 1995; Lohmann et al., 1991). Recent studies carried out by this laboratory and others have provided evidence consistent with three different magnetoreception mechanisms: (1) a light dependent mechanism involving specialized photoreceptors that form long-lived radical pair intermediates (i.e. radical pair mechanism "RPM"; Edmonds 1996, Shcherbakov and Winklhofer 1999, Schulten and Windemuth 1986, Schulten, 1982, Ritz 2000, Schulten et al. 1978, Cintolesi et al. 2003, Deutschlander et al. 1999b, Solov'yov 2007, Henbest et al. 2004), and (2) a non-light dependent mechanism utilizing ferromagnetic particles (i.e. a magnetite/maghemite based system; Kirschvink and Gould 1981, Fleissner 2007), and (3) a non-light dependent mechanism based on electromagnetic induction (Kalmijn 1981, Paulin 1995). A brief review of the characteristics of the geomagnetic field is given here as a background for discussion of these three mechanisms.

**The Geomagnetic Field**

The geomagnetic field approximates a standard dipole field, similar to that produced by a common bar magnet. Therefore, the magnetic field at any place on the earth can be described as a vector with both a direction and intensity (magnitude). The alignment of the geomagnetic field is independent of the rotational axis of the earth. The magnetic vector emanates from the southern magnetic pole at an angle of $90^0$ relative to the horizon, becomes parallel to the earth at the magnetic equator, and reenters the earth at the magnetic north pole, also at an angle of $90^0$.

Magnetic field lines, defined as "lines of flux" can provide animals with a vast amount of information (i.e. polarity, inclination, and intensity) and can be utilized as cues for spatial orientation. As convention states with regards to magnetic inclination, the angle relative to the horizontal is positive (north down) in the northern hemisphere and negative (north up) in the southern hemisphere (Wiltschko, R. and Wiltschko, W. 1995).

The earth's magnetic field is not uniform. Geomagnetic field intensity varies from approximately 60,000 nT at the magnetic poles to around 26,000 nT at the magnetic equator (Skiles, 1985). On a global scale, spatial variation in magnetic field intensity is relatively regular and predictable, but can be much more variable on smaller spatial scales. Geographic anomalies resulting from large mineral deposits can alter field values up to 1,000 nT (2% – 4%) in localized areas (Wiltschko, R. and Wiltschko, W. 1995).

In addition to spatial variation, daily and yearly fluctuations in the geomagnetic field are caused by several mechanisms. Effects of electromagnetic radiation from the sun on the ionosphere cause regular daily fluctuations in the magnetic field, usually within the range of +/- 100 nT, that are greatest during the day. Showers of charged particles emitted from solar flares can also cause magnetic disturbances referred to as "magnetic storms" with varying effects on the earth's field. While temporal fluctuations in the earth's magnetic field can impact estimates of geographic position for organisms utilizing a magnetic map, they should have a negligible effect on organisms utilizing magnetic information to obtain directional (i.e. compass) bearings. Long-term changes in the magnetic field occur over a vast time scale, the most dramatic of which is a reversal of the magnetic poles that occurs at intervals of approximately 200,000 years. While the geomagnetic north/south axis is constantly shifting, annual changes are miniscule (approximately $0.1^0$ in inclination declination, roughly 10 km) having little effect on the magnetic compass however, changes even that small may have a dramatic effect on map orientation. The aggregate effect over long periods (thousands of years), however, is an important factor in understanding evolutionary trends of both the sensitivity of the magnetic compass and the use of alternate compass systems (Olson, 2002).

**Passive Orientation**

Magnetotaxis was first described in anaerobic magnetotactic bacteria (Blakemore, 1975). Bacteria stirred up into the water column from anaerobic

marine silt use the magnetic field to return to the substrate. Single domain chains of magnetite passively rotate the bacteria into alignment with the magnetic field lines, requiring no expenditure of energy by the organism, and require no "sensory" processing of magnetic information. Once the bacteria are aligned with respect to the magnetic field, propulsion by their flagellum returns the bacteria to the anaerobic substrate. Bacteria in the northern magnetic hemisphere have flagellum positioned near the "north seeking" end of the magnetosome chain, while those in the southern magnetic hemisphere possess flagellum positioned near the "south seeking" end of the chain (Blakemore, 1980).

**Active Orientation**

Active magnetic orientation involves the detection and integration of magnetic information at a more complex level. An animal's response to this type of magnetic input is based upon several factors; (1) motivation to orient; (2) variation in information processing; (3) potential use of multiple inputs (i.e. the ranking of alternative cues); and (4) the ability to process spatial information in this context (Wiltschko and Wiltschko, 1995). The simplest magnetic response is the use of the geomagnetic field to derive a compass bearing (angle) with regard to an ecologically relevant goal, i.e. menotaxis (Sawin-McCormack et al., 1995). Magnetic compass orientation is widespread in the animal kingdom, and use of the geomagnetic field can provide a relatively consistent source of directional information.

**Polarity and Inclination Compasses**

There are two types of magnetic compasses responses.  Polarity compasses possess the ability to detect a reversal of magnetic field polarity (like a human navigator's or "dipole" compass) and, therefore, are able to distinguish magnetic north (or south) independent of the field's inclination. Animals shown to have polarity compasses include naked mole rats and mealworm beetles, both of which have a visual ecology with little to no light (Marhold et al., 1997b; Burda et al., 1990; Arendse, 1978; but see Vaccha and Soukopova, 2004). Inclination or axial compasses detect the slope of magnetic field lines, independent of the polarity of the magnetic field.  Since magnetic inclination is positive (North down) in the Northern and negative (North up) in the Southern hemispheres, an inclination compass can only distinguish  "poleward" from "equatorward" (rather than true north from south).  Surprisingly, this type of magnetic compass response has been found in birds, amphibians, and reptiles (Wiltschko, W. and Wiltschko, R. 1972; Phillips, 1986a; Lohmann and Lohmann 1994) and appears to provide reliable magnetic information for both short and long distance movement.

 For example, when the polarity of the magnetic field is rotated by 180 degrees, without changing inclination, European Robins that normally orient to the southwest during the fall migration continue to follow a seasonally appropriate migratory heading.  However, when the vertical component of the magnetic field is inverted, reversing inclination without changing the horizontal polarity, birds reverse their direction of orientation to a North Easterly heading

indicating sensitivity to the magnetic axis (i.e. inclination), but not the polarity (North vs. South), of the magnetic field.  In contrast, mole rats exhibit magnetic compass orientation that is sensitive to polarity, but is unaffected by changes in inclination (Marhold et al. 1997). The available evidence suggests that "polarity magnetic compasses" are mediated by magnetite-based magnetoreception mechanisms, while "inclination magnetic compasses" are theorized to involve a photoreceptor-based magnetoreception mechanism (Edmonds 1996, Ritz et al. 2000).

**Magnetic Maps**

In addition to providing a source of compass information, natural spatial variation in the geomagnetic field ("magnetic gradients") may be used as a source of geographic position ("map") information (Fischer et al., 2001).  To derive map information from the magnetic field, a precise magnetosensory system must be employed to detect minute changes in field intensity or inclination, which change only 5-10 nT per km or 0.01° per km (respectively).  Bi-coordinate position fixing requires non-parallel gradients of two different geophysical parameters, analogous to latitude and longitude.  While total field intensity and inclination both vary relatively predictably along the north-south axis in many parts of the world, it is unclear what parameter of the field is responsible for east and west position fixing.  To date, evidence for the use of natural spatial variation of the magnetic field to derive information about geographic position has been obtained in amphibians, reptiles, and birds (Fischer et al., 2001, 2003; Lohmann and Lohmann 1994, 2004; Phillips et al. 2002).  Theoretical

considerations (Kirschvink 1981, 2001), as well as experimental evidence (Beason and Semm 1996), have implicated magnetite/maghemite based magnetoreception mechanisms as the only potential mechanism capable of the high level of sensitivity necessary to determine true geographic position from spatial variation in the magnetic field.  Moreover map sense has been shown to involve a magnetoreception system distinct from the magnetic compass sense in at least two groups of animals (i.e. urodele amphibians and migratory birds), indicating that they possess both magnetite/maghemite-based and a photoreceptor- based magnetoreception mechanisms used for two distinct tasks (Fischer et al., 2001).

**Magnetoreception mechanisms**

The two most plausible models of the magnetoreception mechanisms in terrestrial animals revolve around use of single domain or super-paramagnetic particles of biologically synthesized magnetite/maghemite (a non-light-dependent mechanism) and photo-induced radical pair reactions involving specialized photopigments (a light-dependent mechanism).  The third theory for magnetoreception, electrical induction, can only operate in a conducting media (i.e. saltwater) and, thus, is not a plausible mechanism for terrestrial animals (Kalmijn 1981, Paulin 1995).  Evidence exists supporting all three of these mechanisms, indicating that different mechanisms for sensing the geomagnetic field have evolved in different groups of organisms, in different environments, and to provide different types of sensory information.

**Magnetite-based magnetoreception mechanisms**

       Biogenic magnetite ($Fe_3O_4$) has been found in a variety of organisms, and once discovered, started an avalanche of research in the field of animal magnetism (Wiltschko, R. and Wiltschko, W 1995). Magnetite is an attractive hypothesis in that an "internal magnet" could provide organisms with a mechanism sensitive to both the alignment and the intensity of the geomagnetic field. Two classes of biogenic magnetite particles that could be used to detect the geomagnetic field have been found in living systems, single domain and super paramagnetic particles (Wiltschko, R. and Wiltschko, W 1995). Single domain particles have a stable magnetic moment that is constrained to lie in one of two opposite directions parallel to the long axis of the particle. Super-paramagnetic magnetite involves particles too small to have a stable magnetic moment. Instead the magnetic moment tracks changes in the alignment of an external magnetic field (Kirschvink et al., 2001) and may be utilized to amplify the signal from the geomagnetic field to a single domain particle of magnetite. The characteristics expected in a magnetite-based magnetic compass can include an ability to orient in the absence of light, sensitivity to the polarity of the magnetic field, and sensitivity to a brief magnetic pulse with an intensity strong enough to remagnetize single domain particles of magnetite (Kimchi and Terkel, 2001; Lohmann and Lohmann, 1993; Marhold et al., 1997b; Munro 1997a).

       Biogenic magnetite was first observed in the radula teeth of chiton, increasing the rigidity of the structure which is used to scrape algae from rocks

(Lowenstam, 1962). In the mid 1970's Richard Blakemore discovered chains of single domain magnetite in magnetotactic bacteria.   The torque produced by the magnetic field's effect on the magnetite chains was large enough to passively orient the bacteria along the magnetic field lines.   This finding from bacteria not only demonstrated the ability of an organism to synthesize magnetite, but also to produce a crude orientation mechanism that, in a non-horizontal field, allowed them to return quickly to their anaerobic habitat on the ocean floor.  In addition, it was found that within the same species of bacteria the polarity of the magnetic moments associated with the magnetite particles varied between hemispheres so that if bacteria in the northern hemisphere were transplanted to the southern hemisphere, they would swim upward away from the sea floor (Blakemore, 1975).  This finding indicated that population specific magnetic responses occurred, and after this discovery, magnetite was isolated in many organisms including sea hares, honey bees, beetles, trout, amphibians, birds, and rodents, to name a few (Wiltschko, R. and Wiltschko, W 1995).

**Evidence for Magnetite-based Magnetoreception**

Evidence for a magnetite-based magnetoreception mechanism originated from experiments utilizing pulse remagnetization (i.e. exposing organisms to a rapid, high intensity magnetic pulse strong enough to remagnetize magnetite particles).   Pulse remagnetization is expected to alter the response of a magnetite-based mechanism, if single-domaine particles of magnetite are fixed in alignment within the animal (Kalmijn 1978).   In contrast, if particles are free to

rotate, after remagnetization they would rotate back to their original alignment relative to the magnetic field. Experiments with African mole rats showed that they were able to orient in the dark and were sensitive to the polarity of the magnetic field (i.e. a polarity compass), and that pulse remagnetization induced an approximate 180-degree deflection in orientation responses (Marhold et al., 1997b).

In Australian Silvereyes, *Zosterops lateralis*, pulse remagnetization can affect the migratory direction of these passerine birds (Wiltschko et al., 2002). Pulse remagnetization of migratory silvereyes induced a consistent Eastward shift in orientation relative to their normal northernly autumn migratory heading. Effects lasted for as long as ten days, after which, birds demonstrated unshifted headings (i.e. North rather than East).

The task of identifying the biophysical mechanisms that underlies an inclination magnetic compass is much more difficult, because insensitivity to polarity may be characteristic of non-magnetite-based (e.g., radical pair; Ritz et al. 2000) and certain types of magnetite-based mechanisms (e.g., mechanisms involving superparamagnetic particles or freely rotating single domain particles; Kirschvink & Walker, 1985). Therefore the absence of an effect of pulse remagnetization on the orientation of an animal does not rule out magnetite as a potential mechanism (i.e. it could simply indicate that freely-rotating or superparamagnetic particles are present). Similarly, Kirschvink and Walker (1995) speculated that the absence of an effect of demagnetization of honeybees (Gould et al. 1980) may be due to the involvement of a magnetoreception

mechanism involving freely rotating magnetite particles (Kirschvink et al., 2001). To date, however, there is no direct evidence for a magnetoreception mechanism involving freely rotating magnetite particles in any organisms.

**Transduction of a Magnetite Based Magnetoreception Mechanism**

The most plausible transduction mechanism for a magnetite-based detector involves either single domain or super paramagnetic particles that are contained in a sensory hair or coupled to a strain receptor.  In a hair receptor containing a linear array of single domain particles, the torque on the particle(s) would cause the sensory hairs to bend, resulting in a depolarization of the sensory cell membrane. Super paramagnetic particles could act in a similar manner. However, their magnetic properties are insufficient to determine field direction in small numbers (Kirschvink & Gould, 1981; Kirschvink & Walker, 1985; Semm and Beason,1990).  Therefore, closely packed arrays of super paramagnetic particles would be required (Kirschvink & Gould, 1981; Kirschvink & Walker, 1985; Semm and Beason, 1990), and have recently been isolated in the beaks of pigeons (Wiltschko & Wiltschko, 2003).  In close proximity to one another, interactions between adjacent super-paramagnetic particles amplify the external magnetic signal.  This type of mechanism could work e.g., in ferrovesicles where a change in the shape of the SPM array could be detected by stretch receptors in the vesicle membrane.

While regions of the brain that contain abnormally high magnetite concentrations have been discovered in specific tissues of some animals such as

honeybees and trout (Gould et al., 1978; Kirschvink et al., 1985), experiments attempting to characterize the transduction mechanism have been inconclusive. While a host of organisms, including humans, have substantial amounts of magnetite, its presence has to this date not been localized to specific cells.

Evidence exists for the involvement of a magnetite-based mechanism in obtaining map information.  Both amphibians and birds obtain geographic position using a non-light-dependent mechanism involving a permanent magnetic material (most likely magnetite or its recently discovered homologue maghemite) in which the particles are fixed in position within the organism (Beason and Semm, 1996; Phillips & Borland 1994; Phillips et al. 2001b; Munro et al. 1997a,b).

While both experimental and theoretical evidence supports the use of magnetite based magnetoreception mechanisms, it's role appears to be limited to processing map information (providing the high sensitivity necessary to detect the subtle spatial variation in the geomagnetic field) or as a compass mechanism in organisms that live in aphotic environments.  Birds, as well as both anuran and urodele amphibians employ a light dependent magnetoreception mechanism for compass orientation and the compass component of homing (Wiltschko and Wiltschko, 2005), and a magnetite based magnetoreception mechanism for the map component of homing (Fisher et al., 2001, Phillips and Borland, 1994).

**Light dependent magnetic compass**

In a majority of animals in which the directional magnetic sense ("magnetic compass") has been characterized, the underlying magnetoreception mechanism appears to be light-dependent (Semm et al. 1984, Semm & Demaine 1986, Olcese et al. 1985, Reuss & Olcese 1986, Phillips & Borland 1992, Wiltschko et al. 1993, Phillips & Sayeed 1993, Wiltschko & Wiltschko 2005, Freake & Phillips 2005, Wiltschko et al. 2007; for exceptions see Burda et al. 1990, Marhold et al. 1997 Lohmann & Lohmann 1993). The available evidence suggests that the light-dependent magnetic compass involves a photo-induced biochemical reaction that forms long lived radical pair intermediates ('radical pair mechanism' or RPM; Schulten & Windemuth 1986; Timmel et al. 1998; Ritz et al. 2000, 2004; Cintolesi et al. 2003; Solov'yov et al. 2007). In a RPM-based magnetic compass, magnetic field alignment modifies the response of a specialized photoreceptor containing an ordered array of light-absorbing molecules by altering the relative populations of photo-excited states that differ in their ability to drive phototransduction. As a consequence, the magnetic field may be perceived as 3-dimensional pattern of light intensity or color superimposed on an animal's surroundings (Ritz et al. 2000, Cintolesi et al. 2003, Solov'yov et al. 2007). A specialized class of photopigments, the cryptochromes, involved in circadian rhythms as well as in a variety of non-visual light responses in both plants and animals (Cashmore et al. 1999, Ivanchenko et al. 2001, Sancar 2003, Giovani et al. 2003, Tu et al. 2004, Thompson et al. 2004, Partch & Sancar 2005, Zeugner et al. 2005, van Gelder 2006), are the only photopigments in animals that form

radical pair intermediates and, therefore, have been implicated in

magnetoreception (Ahman et al., 2007).

**Properties of a Light-Dependent Magnetoreception Mechanism**

Theoretical and empirical studies have both indicated that radical pair

systems can be influenced by the geomagnetic field despite the extremely low

energies involved in these interactions (Timmel et al. 1998; Eveson et al. 2000,

Ritz et al. 2000, Cintolesi et al. 2003; Henbest et al. 2004, Solov'yov et al. 2007).

Evidence for the involvement of a RPM in sensing the geomagnetic field in

animals includes: (1) sensitivity to the axis (i.e. inclination), but not polarity (i.e. N

vs. S), of the magnetic field (Wiltschko & Wiltschko 1972, Phillips 1986a,b), (2)

involvement of a light-dependent magnetoreception mechanism (Phillips &

Borland 1992, Phillips & Sayeed 1993, Wiltschko & Wiltschko 2004), (3)

disruption of magnetic compass orientation outside a narrow window of static

field intensities (Wiltschko & Wiltschko 2005), (4) absence of an effect of pulse

remagnetization (e.g., Beason & Semm 1996, Munro et al. 1997a,b) and (5)

disturbance by low-level radio frequency fields (~0.1% of the static field strength)

at frequencies in the low MHz range (Ritz et al. 2004), predicted to alter the

magnetic field dependent population of energy states in a RPM-based magnetic

compass (Canfield et al. 1994, 1995; Henbest et al. 2004).  In addition to a role in

magnetoreception, the RPM may be involved in other effects of electromagnetic

fields (EMF) on light-dependent biological processes, in particular those involving

cryptochromes, and the closely related plant photolyases, that form photo-excited

radical pair intermediates (Ritz et al. 2000, Cintolesi et al. 2003, Ahmad et al. 2007, Solov'yov et al. 2007).

**Evidence for a Light-Dependent Magnetic Compass**

A recent study involving *Arabidopsis thaliana* has provided evidence for the role of photolyases, specifically cryptochromes, in a magnetic response of a plant specie (Ahmad et al., 2006). Ahmad et al. found that signaling from cryptochrome 1, measured via an anthocyanin production assay and hypocotyls inhibition increased dramatically when seedlings were reared in magnetic field 10 times stronger (5 G) than the geomagnetic field (0.5 G). Cryptochrome deficient mutants showed no effect of a stronger magnetic field implicating either that a circadian rhythm was necessary to produce an effect based on magnetic field intensity or that cryptochrome 1 does in fact mediate magnetic sensitivity in this species.

Evidence exists in Eastern red spotted newts, fruit flies, and multiple avian systems for a mechanism of magnetoreception that is dependent on the wavelength of light (Phillips and Borland 1992a; Phillips and Sayeed, 1993; Wiltschko et al., 1993). The most convincing evidence that light has a direct effect on the underlying magnetoreception mechanism has come from behavioral studies of newts (Phillips & Borland, 1992a; Phillips & Borland, 1992b). Newts were trained to orient relative to an artificial shoreline, and tested in a radially symmetrical testing apparatus in one of four horizontal alignments of an earth-strength magnetic field. Newts trained and tested under natural illumination

respond by orienting in the magnetic direction that coincides with the shoreward direction in training.  However, newts that were trained under white light and then tested under monochromatic long wavelength light  (>500 nm) exhibited a ~ 90-degree counterclockwise shift in the direction of magnetic compass orientation.  Conversely, newts trained under monochromatic long wavelength, and tested under white light exhibited approximately a ~90-degree clockwise shift in behavior.  Training and testing under the same long wavelength light had no effect on the organism's orientation, i.e. the organisms that experienced the same light conditions in testing and training (whether both conditions were natural illumination or long wavelengths) oriented in the trained shoreward direction.  Newts exhibited random orientation when tested in the dark (Phillips & Borland 1992b), although this could have been due to a change in motivation or behavior, and furthermore, and were disoriented under an intermediate wavelength of light (475nm).  Subsequent experiments with newts in which spectral caps were attached to cover the pineal organ have implicated photoreceptors located in or near the pineal as the primary receptor (Deutschlander et al., 1999a).

Two possibilities existed for the interpretation of the wavelength-dependent 90 degree shift in the direction of magnetic compass orientation. Either the change in the wavelength of light was having a direct effect on the underlying magnetoreception mechanism, or, it was having a nonspecific effect on some other aspect of behavior (i.e. motivate newts to swim perpendicular to the shoreline without any change in the directional information from the

magnetgic compass.   The latter is unlikely, given that, in the absence of other cues that would occur under natural conditions, a non-specific effect on magnetic direction would seem unlikely to cause newts to either orient bimodally to the shoreline.  Moreover, a non-specific effect does not explain the random orientation observed under 475nm, as well as the absence of shifted orientation when newts were trained and tested under long wavelength light.

**Antagonistic Photoreceptor Model**

The antagonistic photoreceptor model (Phillips and Borland, 1992) proposes that the magnetic compass receives antagonistic short wavelength and long wavelength inputs.  Phillips and Borland argued that the relative sensitivity of the short wavelength input was higher in newts, because newts tested under full spectrum showed similar orientation to newts tested under short wavelength (< 475 nm) light, suggesting that white light was preferentially exciting the short-wavelength input.  Monochromatic long wavelength light was proposed to excite a long wavelength mechanism that shifted the newts perception of mN ~90 degrees counterclockwise.  If, as the antagonistic input model proposes, perception of magnetic north is rotated ~90 degrees counterclockwise (i.e. Magnetic north at geographic west), animals trained to full spectrum light and then tested under long wavelength light would orient 90 degrees counterclockwise of the expected direction.  In contrast, animals trained to long wavelength light would perceive magnetic north as rotated CCW of its true direction, and thus learn the direction to shore ~90 degrees clockwise of its true

direction, and orient 90 degree clockwise of the trained direction under full spectrum light. Training and testing under long wavelengths provide equivalent (i.e,, shifted) information in both cases, and should eliminate the shift. Finally, equal excitation of both spectral mechanisms by an intermediate wavelength should equalize the output from each photoreceptor, and render the compass non-functional. As predicted, the magnetic compass response of newts was abolished under 475 nm light.

**Wavelength Dependent Changes in Magnetic Orientation**

Further evidence of a 90-degree shift in orientation comes from tests of adult fruit flies, *Drosophila melanogaster*. Phillips and Sayeed (1993) trained adult flies to a directional light source coming from one of the four cardinal compass directions in the ambient magnetic field. They were tested in an eight arm radially symmetrical maze with light intensity from the eight arms equalized (variation less than 0.05 log units). Flies were trained and tested under short wavelength light (365 nm) in the first of two experimental conditions. Analysis of the resulting data showed males exhibited strong unimodal magnetic orientation in the magnetic direction coinciding with the direction of light in training, while females were randomly oriented with respect to the altered magnetic field. In the second condition, flies trained under 365 nm light exhibited a 90-degree clockwise shift in orientation when tested under 500 nm light of the same quantal flux. This orientation pattern resembled the $90^o$ shift exhibited by the Eastern red

spotted newt, although the shift was in the opposite direction (counterclockwise in newts and clockwise in flies).

Although wavelength-dependent effects of light have been demonstrated on several magnetoreception systems, the only studies investigating the effects of changes in light intensity have been carried out in migratory birds.  Wiltschko et al. (2005) has shown intensity dependent effects of narrow band spectral stimuli in both *Erithacus rubecula* and *Zosterops lateralis*.  Birds show a variety of changes from their normal migratory direction when exposed to different intensities of the same monochromatic wavelength of light.  Of particular interest here is that birds exposed to a high intensity of 565 nm light (12.6 log quanta/cm2/s) oriented bimodally along an axis perpendicular to the expected migratory direction, whereas birds tested at lower intensities (10.3,11.3,12.3 log quant/cm2/s) exhibited unshifted (seasonally appropriate) migratory direction.  At longer wavelengths, the Wiltschko group has reported disorientation. Interestingly, however, Muheim et al. (2002) demonstrated seasonally appropriate migratory orientation at three intensities of 561 nm, disorientation under comparable intensities of  568 nm light, and an ~90° CW shifted orientation under the same intensities of 617nm light, although in the latter case only the lower intensities were significantly oriented.  Overall, experiments with birds have revealed a complex dependence of magnetic compass orientation on light.  In general, at low to moderate intensities of short wavelength (i.e. < 550nm) light birds exhibit seasonally appropriate migratory orientation similar to that of birds tested under full spectrum light.  Like newts, therefore, the response

of the avian magnetic compass under short wavelength light appears to be indistinguishable from that exhibited under full spectrum light.  Another similarity is that in both newts and migratory birds, there is an abrupt transition between regions of the spectrum that produced normal, disoriented, and shifted orientation.  Tests of newts and flies under different intensities of monochromatic light are needed to determine the extent to which other light-dependent properties are shared by these taxonomically distinct organisms.

Experimental evidence indicates that the magnetic compass of passerine birds is located in the retina; interestingly the magnetic compass appears to be strongly lateralized, with only the right eye playing a role in magnetoreception (Wiltschko and Wiltschko, 2005).  As aforementioned, several avian species have been shown to possess an inclination compass, based upon the radical pair model (in addition to the presence of magnetite particles).  Studies of amphibian species have also indicated the use of such a radical pair model.

Neurophysiological evidence for a light-dependent magnetic compass in migratory birds was obtained from recordings carried out by Semm and his colleagues.  Nerve recordings from the nucleus of the basal optic root (nBOR), the optic tectum (a homologous structure to the superior colliculus of mammals), and the pineal organ (Semm, 1983; Semm, 1984; Semm, 1986) showed sensitivity to changes in the alignment of an earth strength magnetic field. Responses were dependent on the presence and wavelength of light, strengthening the case that the underlying magnetoreception mechanism is light dependent.

**Radical Pair Mechanism**

Evidence that the light dependent compass is mediated by a radical pair mechanism stems from work involving low-level radio frequency interference (RFI) on the magnetic orientation of migratory birds.  Ritz et al. (2004) demonstrated that RF fields (weak oscillating fields in the low radio frequency range (~1-50 MHz)) disrupt magnetic compass orientation in migratory birds, and furthermore, that this effect is dependent on the alignment (parallel vs antiparallel) of these fields relative to the static, earth-strength magnetic field. In contrast to a light dependent magnetoreception mechanism, an effect of RFI on a magnetite-based magnetoreception mechanism would require RF field intensities several times that of the geomagnetic field (Henbest et al. 2004), thus making RFI interference a specific test for a radical pair mechanism.

The specificity of the effects of low-level RF on the RPM is because the energy of interaction is 4-6 orders of magnitude below kT (the thermal energy of the surrounding media), which sets a lower limit on most biologically relevant processes.  The RPM is not constrained by kT in the same way as other biophysical processes because the interaction occurs in nsecs, whereas thermal collisions require μsecs to msecs (Ritz et al. 2000; Henbest et al. 2004).  This energy differential makes it extremely unlikely that low-level RF fields (i.e., intensities $10^{-2}$ to $10^{-3}$ that of the geomagnetic field) will effect processes in living systems that are unrelated to a RPM-based magnetoreception mechanism or a functionally similar radical pair process.

A recently discovered class of photopigments, cryptochromes, contain flavin chromophores, and are thought to be the best candidate molecule for the RPM (Solov'yov et al. 2007). Homologous photopigments (plant photolyases) that also contain flavin chromophores have been shown to form radical pair intermediates. Evidence for a magnetic effect on cryptochrome-mediated photo-inhibition of stem elongation in the plant *Arabidopsis* (Ahmad et al. 2007) suggests that magnetic sensitivity is an intrinsic property of cryptochrome-based light responses (Solov'yov et al. 2007), and may also be present in the functionally similar photolyases that carry out light-dependent DNA repair (Sancar 2003, Cintolesi et al. 2003). Thus, investigating the RPM's involvement in magnetoreception and, more generally, the effects of magnetic and electromagnetic fields on cryptochrome-based radical pair systems, will contribute to a better understanding of this novel class of biophysical processes in living systems.

While research on light dependent magnetoreception has focused on characterizing the functional properties of the magnetic compass response in a variety of organisms, a better understanding of the underlying biophysical and molecular mechanisms will require studies of model organisms such as laboratory mice and Drosophila. In particular, the development of a robust assay of magnetic sensitivity in Drosophila will make it possible to localize the photoreceptors and corresponding neurological pathways, as well as the molecular basis, of the light-dependent magnetic compass.

**Insect magnetoreception**

Over the last 70 years, studies in insects have demonstrated widespread use of geomagnetic field cues in the orientation and innate responses. While initial studies indicated that cockchafer (*Melolontha meolontha*; Schneider, 1957) potentially utilized magnetic cues during migratory flights on overcast days, ruling out the potential for using a sun or polarized light compass, most of the research on insect magnetoreception has focused on innate (non-goal oriented) response to magnetic cues.

Previous studies indicate that multimodal orientation (i.e. either bimodal orientation to two opposite ends of a magnetic axis, or quadramodal orientation to four magnetic directions separated by ~90°), and alignment behaviors are widespread in insects including termites (Roonwal 1958; G. Becker 1964, 1976; von Frisch 1968; Duelli and Duelli-Klein 1978), hymenopterans (Altman 1981, Martin and Lindauer, 1977; Kirschvink 1981; Towne and Gould 1985), beetles (Schneider 1960; G. Becker 1964), lepidopterans, and flies (G. Becker 1963, 1965; G. Becker and Speck 1964; Wehner and Labhart 1970). While the adaptive significance of these behaviors is mired in debate, these findings clearly indicate that a wide variety of insects are capable of sensing geomagnetic field cues, and that innate magnetic responses show remarkable similarity across taxa.

The first evidence for a learned magnetic compass response by an insect was obtained in studies of the mealworm beetle (*Tenebrio molitar*). Mealworm beetles occupy habitat that is devoid of light. Early studies by Arendse (1978),

later confirmed by Vacha and Soukopova (2004), showed that response of the mealworm beetles to light was dependent on relative humidity. In very high or very low humidities, the beetles exhibited photopositive responses, whereas at intermediate humidities, photonegative responses were observed. Conditions featuring high humidity are likely to prompt growth of mold or fungi on the animals exoskeleton whereas low humidities increase the risk of dessication. Failure to leave unfavorable conditions (e.g. leave the media and orient positively to phototactic cues) provide a strong selection pressure to reinforce this change in behavior (Vacha and Soukopova, 2004). Mealworm beetles that were exposed to a directional light source and subsequently tested in an earth-strength magnetic field showed corresponding humidity-dependent responses, i.e., magnetic orientation towards the direction of the light experienced in training at high and lower humidities, and magnetic orientation opposite the direction of the light at intermediate humidtiies.

**Larval Drosophila**

D. melanogaster molt twice during larval development resulting in three instars (Ashburner et al., 1989) After hatching, 1st instar larvae burrow into their media utilizing a suite of cues ranging from direction of light, chemical and moisture cues, a phenomena referred to as "foraging" (Hassan et al., 2000). Of these cues, the most critical appears to be light. In the 1st through mid 3rd instar, larvae respond photonegatively (Grossfield, 1978), whereas mid 3rd instar larvae switch to being photopositive prior to leaving the media and finding a suitable

pupation site (Goody-Herrera et al., 1992).  This change in behavior corresponding to a cessation in foraging, and removal from the media to search for a pupation site, is referred to as "wandering behavior" (Sokolowski et al., 1984).

Magnetic compass orientation has been demonstrated in adult *Drosophila* (Phillips & Sayeed 1993; Dommer & Phillips, in prep.).  The magnetic compass of adult *Drosophila* exhibits light-dependent properties consistent with those observed is newts (Phillips & Borland 1992, Deutschlander et al. 1999). Larval *Drosophila* lack the sophisticated sensory structures found in adults (e.g., antennae, tympanum, image-forming visual system) and exhibit a correspondingly less complex array of behavioral responses.  The main light sensing organ in these larvae (Bolwigs organ; Sawin-McCormack et al., 1995) is a pair of bilaterally symmetrical photoreceptive organs located anteriorly at the base of a pair of slits on the dorsal surface proximal to the mouthhooks.   Each hemisphere of the Bolwig's organ consists of twelve photoreceptors juxtaposed to the mouth hooks and innervated by the larval optic nerve to the larval brain.

The advantage of characterizing the light-dependent magnetic compass in larval Drosophila is that they approach the minimum number of neurons necessary to implement this sensory mechanism, making this an ideal organism in which to characterize the biophysical, molecular and neural mechanisms underlying this intriguing sensory mechanism.  Experiments laid out in chapters 1-3 have been a step to develop behavioral assays compatible with new approaches in molecular tools in which to genetically dissect the visual system of

larval Drosophila with the express interest of classifying components of the

magnetoreception mechanism in this species.

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**Chapter 1**


**Magnetic Compass Orientation of Adult *Drosophila melanogaster***

**Trained and Tested Under 530 nm Light**


**David H. Dommer and John B. Phillips**


**Introduction:**

Sensitivity of animals to the geomagnetic field is widespread, including members from every major vertebrate class (e.g. amphibians, reptiles, mammals, birds, and fish; Phillips, 1986a; Lohmann et al., 2001; Burda et al., 1990; Wiltschko, W. & Wiltschko, R 1995; Diebel et al., 2000) and a diverse group of invertebrates (e.g. insects, lobsters, and sea hares; Phillips & Sayeed, 1993; Arendse, 1978; Lohmann, et al., 1995; Lohmann, et al., 1991). The available evidence is consistent with at least two different mechanisms of magnetoreception: (1) a light-dependent biochemical reaction that forms long-lived radical pair intermediates (i.e. radical pair mechanism "RPM"); Ritz et al. 2000, Cintolesi et al. 2003, Phillips & Borland 1992, Wiltschko et al. 1992, Phillips & Sayeed 1993, Wiltschko & Wiltschko 2007, Freake & Phillips 2005).and (2) a non-light dependent mechanism utilizing particles of biogenic magnetite (i.e. a magnetite-based system; Phillips 1986, Beason & Semm 1996, Munro et al. 1997a,b, Phillips et al. 2002, Shcherbakov & Winklhofer, 1999; Fleissner et al., 2007).

**Previous studies of light effects on magnetic orientation:**

Amphibians (*Notophthalmus viridescens*—Phillips & Borland 1992, and *Rana catesbeiana*—Freake & Phillips 2005) and adult male flies (*Drosophila melanogaster*--Phillips & Sayeed 1993, *Musca domestica*—Phillips unpubl. data) trained to exhibit a magnetic compass response under full spectrum or short-wavelength light (respectively) undergo a $90^o$ shift in the direction orientation when tested under long-wavelength ($\geq$ 500 nm) light. Surprisingly, however, the $90^o$ shift is counter-clockwise (CCW) in amphibians and clockwise (CW) in flies. The magnetic compass response of bullfrogs is bimodal, so the sense of rotation (CW vs. CCW) cannot be determined (Freake & Phillips 2005).

Experiments in which newts (*Notopthalmus viridescens*) were trained and tested under long-wavelength light were used to demonstrate that the wavelength-dependent $90^o$ shift in magnetic compass orientation was caused by a direct effect of light on the magnetic compass. After training under long-wavelength (> 500 nm) light, newts tested under long wavelength (550 nm, and > 500 nm) light exhibited normal (i.e., unshifted) orientation, while those tested under full spectrum light exhibited a $90^o$ shift in the counter-clockwise direction. These findings indicate that the wavelength-dependent $90^o$ in newts is due to a change in directional information from a light-dependent magnetic compass. Phillips and Borland (1992) proposed that the wavelength dependent 90-degree shift results from an antagonistic interaction of short-wavelength ($\lambda_{max}$ < 450 nm) and long-wavelength ($\lambda_{max}$ > 500 nm) inputs from photoreceptors in the pineal

complex that produce inverse or complementary patterns of response

("antagonistic photoreceptor model"; Phillips & Borland 1992, Deutschlander et

al. 1999; see Fig. 1.1).  They predicted that an intermediate wavelength of light

that equally excited the short- and long-wavelength receptors would cause the

complementary patterns produced by a light-dependent mechanism to cancel

out, and prevent the newts from orienting relative to the geomagnetic field.

Consistent with these predictions, newts tested under 475 nm exhibited random

orientation.

In adult male *Drosophila*, Phillips and Sayeed (1993) found that flies

trained to a directional source of ultraviolet light (365 nm, 10.0 log quanta/cm$^2$/s)

exhibited unimodal magnetic compass orientation in the trained direction when

tested under diffuse light of the same wavelength and intensity of light.

However, flies displayed a ~90-degree CW shift in orientation when tested under

the same intensity of long-wavelength light (500 nm).  The similarity in the

wavelength-dependent effects of light on magnetic compass orientation in flies

and amphibians, as well as recent evidence for wavelength-dependent effects of

light on the magnetic compass response of larval *Drosophila* (Dommer et al.,

2008), point to the possibility of a common underlying magnetoreception

mechanism.

In contrast to the learned magnetic compass orientation of amphibians

and flies, many species of birds exhibit innate magnetic compass orientation

during seasonal migrations[1].  Migratory birds have been shown to exhibit ~90$^\circ$-

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[1] Although naive migratory birds exhibit an innate magnetic compass heading that coincides with
the species and/or population specific migratory direction, this directional heading may be altered

shifted CW orientation under low intensities of long-wavelength light that resembles the responses in flies and amphibians[2]. Higher intensities of long-wavelength light disrupt the magnetic compass orientation of birds. Like the wavelength-dependent 90$^o$ shift in amphibians, however, disorientation of migratory birds under long-wavelength light appears to involve an antagonistic long wavelength input.

While evidence from newts indicates that the ~90º shift in the direction of orientation under long-wavelength light is due to a direct effect on the underlying magnetoreception mechanism, it remains to be determined whether the same is true in adult flies and migratory birds or, instead, to a non-specific effect of lighting conditions on the orientation behavior of these animals (i.e. a change in the type of behavior, rather than a change in directional information).

Findings reported here suggest that the light dependent properties of the magnetic compasses of different taxonomic groups are more similar than is currently realized and suggest that a more complete characterization may reveal a common light-dependent magnetoreception mechanism may be present. Training and testing of adult flies under long wavelength light provides an important first step in understanding the nature of this response (see earlier discussion). As shown in experiments with newts, if the perception of magnetic north under long wavelength light at the intensity used by Phillips & Sayeed (1993) is shifted by ~90-degrees, training and testing of flies under the same

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(i.e., the magnetic compass may be "recalibrated") if the alignment of the magnetic field is rotated so that it is in "conflict" with celestial compass cues (Muheim et al. submitted).

[2] In birds the light-dependent magnetic compass is located in the retina, rather than in the light-sensitive pineal (Wiltschko & Wiltschko, 2007), although the avian pineal appears to retain some sensitivity to magnetic stimuli (Semm & Demaine, 1986).

intensity of long wavelength light should eliminate the directional shift in orientation behavior since the directional information would be the same in both training and testing. Furthermore, in migratory birds, higher intensities of long-wavelength light caused disorientation, rather than 90-degree shifted orientation (Muheim et al., 2002). Therefore, increasing the intensity of long-wavelength light in testing provides an opportunity to investigate if there are intensity dependent changes in the affect of long-wavelength light on magnetic compass orientation in *Drosophila* that parallel those reported earlier in migratory birds (Wiltschko & Wiltschko 1995; and see earlier discussion)

**Methods:**

**Rearing:** Fly stocks (Oregon R x Canton S hybrids) were maintained in our Ecosystem Simulation Laboratory (ESL) at 25 ± 2 ºC in 250 ml glass bottles prepared with premixed Carolina Drosophila Media containing a mold inhibitor (5% Tegosept) and two folded laboratory tissues (Kim wipes) partially submerged in the media to act as a dry substrate. Populations were maintained at approximately 75 adults per bottle, and flies were transferred to new rearing bottles every 5 days to limit bacterial and fungal growth. Flies were placed on a 12:12 L/D cycle, and were reared under indirect incandescent light at an intensity of ~ 12 ± .05 log quanta/cm2/s.

**Training:** Approximately 50 two-day-old male and female *Drosophila melanogaster* were removed from rearing bottles between 4:00pm and 5:00pm

EST and placed into 250 ml Pyrex training vials containing 50 ml of medium. The medium contained 44 ml distilled water, 10 grams of LabScientific Agar, 10 gm sugar, and 0.7ml of 10% Tegosept (Methyl-4-hydroxy benzoate dissolved in 95% Ethanol) as a mold inhibitor. Pyrex training vials transmit both visible and UV light down to approximately 320nm, and were chosen to permit a wide spectrum of light in training. Bottles were removed from cold storage for 6 hours prior to the start of training in order to allow condensation from the surface of the media and the inside walls of the bottles to evaporate. After approximately 50 flies were placed in a training vial they were transported by car in a foam filled, light tight container to the Behavioral Testing Facility (BTF). The BTF was built specifically for magnetic studies, utilizing two non-magnetic fly training and testing buildings that received forced air heating/cooling and filtered power from a central "hub" building. The training and testing buildings were double-walled (a "building inside a building") to minimize disturbance.

The light-tight training building contained a training maze based on the design used in earlier work by Phillips and Sayeed (Fig. 1.2). Precautions were taken to ensure that all bottles were treated similarly prior to being in transport and until they were placed within the training apparatus. The training apparatus consisted of a cross shaped enclosure, with a central frosted aluminum pyramidal diffuser used to reflect light from a central overhead incandescent light source out into each arm of the training apparatus. Each arm of the maze was covered by a hinged lid allowing access to the end of each arm. When the lids were closed they helped to prevent incident light and sound from providing a

directional cue to flies in the training maze.  Each arm was further subdivided into 4 19x19x8 cm corridors open at the end toward the center making it possible to train up to 4 bottles to any one light direction at a given time (Up to 16 total bottles could be trained simultaneously).  A 100 watt tungsten, halogen bulb was centered above the frosted aluminum pyramid powered by a 12 volt regulated DC power supply.  The light passed through two gel filters (peak transmission 530 nm, bandwidth at half maximum intensity ~ 40 nm) and two frosted Pyrex plates. The diffuse light reached the training arena at an intensity of 11.2 log quanta/cm$^2$/s.  Light intensity was measured with a calibrated photodiode (United Detector Technologies), and a picoameter (Keithley model 486).  Each arm of the maze was aligned along one of the four cardinal compass directions, hence light reached the bottles placed at the end of the arms from geomagnetic North, South, East, or West.  Flies were trained for a period of 5 days; therefore, adult flies were between 5 and 7 days old at the time of testing. The temperature in both the training and testing rooms was maintained at 24 $\pm$ 1ºC.

Thirty minutes prior to testing one of the corridor tops was raised in order to minimize disturbance when a bottle was subsequently removed for testing. Training bottles were removed in total darkness, taking care not to bump the bottles against the sides of the training apparatus, placed in a light tight black bag, and taken immediately to the testing building 30 meters to the southwest of the training building.

**Testing:**  The testing apparatus was based on a design by Phillips and Sayeed (Fig. 1.3).  Light was projected down onto the top of the arena through the same model gel filters and frosted Pyrex diffusers used in training.  The light was then reflected off of 8 frosted aluminum diffusers sloped away from the maze at an angle of approximately 50 degrees.  The light source and aluminum diffusers were adjusted so that light reaching the center of the arena from each of the 8 arms of the maze varied by $\pm$ 0.05 log quanta/cm$^2$/s).  The end of each arm was equipped with a 90-degree Pyrex elbow equipped with a funnel trap.  Funnel traps prevented flies that had entered test tubes above the elbows from re-entering the maze.  A beaker of cold water (2° C) was used to chill a Plexiglas holding tube suspended from the bottom of the arena.  Flies were transferred into the testing arena by removing the foam plug from the training bottle, inverting the bottle, and gently tapping the flies into a glass funnel that extended below into the holding tube.  After flies were transferred to the holding tube, a hydraulic release mechanism was immediately put in place, covering the top of the holding tube until the flies were released.  If there was any contact with the testing arena or surrounding coil after flies were in the holding tube, the test was immediately terminated and flies were not scored.  Flies were allowed to acclimate in the holding tube for a period of 60 seconds.  After the acclimation period, the flies were allowed access to the maze by raising the hydraulic release device remotely from the outer room of the testing building.  Flies had free access to the maze for a period 15 minutes.  The release device was then closed to prevent additional flies from entering the maze.  Once the release device was closed the

test tubes were removed starting at the 315-degree glass elbow and working counterclockwise around the arena. Only male flies entering the glass funnel traps were scored. Both 16-test series were completed within a 6 week time period from June 12th, 2002 to August 4th, 2002.

**Testing Fields:** The testing maze was centered within a pair of cube surface coils (Merritt et al. 1983) using a configuration described in Phillips 1986b. A DC current regulated power supply (Lambda Electronics, Inc. model LPD-423A) was used to power the coils. Flies were tested with magnetic north in one of four directions (North, East, South, or West). The South (mN=S), East (mN=E), and West (mN=W) fields were similar to the ambient field (mN=N) in intensity (+/-1%) and inclination (+/- 1 degree).

**Symmetrical Testing Format:** One group of flies from each trained direction was tested in each alignment of the testing field, resulting in 16 total tests (4 trained directions x 4 magnetic alignments). Both the trained direction and the test field were changed between tests to avoid the same expected direction within the testing arena in successive tests. This eliminated the possibility that flies were following odor trails left by flies in the previous experiment. Furthermore, a minimum of 45 min was allowed between tests carried out on the same day, which previous work has shown is sufficient for olfactory cues to dissipate (Phillips unpublished observations). Using previously established

protocols (Phillips & Sayeed, 1993), tests in which 5 or less males scored were not counted.  In addition, tests in which any unusual disturbance such as loud noises, contact with the coil or testing table, or rough handling of the testing or release device were discarded and the testing condition was repeated at a later date.

**Testing light conditions:**  Light intensity within the 8 arm testing maze was measured using a calibrated photodiode (United Detector Technologies model 756AX3) and a picoameter (Keithley model 486).  A specialized Plexiglas device was constructed which allowed light reaching the center of the maze from each of the arms to be measured.  Due to the constraints of only measuring 1 of each 8 arms at a time, light intensity was calibrated to 10.2 log quanta/$cm^2$/s under normal testing conditions.  The integration of light intensity from all 8 arms simultaneously yielded an approximate intensity of 10.9 log quanta/$cm^2$/s, roughly equal to the light intensity experienced in training.  Subsequent experiments under high intensity light were calibrated to 11.2 log quanta/$cm^2$/s in each of the 8 arms, for an approximate total intensity of 11.9 log quanta/$cm^2$/s in the center of the testing maze.

**Data Analysis:**  For each group of adults, a mean bearing was calculated by vector addition, which was analyzed and tested for significance using the Rayleigh test for circular statistics. Previous studies by Phillips and Sayeed (1993) indicate that only males orient relative to the geomagnetic field.  A

Hotelling's Test was performed using the mean vector, and r value from the Rayleigh test in order to determine the mean direction and relative degree of scatter between adults tested under each wavelength condition.  The Watson $U^2$ test was used to compare two distributions.  For a review of circular statistics see Batschelet, 1981.

**Results:**

Groups of flies tested under 530nm light at an intensity of 10.9 log quanta/cm$^2$/s oriented unimodally in the direction opposite of the light in training (183°, r = 0.91, p < 0.001; Rayleigh test; Fig. 1.4A).  Flies tested under 530nm light at an intensity of 11.9 log quanta/cm$^2$/s were disoriented, although there was a bimodal tendency perpendicular to the trained axis that approached significance (169°, r = 0.52, p = 0.06; Fig. 1.4B).  The difference between the two distributions was highly significant (p < 0.05; Watson $U^2$ test).  The distribution of magnetic bearings obtained from males and females was significantly different under both light intensities (p < 0.05; Watson $U^2$ test).

**Discussion:**

Male adult *Drosophila* trained under long wavelength light (530 nm) oriented unimodally in a magnetic direction that corresponds to the "dark" end of the light gradient experienced in training. As in earlier experiments with newts trained outdoors under natural lighting conditions and tested indoors under broad band (> 500 nm, ~ 40nm bandwidth) long-wavelength light (Phillips & Borland 1992, Deutschlander et al. 1999), flies trained and tested under long wavelength light oriented along the trained axis relative to the magnetic field, suggesting that the wavelength-dependent shift in the axis of magnetic compass orientation observed in flies (Phillips & Sayeed, 1993) is due to a direct effect of light on the underlying magnetoreception mechanism.

Interestingly, however, although training under long-wavelength light eliminated the 90$^o$ shift, the direction of orientation along the un-shifted axis was opposite that observed by Phillips & Sayeed (1993). Flies trained and tested under 365 nm light oriented toward the direction of light in training, while flies trained and tested under 530 nm light of the same intensity oriented away from the direction of light in training. Therefore, the ~90-degree CW shift in the orientation of flies trained under 365 nm light and tested under 500 nm light (Phillips & Sayeed 1993) may be due to two separate effects: (1) a 90$^o$ counterclockwise rotation of directional information from the magnetic compass (as previously described in newts), and (2) a change in behavioral/motivational preference from orienting towards the direction of the training light (i.e. flies tested under 365nm light) to orienting away from the direction of the light in

training (i.e. flies tested under 530nm light).  If this reasoning is correct, then the wavelength-dependent effect of light on the directional response of the magnetic compass (the wavelength-dependent 90° shift in the direction of orientation) is the same in amphibians and insects and, thus suggests either: (1) the magnetic compasses of amphibians and flies have evolved similar functional properties due to convergent evolution (presumably in response to similar selective pressures; Phillips et al. 2001 or 2002), or (2) the light-dependent magnetic compass evolved independently in a common ancestor of the vertebrate and invertebrate (i.e. arthropod) lineages.

Given the possibility that the light-dependent magnetic compass may have predated the split between arthropod and vertebrate lineages, is there any evidence that a similar light dependent mechanism underlies the light-dependent magnetic compass of migratory birds? To date, the magnetic compass responses of flies and newts have been tested under a limited range of wavelength and intensity conditions, providing little basis for comparisons with birds.  To begin to remedy this situation, we tested adult Drosophila under a higher intensity (11.9 log quanta/$cm^2$/s) of monochromatic (530nm) light.

Under low intensity (~6-9x$10^{15}$ quanta $s^{-1}$ $m^{-2}$) conditions of full spectrum light, and monochromatic wavelengths of 424nm, 510nm, and 565nm, European robins demonstrate a seasonally appropriate migratory heading (Northeast orientation).  When exposed to 590nm and 635nm light of the same intensity their orientation is random with respect to the appropriate migratory direction.  At light levels of approximately 7 times higher than previous experiments, testing

under full spectrum light revealed normal migratory orientation. However, under monochromatic wavelengths birds exhibited a host of responses from perpendicular bimodal orientation at 424 and 565nm, to a fixed (polar) response at 510nm, and random orientation under both long wavelength conditions (e.g. 590 and 635nm; Wiltschko & Wiltschko, 1998). More careful experiments have revealed the European robins orient with respect to the appropriate migratory heading under 560 nm light at intensities of 11.5, 12.2, and 12.5 log quanta/cm$^2$/s, however, tests at 567 nm revealed random orientation at all three intensities. The abrupt change from seasonally appropriate orientation to disorientation between 560 and 567nm, like the wavelength-dependent 90º shift in newts, has been attributed to an antagonistic interaction of two (or more) magnetically sensitive spectral inputs (Muheim et al., 2002).

In contrast to the response of flies tested under the lower intensity long-wavelength light, flies tested under a ten-fold higher intensity of long wavelength light (11.9 log quanta/cm$^2$/s) were disoriented (Fig. 1.4B), and the distributions of mean vector bearings obtained under the two different intensities of long-wavelength light were significantly different. Interestingly, the distribution of mean vector bearings under the higher light intensity approached significance along a bimodal axis perpendicular to the trained axis (p < 0.06, Hotteling's test on doubled angles) consistent with the finding that migratory birds exhibit bimodal magnetic compass orientation perpendicular to the seasonally appropriate migratory direction under high intensities of monochromatic light in this region of the spectrum (565 nm; Wiltschko & Wiltschko, 1998).

Based on the available evidence there is no way to determine in either birds or flies whether higher intensities of long-wavelength light have a direct effect on the underlying magnetoreception mechanism(s).  Higher intensities of long-wavelength light may have a motivational effect (e.g., flies and birds may be less motivated to orient under high light intensities of long-wavelength light characteristic of direct sunlight), or may induce a non-specific change in behavior (e.g., birds and possibly flies may correct for wind drift that occurs during nocturnal flights by orientating perpendicular to the flight direction, in the upwind direction, when light levels increase at dawn (Wiltschko et al., 1986).  While further work is needed to better characterize the effects of different wavelengths and intensities of light on the magnetic compass orientation of adult *Drosophila*, the present findings indicate that variation in light intensity does affect the flies' orientation response (i.e. producing a change from unimodal orientation under a lower intensity of long wavelength light to either disorientation or perpendicular bimodal orientation under a higher intensity).

In summary, although further work is clearly needed, the findings reported here provide the first evidence in an invertebrate that the effects of exposure to a specific wavelength and intensity of light in testing is dependent on the lighting conditions experienced in training, as shown previously in newts.  Our data in combination with results obtained by Phillips and Sayeed suggest that there is a direct wavelength-dependent effect of light on the magnetic compass (towards or away from the light direction in training) of adult flies, and in addition there is an effect of long wavelength (and possibly other wavelengths of) light on the

strength of response and/or direction of orientation relative to the geomagnetic field, similar to that observed in birds, although the nature of this effect remains to be determined.  The present findings indicate that similarities in the functional properties of the magnetic compasses of flies, amphibians, and birds may be considerably greater than previously recognized (Wiltschko et al., 2004).  Indeed, the available evidence is consistent with the hypothesis that the light-dependent magnetic compass evolved only once in a common ancestor of all the major lineages of multicellular organisms, and that differences in light-dependent magnetic compass orientation in different groups of animals may represent minor modifications of a common underlying mechanism, analogous to the well documented variation in the spectral sensitivity of rhodopsin-based visual pigments.

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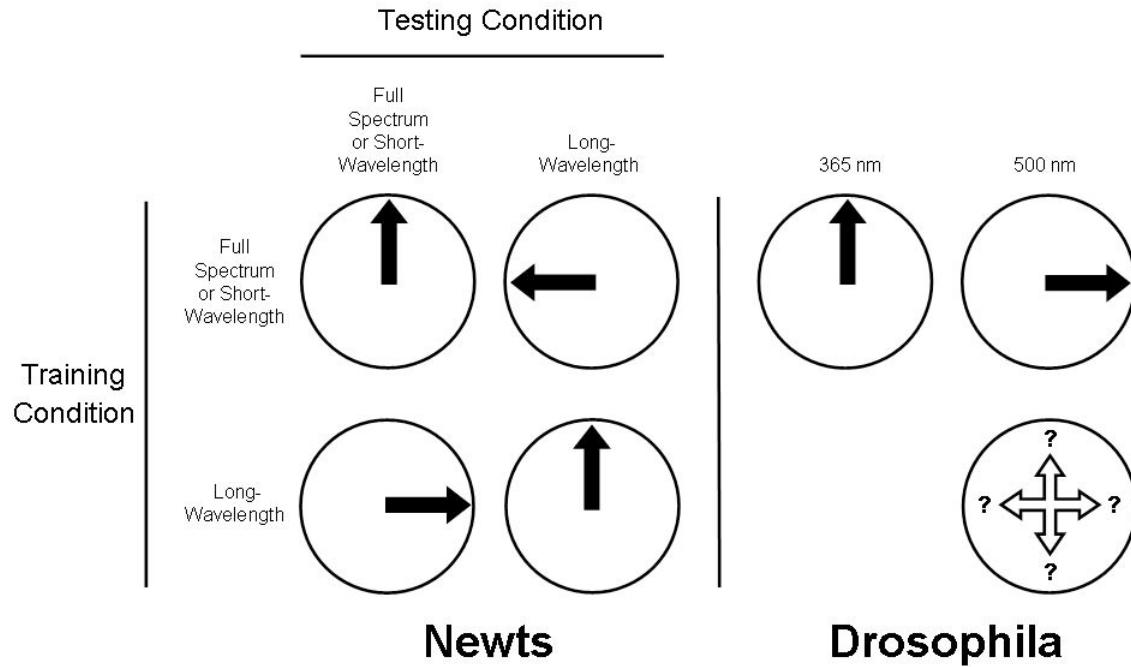
**Figure 1.1) Orientation response of eastern red spotted newts (*Notophthalmus viridescens*; Phillips and Borland 1992); and adult Drosophila melanogaster under differing wavelengths of light.** Black arrows indicate the mean direction of orientation for newts or flies exposed to different lighting conditions in training and testing. Lower right circle diagram in B shows possible responses of *Drosophila* trained and tested under long wavelength light.
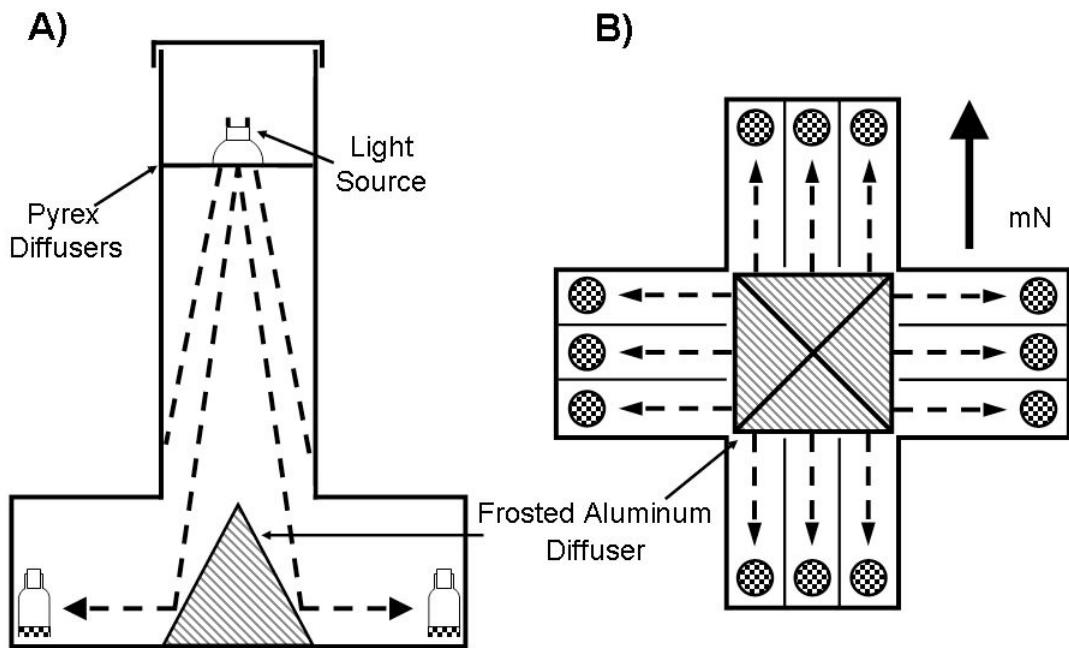
**Figure 1.2) Training apparatus.** (A) Side view of the training apparatus in which adult *Drosophila* were exposed to directional 530nm light (dashed arrows). Light passed down through a pair of Pyrex diffusers and then was reflected by a frosted aluminum pyramidal diffuser (grey slash), horizontally into each training arm. The four arms of the training apparatus were aligned to geomagnetic North, South, East, and West. Groups of flies were placed in Pyrex bottles at the ends of corridors. (B) Top view illustrating the training corridors and their alignment relative to the geomagnetic field with dashed arrows showing the direction of light reflecting off the pyramid shaped diffuser. mN = magnetic North.
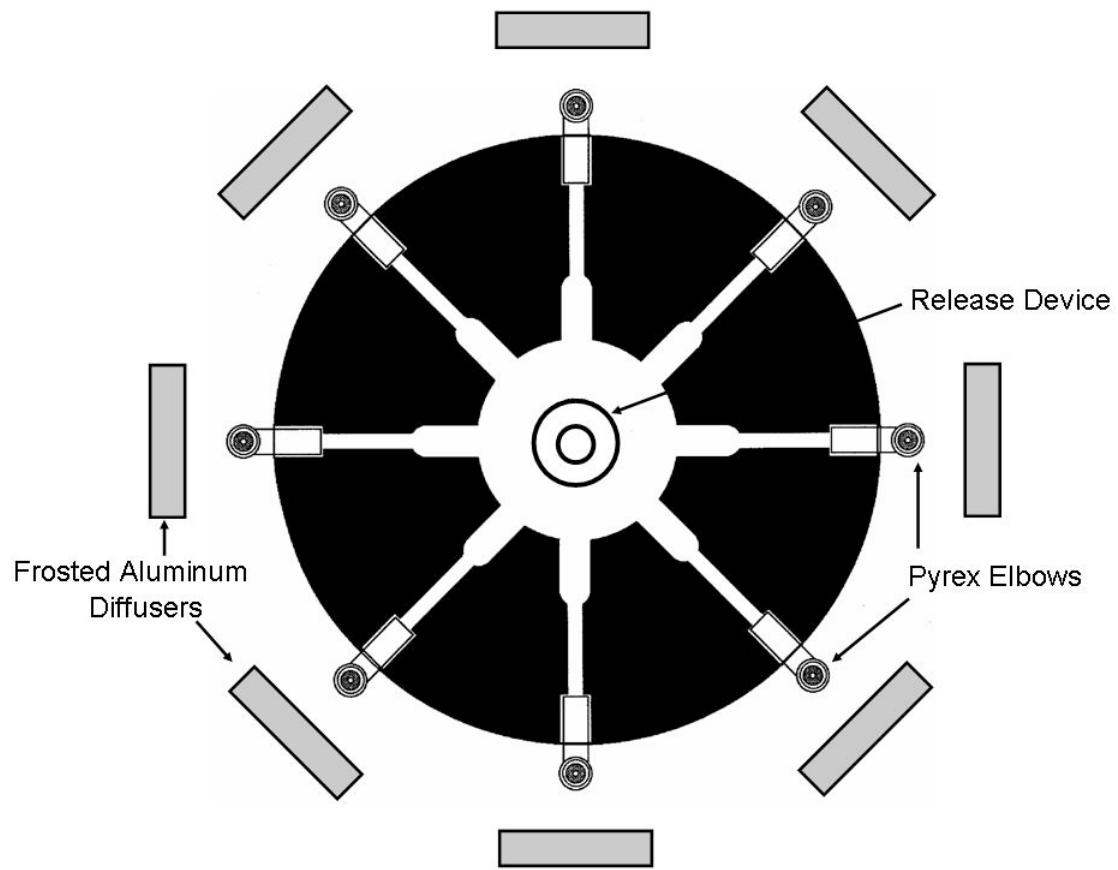
Figure 1.3A
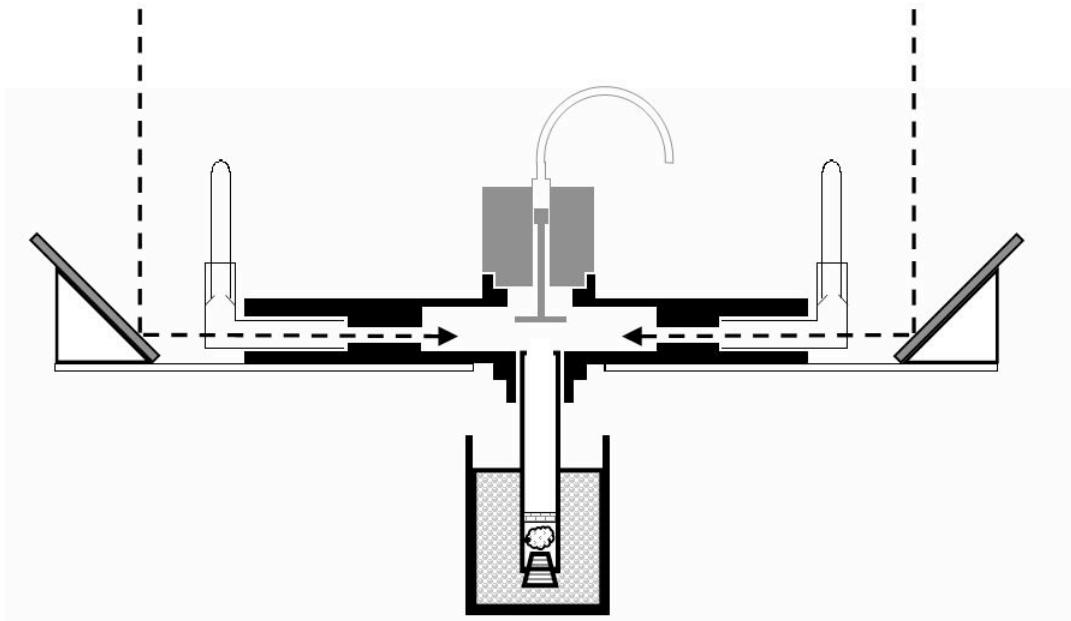
Figure 1.3B

**Figure 1.3) Testing apparatus.** (A) Top view of the Plexiglas 8-arm radial maze used to test adult *Drosophila*. The light source (not shown) was centered over the maze. The maze was painted flat black to ensure that light only entered the maze from the apertures at the end of each arm. (B) Side view. Light was reflected into each arm of the maze by means of a sandblasted aluminum diffuser (Grey rectangles) tilted at an angle of ~50 degrees. Flies were introduced into the maze by tapping them through a glass funnel from their training bottles into a 2cm Plexiglas holding tube suspended below the floor of the maze (see text). The bottom of the holding tube was plugged by a black rubber stopper. A plastic mesh screen separated several damp paper towels which were used to maintain humidity in the holding tube from the flies. The tube was chilled in an ice water bath to motivate flies to exit. A release device was placed over the holding tube to control entry and exit into the maze by adult flies.

Flies had access to the maze for 15 minutes, during which time all flies that had entered the Pyrex funnel traps at the end of each arm were scored.
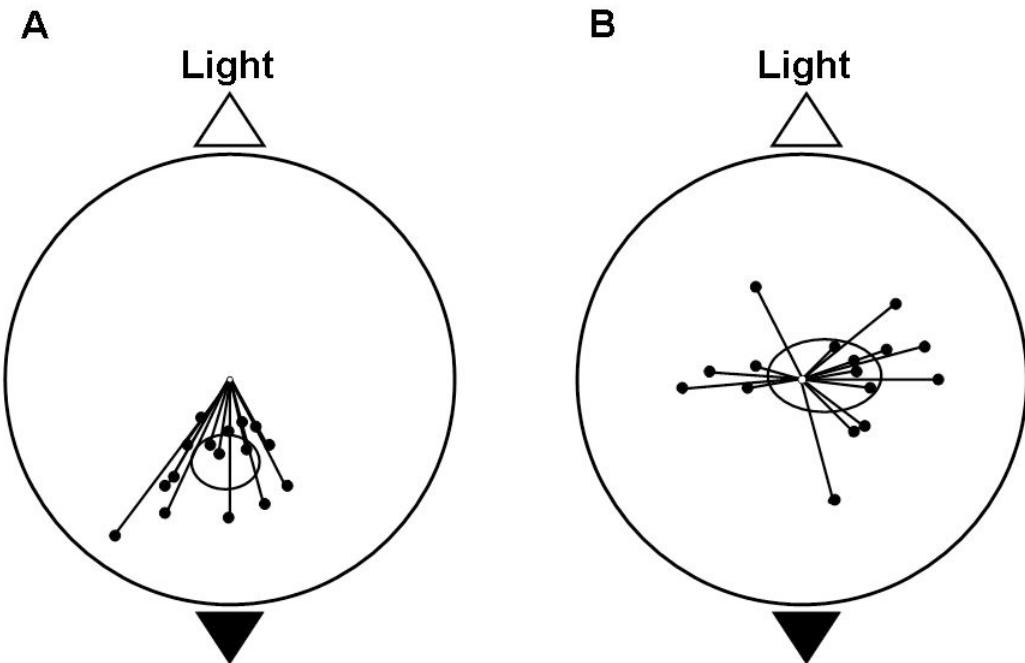
**Figure 1.4) Magnetic compass orientation of *D. melanogaster* tested under 530nm at intensities of 10.9 (A) and 11.9 (B) log quanta/cm2/s.** Each distribution contains data from 16 tests from 4 trained directions (N,E,S,W) x 4 alignments of magnetic North in testing (mN at geographic N,E,S,W; see methods). Each vector represents the mean vector bearing of an individual test calculated from the magnetic bearings of between 5 and 30 adult male flies. Length of the MVB is proportional to the clustering of the bearings ("r" value) with a longer vector indicating a higher r value (radius of the circle corresponds to r = 1). (A) Under low intensity long wavelength light adult *Drosophila* oriented unimodally toward the "dark" end (black triangle) of the trained axis (181°, r = 0.29, P < 0.01;); (B) Under a higher intensity of long wavelength light adults were randomly distributed (94°, r = 0.12, P > 0.05).

**Chapter 2**

**Magnetic compass orientation by larval *Drosophila melanogaster***

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**Abstract:**

We report evidence for magnetic compass orientation by larval *Drosophila melanogaster*.  Groups of larvae were exposed from the time of hatching to directional ultraviolet (365nm) light emanating from one of four magnetic directions. Larvae were then tested individually on a circular agar plate under diffuse light in one of four magnetic field alignments. The larvae exhibited magnetic compass orientation in a direction opposite that of the light source in training.  Evidence for a well developed magnetic compass in a larval insect that moves over distances of at most a few tens of centimeters has important implications for understanding the adaptive significance of orientation mechanisms like the magnetic compass. Moreover, the development of an assay for studying magnetic compass orientation in larval *Drosophila melanogaster* will make it possible to use a wide range of molecular genetic techniques to investigate the neurophysiological, biophysical, and molecular mechanisms underlying the magnetic compass.

**Introduction**

Use of the geomagnetic field for menotactic ("compass") orientation was first described in birds, and was once thought to be a navigational aid used only in long distance migration and homing. Subsequent research, however, has shown that magnetoreception is far more widespread than originally thought, having now been demonstrated in every major class of vertebrates, as well as many classes of invertebrates (Wiltschko & Wiltschko 1995). Moreover, magnetic compass cues have been shown to play a role in the spatial behavior of animals that move over much shorter distances (e.g., salamanders, rodents, ants; Burda et al. 1990; Phillips & Borland 1992; Banks & Srygley 2003). Here we report the first evidence of magnetic compass orientation by a larval insect, 2nd instar *Drosophila melanogaster*.

Studies by our laboratory have demonstrated the use of a magnetic compass by adult male *Drosophila* (Phillips & Sayeed 1993; Dommer & Phillips in prep.). Magnetic compass orientation by adult flies exhibits light-dependent properties similar to those observed earlier in amphibians (e.g., Phillips & Borland 1992; Deutschlander et al. 1999a; Freake & Phillips 2005) and consistent with theoretical models that have implicated a light-dependent biochemical process involving radical pair intermediates in magnetoreception (Ritz et al. 2000). Magnetic compass orientation in birds has been shown to be sensitive to both the wavelength and intensity of light, as well as to low-level radio frequency fields, both of which are consistent with a radical pair mechanism (Wiltschko & Wiltschko 1995; Ritz et al. 2000, 2004; Thalau et al. 2004).

During the larval stage, *Drosophila* move over distances of at most a few tens of centimeters. Female *Drosophila* oviposit on or near media suitable for larval development, usually fermenting plant material (Cadieu et al. 2000). Once hatched, 1st instar larvae orient towards and burrow into the media. This "foraging" behavior involves orientation to gradients of light intensity, chemical

cues, and humidity (referred to as phototaxis, chemotaxis, and hygrotaxis, respectively; Hassan et al. 2000). Of these cues, phototaxis has been studied most extensively (Sawin-McCormack et al. 1995). In the 1st through mid 3rd instar, larvae respond photonegatively (i.e., orient away from light; Grossfield 1978). During the middle of the 3rd instar, however, the larvae become photopositive, leaving the media in search of pupation sites (Godoy-Herrera et al. 1992). This change in behavior, which coincides with a cessation in feeding, is referred to as "wandering behavior" (Sokolowski et al. 1984).

Here we provide behavioral evidence for a robust magnetic compass response in 2nd instar *Drosophila melanogaster* larvae. We show that larvae exposed for several days to a directional source of ultraviolet (360 nm) light in the presence of an earth-strength magnetic field, when subsequently tested under non-directional UV light, exhibit magnetic compass orientation that coincides with the direction away from the light source in training (photonegative direction).

**Methods**

**Stocks:**

A hybrid of both Oregon-R and Canton S. adult wild type *Drosophila melanogaster* were reared in 250mL Pyrex bottles containing 50mL of Instant Carolina Drosophila Media (Formula 424 Blue). Dry active granulated yeast was sprinkled onto the surface of the media (~30% coverage), in combination with 2 mL of 5% tegosept solution to inhibit mold and bacterial growth within the rearing media. Two folded laboratory tissues (Kim wipes) were partially submerged in the media to provide a dry substrate for the adult flies. Flies were reared at $23 \pm 3^0$ C, relative humidity of $35 \pm 10\%$, and maintained under incandescent light produced by two, 60W soft white bulbs, on a 12:12 light/dark cycle. Every 5 days 75 – 100 adult flies were transferred into bottles containing fresh media to maintain colony health.

**Training/Testing Enclosure:**

Both training and testing took place in a light-tight radio-frequency shielded enclosure (Lindgren Enclosures, Inc. Model #12W-2/2-I).  In the testing enclosure, a "floating" floor, supported at the edges of the experimental chamber, helped to uncouple vibrations caused by the observer from the experimental apparatus. The training apparatus and testing apparatus (see below) were supported on separate tables each sitting in a sand box containing a ~ 40 cm deep layer of sand. The equipment was further isolated from vibrations by several layers of vibration dampening material underneath the training and testing equipment (Fig 2.1, 2.2).  All power supplies were located outside of the enclosure and lines were filtered through the enclosure prior to directly powering either light sources or magnetic coil elements.

**Training Vials:**

Training media was prepared following instructions provided by the Bloomington Stock Center consisting of 266ml distilled water, 1.3g Lab Scientific agar, 3.3g dry active granular yeast, 16.3g Quaker degerminated yellow cornmeal and 10ml unsulphured molasses. The media was distributed into 35ml Pyrex vials (which transmit UV light down to ~ 320 nm) in 10 ml aliquots. Training vials were allowed to cool at room temperature for approximately 2 hours before being plugged with a cotton stopper and placed in the refrigerator for a period of no longer than 7 days ($13 \pm 0.05^0$ C). Prior to placing flies in training, vials were removed from the refrigerator for 24 hours and allowed to acclimate to room temperature ($22 \pm 2^0$ C). The surface of the media was scored with a probe to facilitate egg laying by adults. Approximately 15 – 20, 5 - 7 day old adult flies were transferred into each vial. Vials were immediately plugged with a fresh cotton stopper and cleaned to remove fingerprints before being carried into the testing enclosure (below).

**Training:**

An incandescent headlamp equipped with a 665 nm long-pass filter provided long-wavelength light for the experimenter (undetectable by the larvae) during movement within the testing enclosure (Ashburner, 1989). Once inside the experimental enclosure, individual vials were inserted horizontally into holes drilled into the sides of a matte black wood block in the center of the training apparatus ("training block"; Fig. 2.1). The vials were aligned with the larval media towards the inner (dark) end and the cotton plug toward the outer (light) end. Vials were placed in training between 1600 and 1800 hours EST. Adult flies were left in the training vials with the developing larvae for the duration of training. The training room was maintained at $21 \pm 2^0$ C (relative humidity $35 \pm 5\%$) and flies were allowed to develop to the late $2^{nd}$ instar stage (~140-150 hrs). Larvae were trained under constant light (24L:0D) to avoid any differences in access to magnetic cues while the larvae were in training (e.g., if the magnetic compass does not operate in the absence of light; Phillips & Sayeed 1993, and see earlier discussion).

The training block centered in the middle of a 72cm square table (Fig. 2.1 A,B,C). Groups of larvae were trained to four different directions relative to the geomagnetic field: with the dark ends of the vials at magnetic northeast ($45^o$), northwest ($315^o$), southeast ($135^o$), and southwest ($225^o$). The expected direction of orientation for each group was toward the dark (photonegative) end of the vial.

Monochromatic ultraviolet (UV) light (365 nm, 12nm bandwidth @ 50% maximum) was supplied by four Nichia UV LED's (NSHU #550A) from a height of 95cm. Training and testing LED's were wired in parallel and powered by a voltage regulated, DC power supply (RSR - HY3005). Light from the diodes passed through four diffusers separated by 2.5 cm each (two made of Pyrex glass frosted on both sides, and two made of frosted Mylar sheets). The UV light was reflected towards the sides of the training block by four $45^o$ angled pieces of

sand-blasted aluminum, located at the same height as the training block (Grey dot fill, Fig. 2.1 A,B,C). Cotton plugs were in place throughout the training regime, and provided a diffuse surface to reflect the overhead light.  Both the angled diffusers and the cotton stopper reflected diffuse light onto the surface of the media.  Light intensity at the surface of the media with a cotton stopper in place was 10.1 ± 0.05 log quanta/cm$^2$/s, measured using a Keithly RFA 486 picoammeter with a calibrated United Detector Technology photodiode (UDT Sensors Inc.10DP/SB).

**Testing Fields:**

Tests were conducted within a pair of perpendicularly aligned cube surface coils (Merritt et al. 1983) using a configuration described in Phillips (1986). A current regulated DC power supply (Lambda Electronics, Inc.) was used to power both coils.  Flies were tested with magnetic north (mN) in one of four geographic directions (North, East, South, or West). The South (mN=S), East (mN=E), and West (mN=W) fields were similar to the ambient field (mN=N) in intensity (+/-1%) and inclination (+/- 1 degree).   The two coils were connected in parallel and powered by a single power supply, so the north field was the only testing condition in which the connection to the power supply was interrupted[3].   A full testing series consisted of 16 larvae, one individual from each trained direction tested in each alignment of the testing field (4 trained directions x 4 magnetic field alignments). Two test series were carried out, resulting in 32 total bearings from individual larvae, eight bearings from larvae trained in each of the four directions (i.e., 45$^o$, 135$^o$, 225$^o$ or 315$^o$).

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[3] The north field was the only testing condition in which the connection of the power supplies to both coils was interrupted.  The resulting open circuit may have increased the electrical noise level and contributed to the scatter of bearings in this testing condition. Extremely weak AC fields in the low MHz range have been shown to disrupt magnetic compass orientation in migratory birds (Ritz et al. 2004).

**Testing:**

The directional responses of larvae were observed on 150mm Petri plates filled with 60mL grape juice agar (17.5g agar, 750ml distilled water, 12.5g sucrose and 250ml grape juice made from concentrate). Plates were covered and allowed to cool to room temperature prior to testing. A new plate was used for each trial to eliminate the possibility that a larva could follow chemical cues left by the larva tested in the previous trial. The concentration of agar in the testing plate media prevented larvae from burrowing into the surface, and facilitated horizontal movement of larvae along the surface of the media.

For testing, a training vial was removed from the training block using the 665 nm headlamp. A non-magnetic stainless steel probe was used to remove media from the dark end of the training vials, which was then placed into a 40% sucrose solution to separate larvae from the training media. The solution was agitated by using fine bristle paint brushes. Larvae are less dense than the sucrose solution, and floating larvae were removed individually from the sugar bath using a fine paint brush. Isolated larvae were cleaned of residual media in a distilled water bath and placed near the center of an acclimation plate identical to the testing plates. Once 10-12 larvae had been isolated and placed onto the acclimation plate, the plate was covered with an opaque lid and placed in a light tight black felt bag to minimize light exposure from the UV testing light. The larvae remained on the acclimation plate until they were tested, not to exceed 40 minutes of total time on the acclimation plate.

Prior to testing, a fresh testing plate was aligned on a circular template (visible through the agar). One of the larvae from the acclimation plate was transferred to the center of the testing plate. The larva was immediately covered with an opaque 3 cm high x 2 cm diameter plastic cap, and the testing plate was lowered into a level holder centered inside the testing coils (Fig. 2.2 A,B), and aligned with a reference mark at Geomagnetic North (= 0º topographic). The

testing plate holder was illuminated bv a diffuse, non-directional 365 nm monochromatic light source directed downward onto the testing plate with a light intensity (10.1 log quanta/cm$^2$/s at the surface of the testing plate) equal to that in training (Fig. 2.2A,B). After 60 seconds, the opaque cap covering the larva was removed, and the larva was allowed to move freely on the surface of the plate. In all trials, the larva was observed from a fixed position at approximately 135º and at a distance 0.5 m from the center of the testing coil.  A directional response was recorded where the larvae crossed a 60 mm radius circle, 15 mm in from the edge of the plate. Once the larva had scored, the plate was immediately removed from the holder, and positioned so that the 0º indicator on the plate was aligned with a paper template. The position where the larvae contacted the criterion circle was then transferred to the paper template to create a permanent record of the larvae's topographic directional response. After completion of the entire test, the directional responses of the larvae ("topographic bearings") were measured with a protractor to the nearest degree. The alignment of the test field was changed between trials to avoid the same expected direction within the testing arena in successive trials (see below). If the investigator had difficulty removing a larva from the holding plate for testing, that larva was not tested.  In addition, larvae that climbed inside the opaque cap, failed to reach the 60 mm boundary within 15 minutes, or burrowed down into the testing media were not scored, i.e., the trial was terminated, and no bearing was recorded for that larva (approximately 20% of the larvae tested).

**Results:**

The distribution of absolute or "topographic" bearings at the 60 mm criterion boundary, ignoring both magnetic field alignment and trained direction, was indistinguishable from random (    = 231°, r = 0.09, p > 0.10, Rayleigh Test; Fig. 2.3A, Table 1), indicating that the larvae were not orienting relative to a fixed directional cue present in the testing arena. The distribution of magnetic bearings (bearings relative to magnetic North, ignoring trained direction) was also

indistinguishable from random ( = 299°, r = 0.22, p > 0.10), indicating that the larvae did not show a consistent directional response relative to the magnetic field independent of the trained direction (Fig. 2.3B, Table 1). The distribution of topographic bearings plotted as deviations from the trained topographic direction (ignoring magnetic field alignment) was also indistinguishable from random ( = 333°, r = 0.07, p > 0.10), indicating that the larvae were not orienting in the trained direction using a non-magnetic directional cue (Fig. 2.3C; 2.5 A-D, Table 1). In contrast, the clustering in the distribution of bearings plotted relative to the trained magnetic direction, taking into account both the testing field and trained direction, was highly significant ( = 7°, r = 0.56, p = 0.001, n = 32), and the 95% confidence interval for the mean vector bearing included the trained magnetic direction (Fig. 2.3D, Table 1).

Figure 2.4 shows the distribution of magnetic bearings obtained from larvae trained to the four different directions (Fig 2.4; Table 2A, and see Methods); the distribution for each trained direction includes two bearings from larvae tested in each of the four field alignments (mN = N, E, S, W; see Methods). Figure 2.5 shows the distribution of bearings plotted as deviations from the trained topographic direction for each of the four magnetic field alignments used in testing; the distribution for each testing field alignment includes two bearings from larvae in each of the four trained directions (45°, 135°, 225°, 315°; see Methods). In both Fig. 2.4 and 2.5, one of the four distributions was not significant relative to the trained direction (p > 0.05; Rayleigh test); when the bearings were grouped by trained direction the distribution of larvae trained to 225° was indistinguishable from random (Table 2.2A, Fig. 2.4B), and when the bearings were grouped by testing field the same was true of the distribution of larvae tested in the north field (Table 2B, Fig. 2.5A). In neither case, however, were the distributions of bearings plotted as deviations from the trained magnetic directions (open triangles) significantly different from those obtained in any of the other experimental conditions, or from that of the three other experimental conditions combined (p > 0.10; Watson $U^2$ test).

Although there were no significant differences between any of the experimental conditions in Fig 2.4 and 2.5, the absence of significant orientation in the north testing field is noteworthy because in this condition both coils were turned off leaving the horizontal component of the ambient magnetic field unaltered (see Methods).  However, as shown in Fig 2.3, the distribution of bearings of larvae tested in the north field (open symbols) was indistinguishable from those of larvae tested in the three other fields (solid symbols) when the data are plotted as absolute or topographic bearings (Fig. 2.3A), as magnetic bearings (Fig. 2.3B), as deviations from the trained topographic direction (Fig. 2.3C), or as deviations from the trained magnetic direction (Fig. 2.3D).  Moreover, only the distribution of bearings plotted relative to the trained magnetic directions exhibited significant orientation (Fig. 2.3D).

Furthermore, the evidence for a learned magnetic compass response does not depend on a comparison of the north field (coils off) and east, west, and south fields (coils on). If the north field bearings are excluded, significant orientation is evident when the bearings are plotted as deviations from the trained magnetic directions, both in the distributions of bearings from each of the three remaining testing fields (Fig. 2.5B,C,D), and in the distribution of bearings pooled from all three fields (Fig. 2.3, solid symbols;    = 9°, r = 0.63, p = 0.001, n = 24).   Moreover, the three distributions of bearings plotted as deviations from the trained topographic directions (Fig. 2.5B, C, D), each differ significantly from the other two distributions (B vs. C; C vs. D; B vs. D; $p < 0.05$, Watson $U^2$ test), and in each case the 95% confidence interval includes the trained magnetic direction (open triangle) but not the trained topographic direction (solid triangle). [Note in Fig. 2.5, the bearings of larvae trained to the four different directions (45°, 135°, 225°, and 315°) are rotated so that the topographic trained direction is as the top of each distribution. Consequently, the orientation in Fig. 2.5B, C, D reflects both the trained directions and the alignments of the testing fields.]

**Discussion:**

The findings presented here (Fig. 2.3-2.5) show that $2^{nd}$ instar larvae of *Drosophila melanogaster* have a well-developed magnetic compass response and are capable of learning the alignment(s) of important environmental gradient(s) (i.e., light, food, and/or humidity) with respect to the geomagnetic field. Although magnetic orientation has been demonstrated previously in tadpoles of the bullfrog *Rana catesbeiana* (Freake & Phillips 2005) and the Iberian green frog *Rana perezi* (Diego-Rasilla & Phillips submitted), this is the first demonstration of magnetic compass orientation by a larval invertebrate.

Further research is needed to determine if there is, in fact, greater scatter in the north testing field. It is important to emphasize, however, that the response of larval flies was not simply an effect of the coils being off (north field) or on (east, south, west fields); see Results. Indeed, the case for a learned magnetic compass response is even more compelling if data obtained in the north testing field are excluded.

Evidence for magnetic compass orientation in these larval insects suggests that the use of magnetic compass cues may be adaptive for extremely short distance movements. Movement by limbless arthropods is among the most costly per stride length in the animal kingdom due to friction between the ventral surface of the animal and the substrate. Immersion of larvae in their food source adds additional resistance to movement, placing further energetic demands on the organism (Berringam & Lighton 1993). The high cost of larval locomotion is likely to have increased selection for orientation mechanisms, such as the magnetic compass, that facilitate straight line movement and thus, minimize path length between locations. Regardless of the type of behavior a reliable compass cue would decrease the cost of movement for the larvae of this and many other species of insects.

Larval *Drosophila* have a relatively simple nervous system that lacks many of the sophisticated sensory mechanisms present in adults, and exhibit a correspondingly less complex array of behavioral responses (Ashburner 1989).

Consequently, the magnetic compass response of these larvae (Fig. 2.3-2.5) provides an opportunity to study a magnetoreception system in which the numbers and types of neurons are likely to approach the minimum necessary for the implementation of this type of sensory system. Characterizing the functional properties of magnetic compass orientation in such an organism may help to distinguish fundamental properties of the underlying biophysical process from those arising as a consequence of secondary processing of sensory input, which has proven difficult in other systems (e.g., Phillips et al. 2001, 2004; Freake & Phillips 2005). Moreover, the magnetic compass response of larval *Drosophila* is well-suited for behavioral genetic analysis. Use of molecular genetic techniques to "dissect" the molecular mechanisms and neural pathways underlying the magnetic compass will be especially powerful when applied in an organism with such a simple behavioral repertoire.
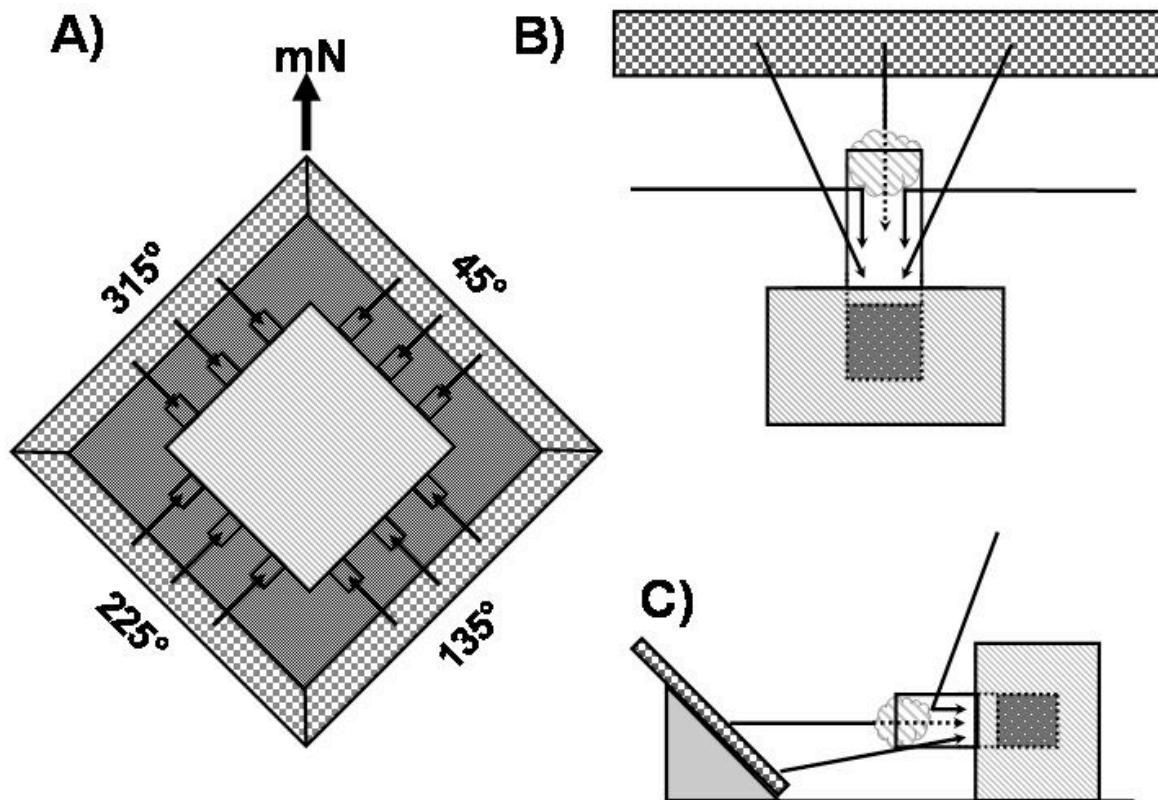
**Figure 2.1) Training apparatus**. A) Top view of apparatus showing the alignments of training vials inserted into holes in the sides of the training block (gray square with parallel lines at center of diagram). Flies were exposed to directional 365 nm light (black arrows) from an overhead light source identical to that used in testing (see Fig 2) scattered toward the light end of the vials by 45° angle aluminum diffusers (gray diamond fill). Larval media provided a dark refuge at the inner (dark) end of the vials. Vials were aligned in one of 4 magnetic directions (dark end at 45°, 135°, 225°, 315° magnetic). B) Overhead view of an individual vial showing light (black arrows) reflecting to the surface of the training media (dark gray fill) off of the diffuser and the cotton stopper. C) Side view of training vial showing light (black arrows) scattered from the 45° angle aluminum diffuser and the cotton plug.

Figure 2.2A
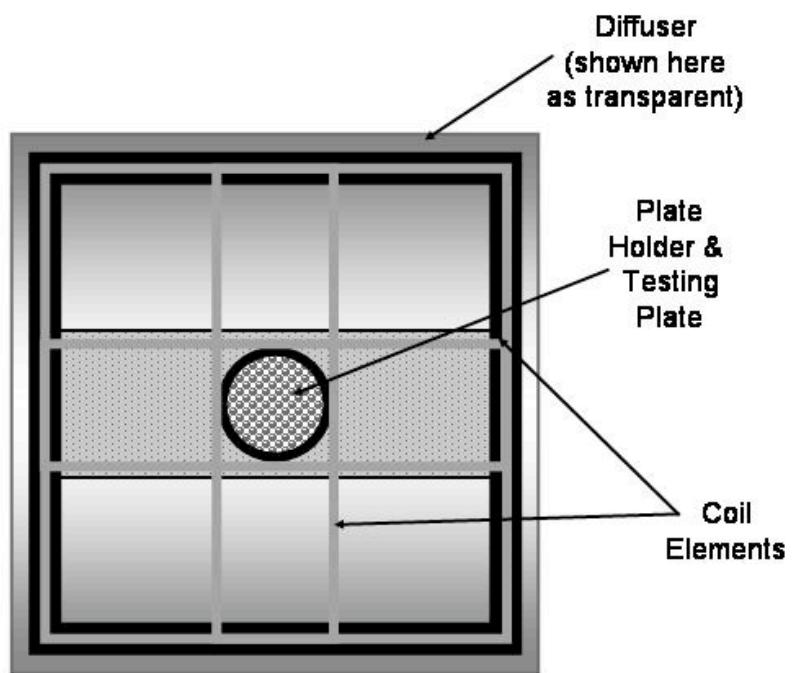
Figure 2.2B
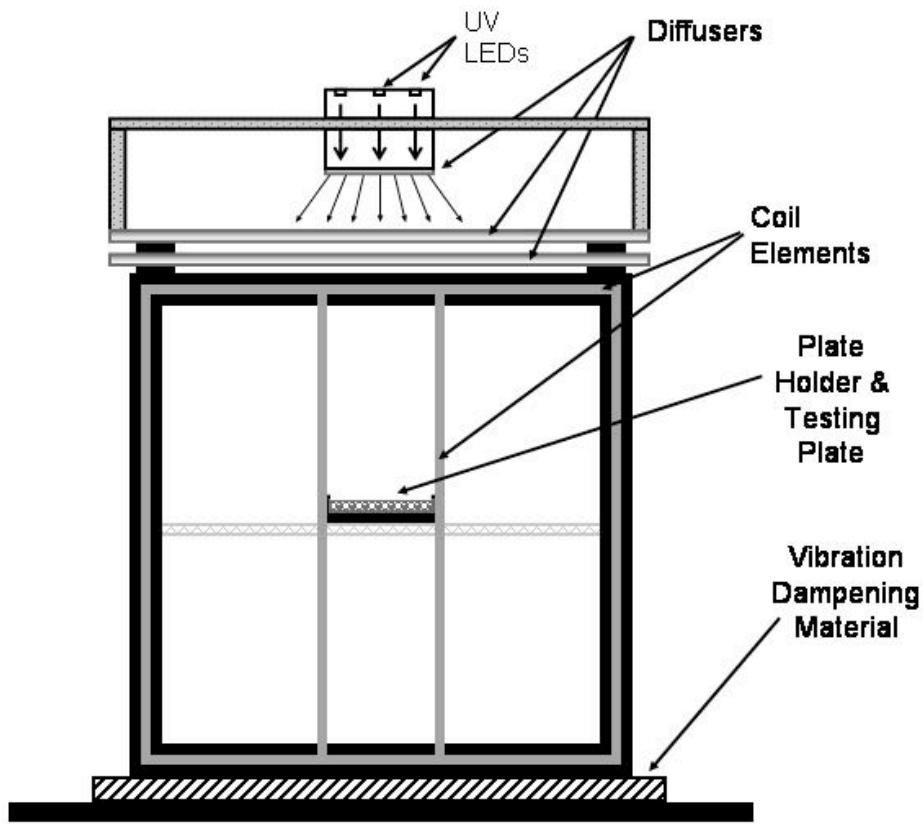


**Figure 2.2) Testing apparatus.** A single larva was placed at the center of a fresh 150mm diameter Petri plate and immediately covered with an opaque cap. The plate was then placed in a matte black plexiglas holder on a platform at the center of the testing coil. A) <u>Overhead view</u> of testing apparatus (overhead light source removed) showing the position of the testing plate in the center of the pair of orthogonally aligned cube-surface-coils (Merrit et al. 1983). Larvae were tested in the ambient field with magnetic north at North (mN = 0°), or in one of three altered fields with magnetic north at East (mN = 90°), South (mN = 180°), or West (mN = 270°) ; see Phillips (1986) for explanation of how testing fields are generated. B.) <u>Side view</u> of the testing apparatus showing the location of the testing plate in the center of the coil system. The UV (365 nm) light source, consisting of four light-emitting diodes (LEDs) and 15 cm diameter frosted Pyrex diffusers, was suspended approximately 10 cm above two 76 x 76 cm diffusers (each consisting of one layer of frosted pyrex and one of frosted mylar) that were

separated by 2.5 cm. The two large diffusers rested on the top of the coil frame. The position and alignment of the light source was adjusted so that the diffuse overhead illumination reaching the surface of the testing plate was non-directional. All surfaces surrounding the testing plate were painted flat black to minimize reflection.

Figure 2.3
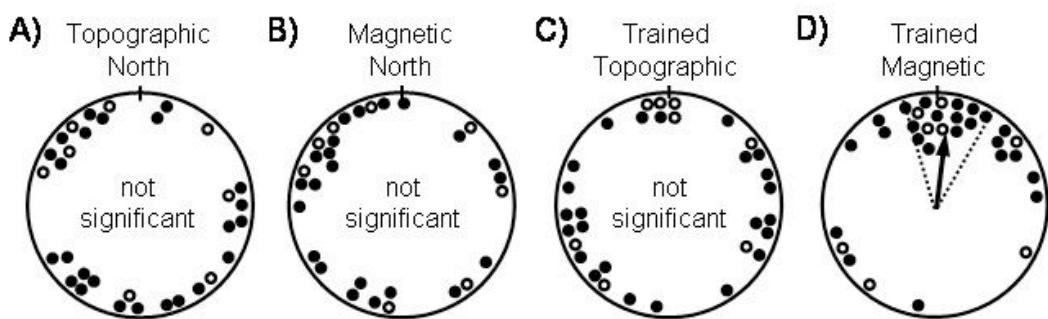


**Figure 2.3** Orientation of 2nd instar *Drosophila melanogaster* larvae tested under diffuse 365 nm light. Each point represents the bearing of a larva tested individually on a fresh testing plate. A) The distribution of absolute or topographic bearings (topographic N = 0°) is indistinguishable from random ( = 231°, r = 0.09, n = 32, p > 0.10; Rayleigh test; Batschelet, 1981). B) When the same bearings are plotted relative to the alignment of the testing field (mN = 0°), the distribution of magnetic bearings is also randomly distributed ( = 299°, r = 0.22, n = 32, p > 0.10). C) Bearings of larvae plotted as deviations from the trained topographic direction (ignoring magnetic field alignment) was also indistinguishable from random ( = 333°, r = 0.07, p > 0.10), indicating that the larvae were not orienting in the trained direction using a non-magnetic directional cue (see also Fig 5). In contrast, D) when the bearings are plotted relative to the trained magnetic direction (Trained Direction = 0°), the bearings are non-randomly distributed and the 95% confidence interval includes the expected direction ( = 7° ± 23°, r = 0.56, n = 32, p < 0.001). Arrows at the center of circular diagrams indicate the mean vector bearings ( ), with the length of the arrow proportional to the mean vector length ("r"; radius of the circle corresponds to r = 1). Dashed lines show the 95% confidence intervals for mean vectors that are significant by the Rayleigh test (p < 0.05). Open symbols are bearings of larvae tested in the north field; solid symbols are bearings of larvae tested in east, west, or south fields.

Figure 2.4
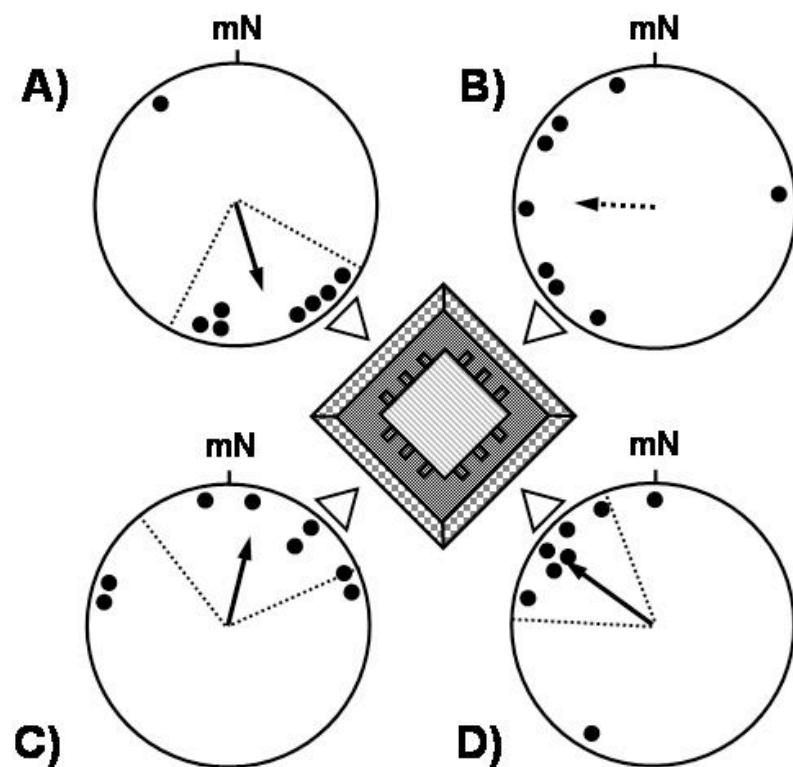


**Figure 2.4** Distribution of individual magnetic bearings obtained from groups of larvae trained to different magnetic directions; each distribution contains two bearings from larvae tested in each of the four testing fields (mN = 0°, 90°, 180°, 270°).  Circular diagrams show the distribution of magnetic bearings obtained from larvae trained with the dark end of their training vial at: A) 135°, B) 225°, C) 45°, and D) 315°.  Symbols as in Figure 2.3.  Open triangles outside of each circular diagram indicate expected direction of orientation relative to the magnetic field.

Figure 2.5
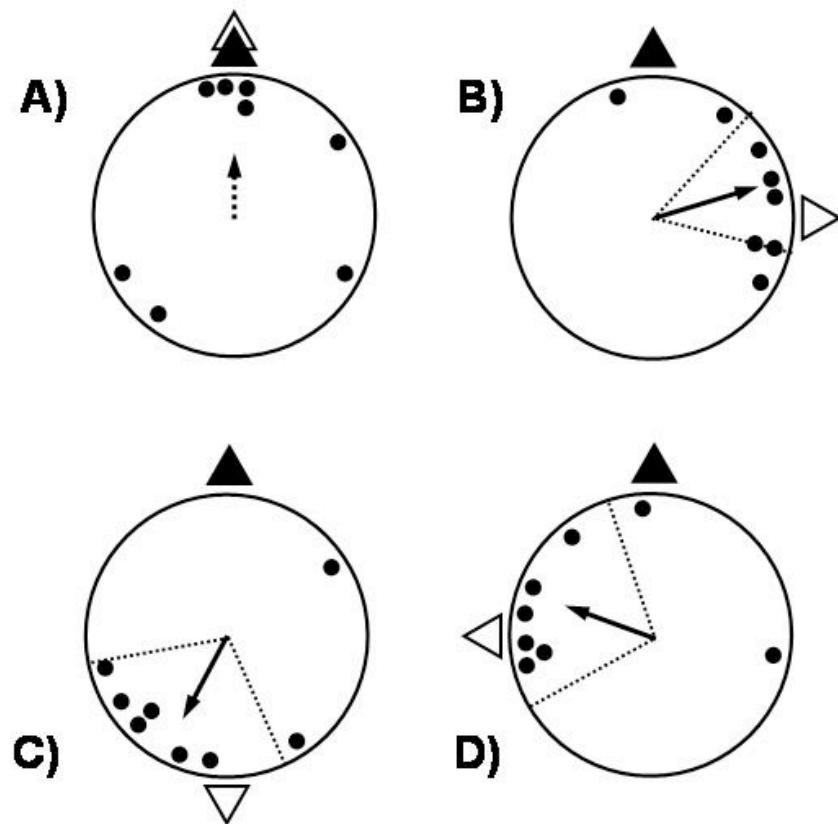


**Figure 2.5** Distribution of bearings plotted as deviations from the trained topographic direction for each of the four magnetic field alignments used in testing [mN at topographic A) north , B) east, C) south, or D) west]. The distribution for each testing field (A-D) includes two bearings from larvae trained in each of the four directions (45°, 135°, 225°, 315°; see Methods). Symbols as in Figure 2.3. Solid triangles outside of each diagram indicate the trained topographic direction of orientation; open triangles indicate the trained magnetic direction for each distribution.

| Table 1) Directional Responses of Drosophila melanogaster Larvae | | | | | | |
|---|---|---|---|---|---|---|
| Date of Test (Mo/Day/Yr) | Direction Magnetic North | Trained Direction (direction away from light in training) | Topographic Bearing | Magnetic Bearing | Bearing Relative to Trained TOPOGRAPHIC Direction | Bearing Relative To Trained MAGNETIC Direction |
| 3/8/2005 | 0 | 45 | 289 | 289 | 244 | 244 |
| 3/23/2005 | 0 | 45 | 40 | 40 | 355 | 355 |
| 3/21/2005 | 90 | 45 | 165 | 75 | 120 | 30 |
| 3/23/2005 | 90 | 45 | 80 | 350 | 35 | 305 |
| 3/8/2005 | 180 | 45 | 191 | 11 | 146 | 326 |
| 3/23/2005 | 180 | 45 | 102 | 282 | 57 | 237 |
| 3/8/2005 | 270 | 45 | 310 | 40 | 265 | 355 |
| 3/23/2005 | 270 | 45 | 336 | 66 | 291 | 21 |
| 3/6/2005 | 0 | 135 | 188 | 188 | 53 | 53 |
| 3/23/2005 | 0 | 135 | 137 | 137 | 2 | 2 |
| 3/6/2005 | 90 | 135 | 214 | 124 | 79 | 351 |
| 3/23/2005 | 90 | 135 | 238 | 148 | 103 | 13 |
| 3/21/2005 | 180 | 135 | 14 | 194 | 239 | 59 |
| 3/23/2005 | 180 | 135 | 322 | 142 | 187 | 7 |
| 3/21/2005 | 270 | 135 | 98 | 188 | 323 | 53 |
| 3/23/2005 | 270 | 135 | 234 | 324 | 99 | 189 |
| 3/9/2005 | 0 | 225 | 84 | 84 | 219 | 219 |
| 4/4/2005 | 0 | 225 | 343 | 343 | 118 | 118 |
| 3/29/2005 | 90 | 225 | 298 | 208 | 73 | 343 |
| 4/4/2005 | 90 | 225 | 330 | 240 | 105 | 15 |
| 3/29/2005 | 180 | 225 | 120 | 300 | 255 | 75 |
| 4/4/2005 | 180 | 225 | 89 | 269 | 224 | 44 |
| 3/9/2005 | 270 | 225 | 221 | 311 | 356 | 86 |
| 4/5/2005 | 270 | 225 | 144 | 234 | 279 | 9 |
| 3/20/2005 | 0 | 315 | 306 | 306 | 351 | 351 |
| 3/25/2005 | 0 | 315 | 318 | 318 | 3 | 3 |
| 3/20/2005 | 90 | 315 | 299 | 209 | 344 | 254 |
| 3/25/2005 | 90 | 315 | 12 | 282 | 57 | 327 |
| 3/10/2005 | 180 | 315 | 157 | 337 | 202 | 22 |
| 3/20/2005 | 180 | 315 | 181 | 1 | 226 | 46 |
| 3/10/2005 | 270 | 315 | 217 | 307 | 262 | 352 |
| 3/20/2005 | 270 | 315 | 211 | 301 | 256 | 346 |

**Table 1.)** 'Topographic', 'magnetic', 'relative to trained topographic direction', and 'relative to trained magnetic direction' bearings obtained from *Drosophila* larvae; each larva tested only once in one of the 16 experimental conditions (4 trained directions X 4 magnetic field alignments).

**Table 2A)  Orientation of larvae plotted as deviations from magnetic north (= 0°).**

| Magnetic orientation--<br>Trained Direction | MVB | r | P | N |
|---|---|---|---|---|
| 45°   (Northeast) | 14° | 0.61 | 0.05 | 8 |
| 135° (Southeast) | 163° | 0.66 | 0.025 | 8 |
| 225° (Southwest) | 273° | 0.51 | n.s. | 8 |
| 315° (Northwest) | 306° | 0.77 | 0.01 | 8 |

**Table 2B)  Orientation of larvae plotted as deviations from trained topographic directions**

| Magnetic Field<br>Alignment | MVB | r | P | N |
|---|---|---|---|---|
| Magnetic North at 0° | 359° | 0.36 | n.s. | 8 |
| Magnetic North at 90° | 73° | 0.77 | 0.01 | 8 |
| Magnetic North at 180° | 208° | 0.62 | 0.05 | 8 |
| Magnetic North at 270° | 290° | 0.61 | 0.05 | 8 |

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# Chapter 3

## The Light-Dependent Magnetic Compass of Larval *Drosophila*

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**Abstract**

Use of a light-dependent magnetic compass is wide spread in animals and has been proposed to involve a photo-excited radical pair mechanism (RPM). As predicted by the RPM, findings indicate the magnetic compass of $2^{nd}$ instar larval *Drosophila* is sensitive to the axis, not polarity, of the magnetic field; the first such mechanism described in an invertebrate. Furthermore, larvae exhibit a complex, axially-symmetrical pattern of response under 365 nm light, while under the same intensity of 490 nm light larvae exhibit the inverse (complementary) pattern. This demonstrates an antagonistic interaction of short- and long-wavelength inputs proposed to explain wavelength-dependent effects of light on magnetic compass orientation in vertebrates. These findings indicate the larval response is mediated by a simple detector with little secondary processing, so the behavior of the whole organism can be used to 'visualize' fundamental properties of the biophysical process underlying the light-dependent magnetic compass.

**Introduction**

Sensitivity to the geomagnetic field is widespread in animals and appears to involve at least three distinct biophysical mechanisms[1-14]. The "radical pair mechanism" (RPM[9,11]) proposes that directional (i.e. compass) information is derived from the magnetic field by means of a light-dependent biochemical reaction that forms long-lived radical pair intermediates. According to the RPM, the alignment of an earth-strength magnetic field modulates the response of a photoreceptor containing an ordered array of light-absorbing molecules by altering the relative population of energy states that influence the lifetime of the photoexcited form of the photopigment and/or its ability to drive phototransduction[9,13]. The response to light depends on the alignment of the photopigment array relative to the magnetic field. If photoreceptors involved in magnetoreception are located in the retina, the magnetic field may be perceived as an axially symmetrical, three-dimensional pattern of light intensity or color superimposed on the organism's visual field[9,11] (Fig. 3.1a,b,c, S1). Alternatively, the complex pattern produced by the magnetic field may be sensed by a separate light-dependent pathway independent of vision, e.g. in organisms in which the light-dependent magnetic compass is mediated by non-image forming and/or extraocular photoreceptors[17].

Experimental support for the RPM includes: (1) involvement of a light-dependent magnetoreception mechanism[18, 19]; (2) sensitivity to the axis, but not the polarity (i.e. N vs. S) of the magnetic field [animals with this type of

"inclination compass" use the slope of the magnetic field to distinguish between the two ends of the magnetic axis][19,20]; (3) disruption of magnetic compass orientation outside of a narrow window of static field intensity[21]; (4) failure to find an effect of brief, high intensity magnetic pulses (pulse remagnetization), shown to disrupt mechanisms involving magnetite particles fixed with respect to the surrounding tissue[22,23]; and (5) disruption of magnetic compass orientation by weak radio frequency fields (~0.1% of the static field strength)[24,25].

Models of a RPM vary considerably in complexity[6,9,11,26-29], but share a 3-dimensional pattern that exhibits axial symmetry (i.e., identical components of the pattern 180° apart; Fig. 3.1a,b,c). Consistent with the response predicted by a simple RPM, adult fruit flies[16] and C57BL/6J mice[15] trained to the north (N) or south (S) exhibited similar unimodal magnetic compass orientation, while the orientation of flies and mice trained to the east (E) or to the west (W) was split into two modes separated by ~90º (Fig. 3.1 d,e). Although these findings suggest that the same type of magnetoreception mechanism may be present in flies and mice, alternative explanations are difficult to rule out because of the behavioral and neural complexity of these organisms. Ideally, a behavioral assay used to characterize a biophysical process as complex as the RPM should involve: (1) an organism with a limited behavioral repertoire, (2) a magnetic compass response mediated by a simple detector (e.g. a single receptor or a small group of similarly aligned receptors), (3) little or no secondary processing that alters or masks characteristics of the primary magnetic input, and (4) experimental manipulation(s) that can be used to determine whether characteristics of the

response reflect fundamental properties of the underlying mechanism. Here, we report that larval *Drosophila* meet these requirements and, moreover, that the response of larval flies demonstrates the light-dependent, axially-symmetrical pattern of response unique to the RPM.

**Methods Summary:**

In the present experiments, larvae of *Drosophila melanogaster* were trained from hatching until the late 2nd instar to orient relative to the magnetic field. Larvae of this stage are strongly photonegative. Previous work has shown that larvae exposed to a directional light source learn to orient in the magnetic direction away from light[29] (see Methods). Separate groups of larvae were trained in vials with the end containing media inserted into a horizontal cavity (dark end). The opposite end of the vial was illuminated with ultraviolet (365 nm) light. The dark end of the vial was aligned in one of eight directions relative to the magnetic field. Larvae were tested one at a time on an agar plate in a diffuse isotropic light field. The testing plate was centered in a system of cube-surface-coils[30] allowing magnetic North (mN) to be rotated to one of four alignments (i.e. mN at geographic N, E, S, or W).

**Results:**

**Larval Magnetic Compass Exhibits Axial Symmetry:**

When tested under 365 nm light, larvae trained E or W (Fig. 3.2a,e) exhibited robust unimodal magnetic compass orientation in a direction opposite that of the light in training. Larvae trained Northeast (NE), Northwest (NW),

Southeast (SE), or Southwest (SW) ("45° axes"; Fig. 3.2b,d,f,h) showed similar

unimodal orientation. In contrast, larvae trained N or S (Fig. 3.2c,g) failed to

orient in the trained direction and instead exhibited tightly clustered quadramodal

orientation along the 45° axes. The similar responses of larvae trained in

opposite directions along the same magnetic axis (Fig. 3.2a-h) are consistent

with the axial symmetry predicted by the RPM, suggesting that the underlying

magnetoreception mechanism is insensitive to the polarity of the magnetic field.

We predicted, therefore, that larval *Drosophila* would have a magnetic inclination

compass.


**Inclination Compass in Larval *Drosophila***

To determine if larval flies have an inclination compass, E and W trained

larvae were tested in one of four horizontal alignments of the magnetic field with

the ambient vertical component (Fig. 3.3a) or with an inverted vertical component

(Fig. 3.3b). Inversion of the vertical component of the field leaves the horizontal

polarity unchanged, while reversing the inclination of the magnetic field. As

shown in previous studies of amphibians and birds[19,20], inversion of the vertical

component caused larval flies to reverse their direction of orientation relative to

the magnetic field (Fig. 3.3). This confirms that the magnetic compass response

of larval flies is insensitive to the polarity of the magnetic field, as predicted by

the RPM, and provides the first demonstration of a magnetic inclination compass

in an invertebrate.

**Complex Pattern of Response Consistent with RPM**

The simple nervous system and limited behavioral repertoire of larval flies makes them well suited to characterize the mechanism of magnetoreception. If the larval behavior exhibited in Fig. 3.2 is mediated by a RPM, the magnetic compass response should show a strong dependence on the alignment of the detector (i.e. the alignment of the underlying photopigment array) relative to the magnetic field. Larvae should exhibit the strongest unimodal orientation when the detector is aligned to respond to the least ambiguous regions ("primary axis") of the pattern produced by the RPM (Fig. 3.1a-c), and/or to regions that show the strongest dependence on the inclination of the pattern. The strongest unimodal orientation was exhibited by larvae trained E and W (Fig. 3.2i), indicating that the detector alignment is perpendicular to the body axis of the larvae (Fig. 3.4a, Fig. S2). Moreover, E and W trained larvae exhibited comparable, inclination-dependent orientation (Fig. 3.2a,e; Fig. 3.3), suggesting that the vertical alignment of the detector(s) must vary symmetrically up (or down) to the larvae's right and left (Fig. S3). If this reasoning is correct, the pattern of response exhibited by a RPM suggests that N and S trained larvae may orient quadramodally because they are aligning the detector to obtain a response that contrasts maximally with the response of the detector when it is aligned along the primary axis, i.e., when larvae are aligned along the E-W axis (Fig. 3.4b, Fig. S2). Regardless of whether the quadramodal orientation of N and S trained larvae is a learned or innate response (see below), the simple nervous system of larval *Drosophila* appears to only detect the maxima and minima of the pattern

produced by the RPM and is consistent with a detector that is perpendicular to the long axis of the larvae. Larvae trained along the 45° axes (Fig. 3.2) exhibit a response that is intermediate between that of larvae trained along the N-S or E-W axes, suggesting this may be a mixture of the two responses[29].

**RPM with Two Antagonistic Spectral Inputs**

To determine whether the larval response is mediated by a light-dependent magnetoreception mechanism, we compared magnetic compass orientation under 365 nm and 490 nm light. In an earlier study of a salamander, we demonstrated a 90° shift in the direction of magnetic compass orientation under comparable intensities of short- ($\leq$ 450 nm) and long- ($\geq$ 500 nm) wavelength light, and proposed that this wavelength-dependent shift was caused by an antagonistic interaction of short- and long-wavelength inputs that produced inverse ("complementary") patterns of response to the magnetic field[36]. Similar wavelength-dependent shifts in magnetic compass orientation have been observed in adult *Drosophila*[16], and migratory European robins[18] pointing to the possibility that a common light-dependent magnetoreception mechanism underlies the magnetic compass in these taxonomically diverse organisms.

If antagonistic responses to the magnetic field under short- and long-wavelength light are fundamental properties of the light-dependent magnetic compass, we predicted that larvae tested under the same intensity of 365 nm and 490 nm light (see Methods) would exhibit complimentary patterns of response (Fig. S1). Specifically, if E and W trained larvae are aligning a simple

detector to respond to the primary axis of the pattern produced by a RPM under 365 nm light (Fig. 3.4a; Fig. 3.5a,b), an equivalent match to the complementary pattern under 490 nm light should occur when the detector is aligned along the 45° axes (open triangles; Fig. 3.5c,d), similar to the response of N and S trained larvae under 365 nm light (Fig. 3.5e,f). Correspondingly, if larvae trained to the N or S under 365 nm light are aligning the detector to contrasting regions of the pattern along the 45° axes (Fig. 3.4b, Fig. 3.5e,f), an equivalent match to the inverse pattern under 490 nm should occur when the detector is aligned along the primary axis of the pattern, resulting in E or W orientation. Because the response of N and S trained larvae under 365 nm light is independent of inclination (Fig. 3.2c,g; Fig. 3.5e,f), it is unlikely that N and S trained larvae learn to distinguish between the two ends of the magnetic axis and, therefore, were predicted to orient bimodally along the E-W axis under 490 nm light.

As predicted, E and W trained larvae tested under 490 nm light oriented quadramodally along the 45° axes (Fig. 3.5c,d), while larvae trained N and S and tested under 490 nm light oriented bimodally along the E-W axis (Fig. 3.5g,h). These findings indicate that both E-W trained and N-S trained larvae had learned to orient along a particular axis (or axes) relative to the magnetic field under 365 nm light (see below). Moreover, the responses observed under 365 and 490 nm light (Fig. 3.5a-h) directly demonstrate the complementary patterns of response proposed to explain wavelength-dependent effects of light on magnetic compass orientation in other, more complex, organisms[16-18]. At a more fundamental level, the response of larval flies appears to involve a simple detector with little, if any,

spatial or temporal integration of magnetic input. As a consequence, fundamental properties of the underlying magnetoreception mechanism can be visualized using the behavioral response of the entire organism.

**Spontaneous Orientation of Untrained Larvae**

To determine that N and S trained larvae were exhibiting a learned response (Fig. 3.4; Fig. 3.5a,b; and see earlier discussion), we tested the magnetic compass orientation of untrained larvae under both 365 nm and 490 nm light (Fig. 3.6). Under 365 nm light, the response of untrained larvae was indistinguishable from that of N or S trained larvae, i.e. in both cases, larvae oriented quadramodally along the 45° axes (Fig. 3.5 e,f; Fig. 3.6a). Therefore, the response of N and S trained larvae tested under 365 nm light is compatible with either a learned (Fig. 3.4; Fig. 3.5a,b) or a spontaneous (i.e. default) response. Under 490 nm light, however, untrained larvae oriented quadramodally along the cardinal compass directions (i.e. N, S, E, W; Fig. 3.6b), while larvae trained to N and S under 365 nm light were bimodally distributed along the predicted E-W axis (Fig. 3.5g,h). The difference in response of trained and untrained flies under 490nm light indicates that the N and S trained larvae had learned to orient in a specific direction relative to the magnetic field axis in training (Fig. S4).

Like the responses of trained larvae, the quadramodal orientation of untrained larvae exhibited complementary patterns of response under 365 and 490 nm light (Fig. 3.6). Spontaneous quadramodal magnetic orientation has been reported in many other insect groups[35], raising the possibility that a light-

dependent magnetoreception mechanism similar to that found in *Drosophila* is widespread in insects.

**Discussion:**

**Using a Simple Organism to Visualize a Complex Pattern**

Findings indicate that the magnetic compass of larval *Drosophila* is light-dependent and exhibits the axially symmetrical pattern of response predicted by the RPM (Fig. 3.2-3.6). In the preceding discussion, the simple RPM modeled by Ritz et.al.[9] (Fig. 3.1a-c) was used to illustrate properties expected from the RPM. The larval behavior is also consistent with the properties of more complex radical pair systems, e.g., in cryptochromes, which have been proposed to play a role in the light-dependent magnetic compass[2, 11]. Interestingly, although not an *a priori* prediction of the RPM, similar wavelength-dependent effects of light on magnetic compass orientation in larval and adult flies, amphibians, and birds[16,12,17,18] suggest that magnetic compasses in these diverse taxa have a common functional architecture and, more specifically, that spectral antagonism may be an intrinsic property of the RPM.

The findings reported here indicate that the behavior of larval *Drosophila* can provide a detailed picture of the effects of experimental treatments on the underlying magnetoreception mechanism (Fig. 3.5, Fig. 3.6), which is rarely possible in more complex organisms[9,26]. Thus, the larval response provides a critical link between the properties derived from theoretical models[9,11] and artificial radical pair systems[39,] and those observed in the behavioral responses of more complex organisms. Moreover, this provides a unique "signature" of the

underlying biophysical process that can be used to identify input from a similar light-dependent magnetic compass in other behavioral and neural responses where the involvement of magnetic cues has not been previously suspected.

**Methods:**

**Training and Testing:** A detailed description of training and testing protocols is given in Dommer et al.[29]. Pyrex vials with 10 ml of larval media containing 4-5 day old adults, plugged with cotton stoppers, were placed in training at 21°C (see below). Adults were allowed to oviposit, and larvae were tested after ~144 hours, insuring that the oldest larvae were in the late second / early third instar, which are photonegative. The training and testing apparati were located in the same RF shielded room (Lindgren enclosures #12W-2/2-I), and all observations were made with a headlamp equipped with a 665 nm long-pass filter ("red light") at an intensity below the detection threshold of the larvae[37].

   In the training apparatus, vials were inserted horizontally into a cavity in the side of a flat black wooden block, so that the ends of the vials containing the larval media faced inward. The cotton plugs in the mouth of the vials ("open ends") were illuminated by overhead diffuse 365 nm light (Nichia model LED's NSHU #550A) as well as from the sides and open ends by light reflected from aluminum diffusers. Separate groups of vials were aligned with the open ends of the vials facing each of the cardinal compass directions, with light entering the vials from the N, S, E and W with an intensity of $10.1 \pm 0.1$ log quanta/cm$^2$/s at the surface of the larval media with the cotton plug in place (i.e. a vial placed in

the W side of the block had light entering from the W, and the "dark" end of the vial was directed to the E). After removal of a vial for testing, larvae were sorted in a 45% sucrose solution, individually washed, and placed onto a holding plate containing a grape juice agarose media (identical to plates used in testing).

Larvae were tested on a new agar plate positioned in the center of a non-directional light field of equal intensity at the surface of the agar (10.1 $\pm$ 0.1 log quanta/cm$^2$/s) as larvae were exposed to in training. Larvae were placed individually in the center of a test plate, immediately covered with an opaque cap (1.5 cm diameter x 2 cm high), and the plate was placed in the center of the testing apparatus. The cap was removed after 1 minute. Larvae were observed every 30 seconds under red light and were scored once they had crossed a 6 cm radius circle from the center of the testing plate. The plate was immediately removed from the testing arena, and the bearing of the larvae was recorded on a template. Trials were discontinued if the larva did not score in 10 min. A new plate was used for each larva to eliminate chemical cues as a possible source of bias. For each of the eight trained directions, an equal number of larvae were tested in each of the four magnetic field alignments (each larvae tested once in one of the four fields)[15,29]. Pooling equal numbers of magnetic bearings from the four magnetic field alignments factors out any non-magnetic bias so that the resulting distribution retains only the component of orientation that is a consistent response relative to the magnetic field[15,29]. Larvae were Oregon-R x Canton-S wildtype stocks.

**Test Fields:** Training and testing apparati were both enclosed in systems of three horizontally aligned single wrapped Merritt cube-surface coils[30] powered by DC power supplies (RSR Hy3005). One N/S coil was used to cancel the horizontal component of the geomagnetic field (< 2% ambient intensity), leaving the vertical component unchanged. The remaining two coils, which were aligned along the N/S and E/W axes, were used to produce a new horizontal component of the same intensity, but aligned in one of four directions relative to the testing enclosure (i.e. mN positioned at topographic N, S, E or W). The horizontal components added to the ambient vertical field to produce artificial fields of the same total intensity and inclination ($\pm$ 2%) and direction ($\pm$ 2°) as the geomagnetic field. An identical coil system surrounding the training apparatus was used to produce an artificial N field. In testing, magnetic field alignment was changed between each trial to control for any topographic biases within the testing arena.

**Axial Compass Experiments:** The vertical component of the magnetic field was altered using a pair of double wrapped Helmholtz coils, producing a magnetic field equal in intensity ($\pm$2%) to that of the geomagnetic field, with mN pointed down (inclination +64° $\pm$1°; control condition) or up (inclination -64° $\pm$1°; experimental condition). Conditions were changed after each larvae, interspersing control and experimental animals. Both training and testing were carried out under ultraviolet (365 nm, 20 nm bandwidth, 10.1 $\pm$ 0.1 log quanta/$cm^2$/sec) light).

**Long Wavelength Experiments:** Larvae were illuminated with 490 nm light (10 nm bandwidth, 10.1 $\pm$ 0.1 log quanta/cm$^2$/s) during testing. Light was produced by 4 white LED's (Radio Shack #276-320) powered by a DC power supply (RSR HY3005) and led through an interference filter (Oriel). Light properties were measured using a calibrated photodiode (United Detector Technologies 10DP/SB) and a picoameter (Keithley #486). As in the axial compass experiments, control and experimental larvae were tested alternately within the same test. Half of the sample (32 larvae, 8 from each trained direction) were carried out double blind. There was no statistical difference on the quadruple angles from either sample (p > 0.05, Watson U$^2$ test) so the data are combined (Fig. 3.5 c,d,f,g).

**Untrained Larvae:** Larvae were allowed to develop in a vertically aligned vial centered in an isotropic 365 nm (20 nm bandwidth, 10.1 $\pm$ 0.1 log quanta/cm$^2$/s) light field. Larvae were tested under 365 or 490 nm light identical to that used in previous experiments.

**Data Analysis:** For each group of larvae, a mean bearing was calculated by vector addition, which was analyzed and tested for significance using the Rayleigh test for circular statistics. Bimodal and quadramodal data were analyzed using a Rayleigh test on doubled or quadrupled angles respectively[31]. The Watson U$^2$ test was used to compare two distributions.
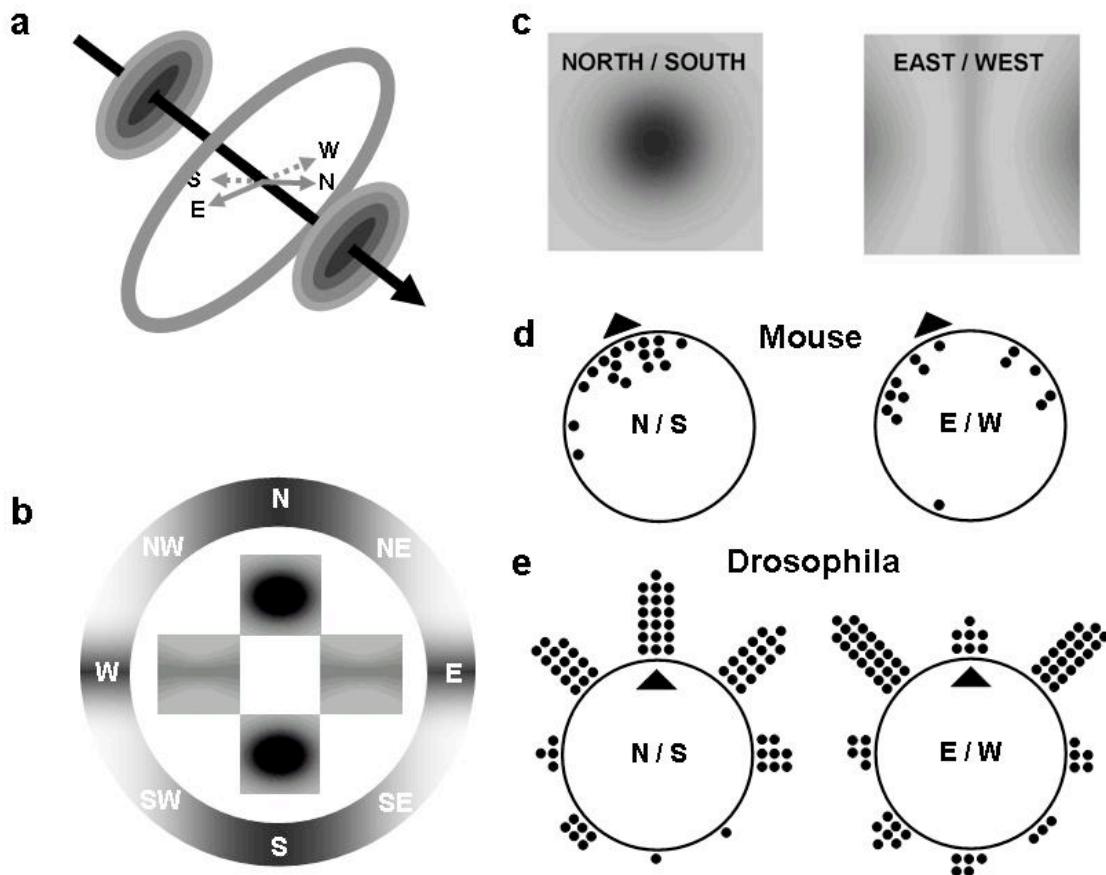
Figure 3.1. RPM and magnetic orientation of mice[15] and adult *Drosophila*[16]. (a) 3-D pattern of response in a simple RPM[9]; black arrow shows positive magnetic inclination in northern hemisphere. (b & c) "Snap shots" and circular panorama of pattern produced by simple RPM[9] in plane of magnetic field axis. Mice[15] (d) and adult male *Drosophila*[16] (e) trained N or S show a single cluster of bearings in the trained direction (left diagrams), while those trained E or W split on either side of E-W axis (right diagrams); bearings pooled from four symmetrical magnetic field alignments (mN = N, E, S or W)[15].
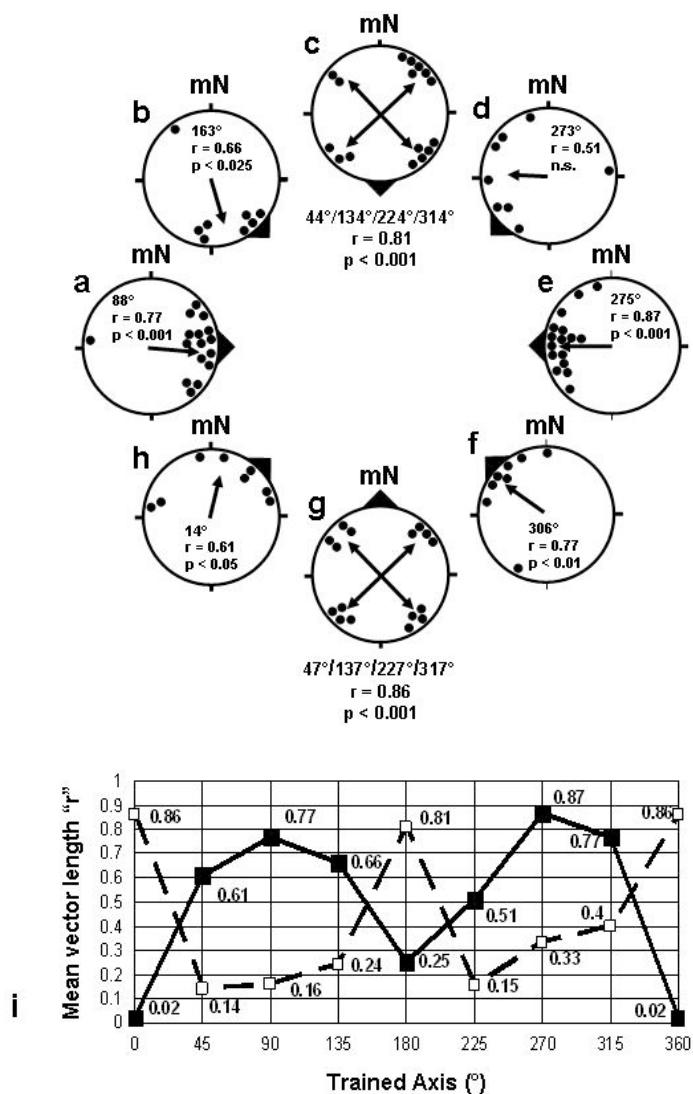
Figure 3.2. Training direction effects magnetic compass orientation of larval _Drosophila_. (a-h) Magnetic bearings of 2nd instar fly larvae trained and tested under 365 nm light; data pooled from four magnetic field alignments (see Methods). Expected direction (black triangles) is opposite direction of light in training. Arrows show mean vectors; mean vector length (r) scaled to radius of circle (r = 1). (i) Unimodal (solid symbols) and quadramodal (open symbols) mean vector lengths "r" plotted relative to the direction of training (a-h).
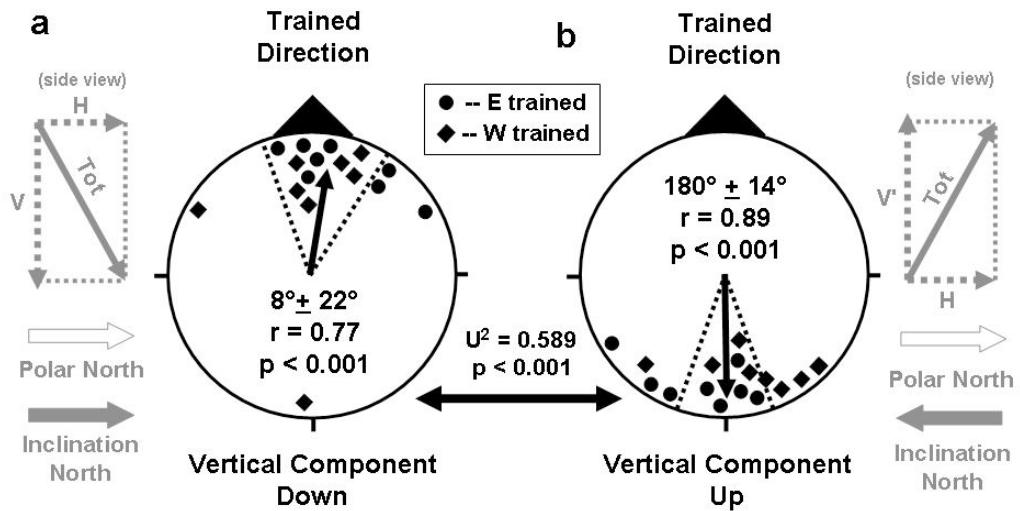
Figure 3.3. Inclination magnetic compass. a) Larvae tested in ambient magnetic inclination (+64°; grey inset) oriented in the trained magnetic direction, while b) larvae tested with the vertical component inverted (-64°; grey inset) reversed their direction of orientation. Bearings pooled from larvae trained to E (round symbols) and W (diamond-shaped symbols), and from four different magnetic field alignments in testing. Insets (side views): "**V**" or "**V'**", "**H**", and "**T**" are the vertical, horizontal and total field components, respectively. 'Polar north' is the direction indicated by a dipole compass. "Inclination north" (in the northern hemisphere) by the downward slope of the magnetic field[17].
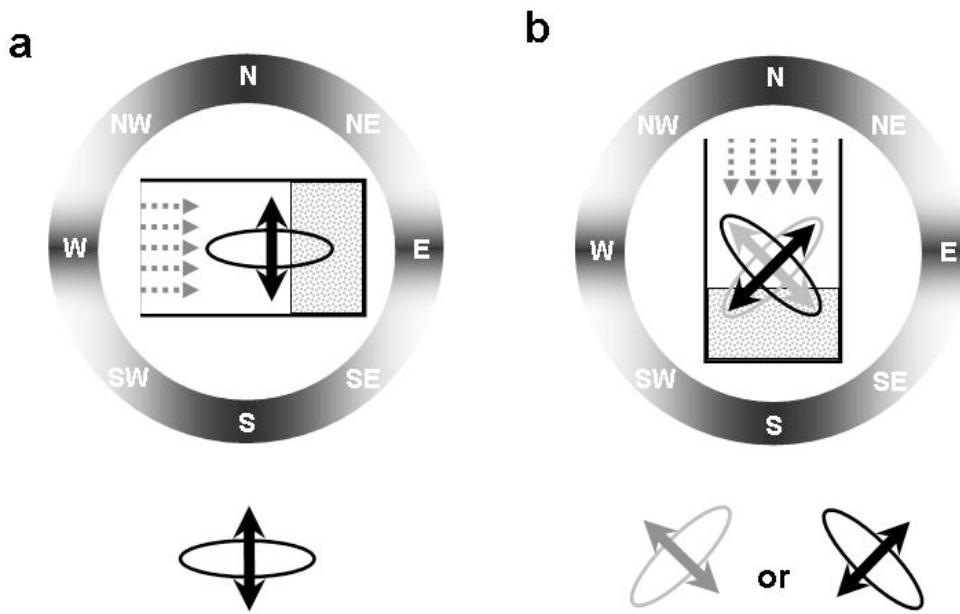
Figure 3.4. Alignment of hypothetical magnetic detector during training. Schematic of training vials shows larva (oval) entering substrate. Grey dashed arrows are incoming light; black double-headed arrows indicate alignment of hypothetical detector (Fig. 3.1). (a) E or W trained larvae. (b) N or S trained larvae.
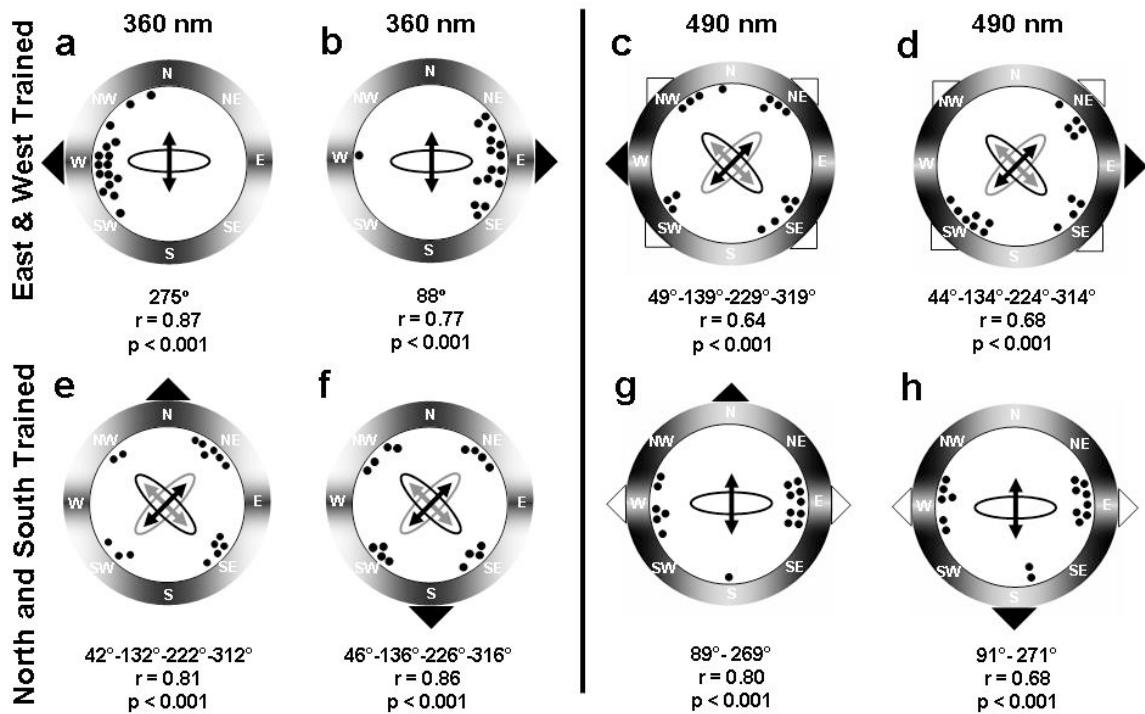
Figure 3.5. Magnetic orientation of 365 nm trained larvae tested under 365 nm (a,b,e,f; data from Fig. 3.2) or 490 nm (c,d,g,h) light. Under 365 nm light, larvae trained W (a) and E (b) oriented in the trained direction, while larvae trained N (e) and S (f) oriented quadramodally (Fig. 3.2). Consistent with predictions, under 490 nm light, larvae trained W (c) and E (d) oriented quadramodally, while larvae trained N (g) and S (h) oriented bimodally perpendicular to the trained direction (see text). Under 490 nm light, half of the larvae (n = 8) from each of the trained directions (c,d,g,h) were tested double blind. There was no significant difference between the two samples in any of the trained directions (Watson $U^2$ > 0.05), so the data were combined. Panoramas in a,b,e,f show the pattern of response expected in a simple RPM[9] (Fig. 3.4). Panoramas in c,d,g,h are the inverse or compliment of the patterns in a,b,e,f. Black triangles indicate the trained magnetic directions, open triangles show the predicted orientation (see text).

Ellipses and double-headed arrows at the center of each diagram show larval

and detector alignments proposed to explain the observed orientation (see Fig.

3.4).
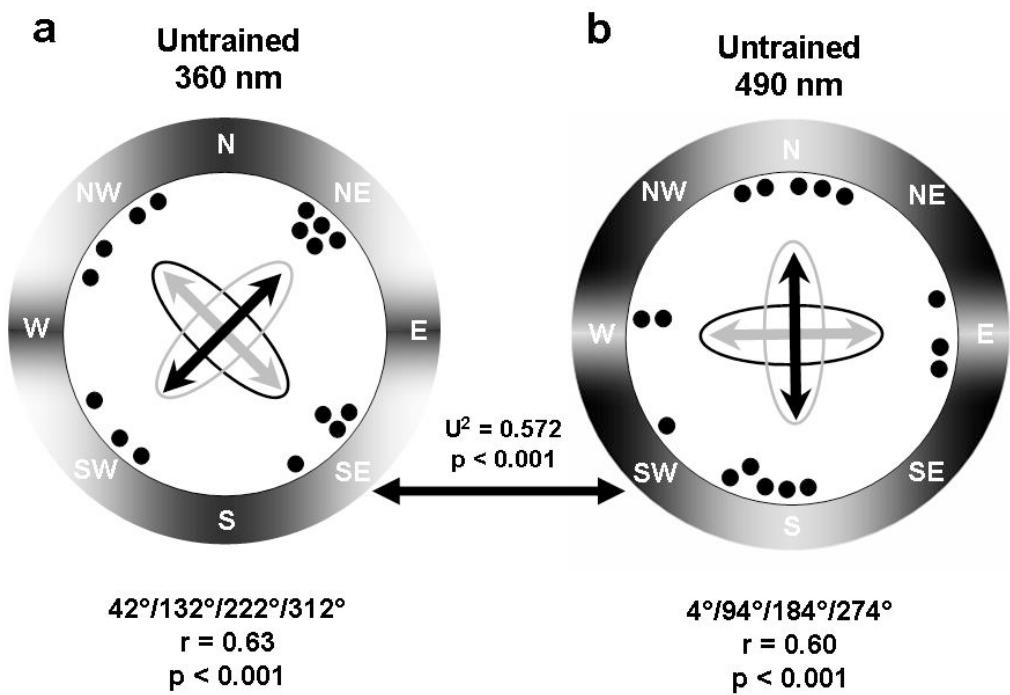
Figure 3.6. Spontaneous (untrained) magnetic compass orientation of larval

*Drosophila*. Larvae exhibited quadramodal orientation (a) along the 45° axes

under 365 nm light, and (b) along the cardinal compass axes under 490 nm light.

**Figure S1.) Yellow and Blue Color Inversion.** A current link to figure S1 can be found at http://genbio.biol.vt.edu/tapages/dommer/nature.html. Circular panorama of a simple RPM modeled by Ritz et al[9]. Previous research on the light-dependent magnetic compasses of amphibians, birds and adult Drosophila has provided evidence for an antagonistic interaction of short- and long-wavelength inputs that produce complimentary patterns of response[12,16,17,18]. To illustrate the predicted effects of short-and long-wavelength light on the pattern of response in such a system, we exploited the color opponent system in the human eye; yellow and blue were chosen because they are complementary colors, not because there is any reason to believe that perception of the magnetic field by animals is color dependent. **Instructions:** Cover one eye and stare at the fixation point (black dot) in the center of the first diagram. Open double-headed arrows show the alignment of the hypothetical receptor that would mediate the orientation of larvae trained to the E or W and tested under short-wavelength (365 nm) light (Fig. 3.4a). After 10 seconds, the pattern will switch to the second slide. Continue to fixate with one eye on the black dot at the center of the page. The after image should be the complement of the first diagram (each area of the first pattern should be seen in its complementary color). Four-headed arrow shows the corresponding detector alignments to the complementary pattern. The third slide shows the alignment of the receptors for larvae trained N or S with respect to the original pattern (See Fig. 3.4b). The after images seen by the observer show how a pattern produced by changes in the relative sensitivity of retinal photoreceptors can be superimposed on the observers visual surroundings.
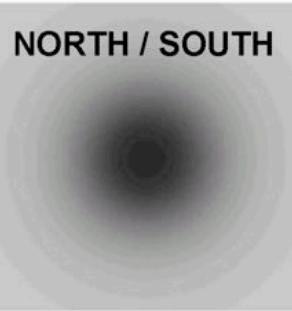
NORTH / SOUTH
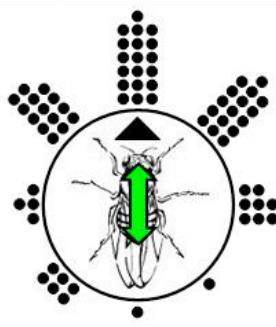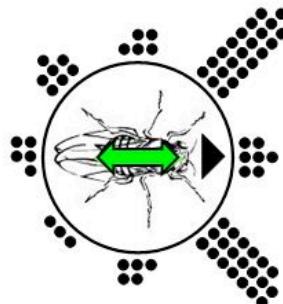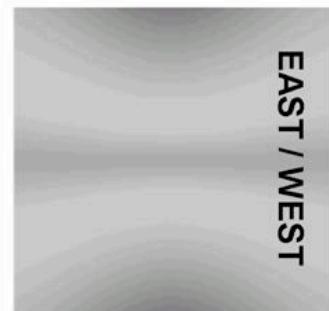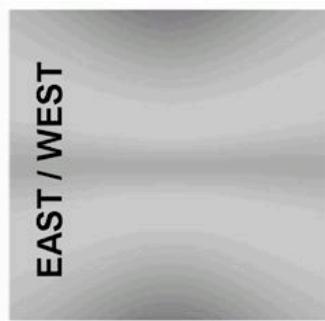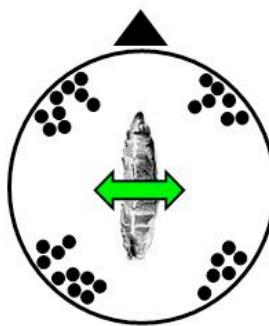
EAST / WEST

(north & south trained combined)
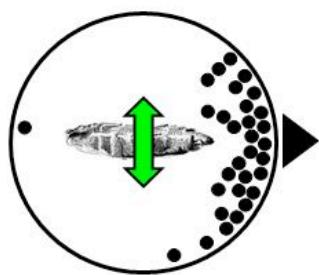
(east & west trained combined)

NORTH / SOUTH

EAST / WEST

(north & south trained combined)

(east & west trained combined)
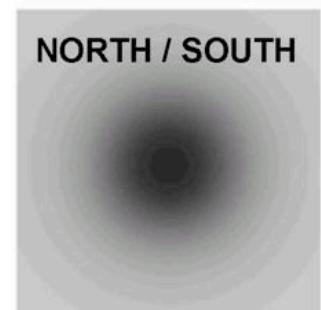
**Figure S2.) Hypothetical alignment of magnetoreceptors in *D. melanogaster* (a) adults and (b) larvae.** According to theoretical models of an RPM based magnetic compass, animals should exhibit the strongest magnetic orientation when the detector is aligned to respond to the least ambiguous axis of the pattern ("primary axis"), and/or the detector can be used to distinguish between the two ends of the axis that provide information about magnetic field inclination. (a) Left circle diagram shows bearings of adult *D. melanogaster* trained N or S oriented unimodally in the trained direction (N- and S-trained larvae combined with respect to the trained direction; black triangle). Right circle diagram indicates adults trained E or W show two clusters of bearings split on either side of the E-W axis (bearings pooled from E- and W-trained larvae, with respect to trained direction; black triangle). Drawings of an adult *D. melanogaster* (in the circular diagrams) show the predicted alignment of the magnetic detector (black double-headed arrow) that would mediate the observed responses of N-S and E-W trained flies[16]. (b) Left circle diagram shows bearings of *D. melanogaster* larvae trained N or S (two groups combined; Fig. 3.2c,g;Fig. 3.5e,f) demonstrated strong unimodal orientation and used inclination to distinguish between the two ends of the magnetic axis (Fig. 3.3a,b). Right circle diagram shows that larvae trained E or W oriented quadramodally (two groups combined; Fig. 3.2a,e). Differences in the responses of larvae trained along the N-S and E-W axes are consistent with larvae aligning the receptor to contrasting regions of the pattern (see text). Square panels in a and b show response of a simple RPM modeled by Ritz et al.[9] when the primary axis of the detector is aligned parallel (top panel) or

perpendicular (right panel) to the N-S axis of the magnetic field.
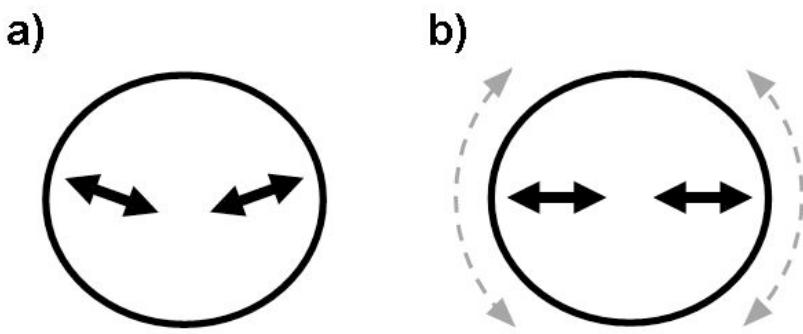
**Figure S3.) Orientation of detector alignment in *Drosophila* larvae.** The comparable orientation of E and W trained larvae (Fig. 3.2a,e) indicates that the magnetic detector is equally sensitive to inclination of the magnetic field to the right or left of the trained direction. Consequently, the larvae's response is mediated by paired detectors (e.g., in the Bolwig's organ or associated small lateral neurons ventral (s-LNvs)[32]), that are (a) inclined symmetrically up (or down) to the animal's right and left, or (b) aligned horizontally and alternately tilted back and forth to the right and left (e.g., during "head-swinging" exhibited by larvae between bouts of locomotion[33]).
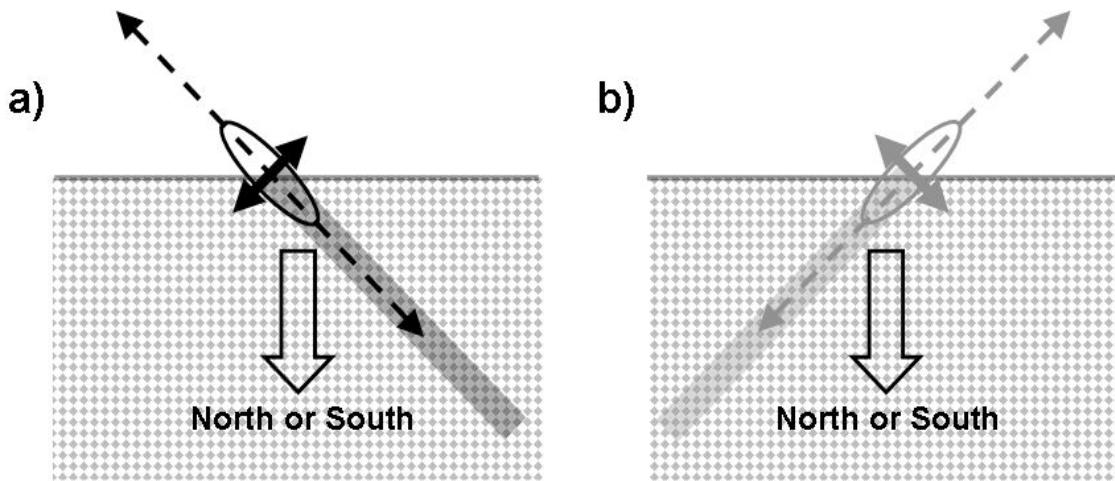
**Figure S4.) Angular movement by larval *Drosophila*.** Learned magnetic compass orientation at an angle of $\pm$ 45$^o$ relative to the surface of the substrate may be adaptive if a) straight line movement is move efficient than random movement, even if path alignment is not orthogonal to the surface of the substrate (shown previously in magnetotactic bacteria[34]), and/or b) if the substrate occupied by the larvae (gray shaded rectangles) is comparable in quality to the substrate the larva would occupy if it oriented orthogonal to the surface.

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## Chapter 4
## Conclusions

Findings outlined in the previous chapters show that *Drosophila* larvae have a light-dependent magnetic compass that demonstrates attributes (i.e. sensitivity to light, axial symmetry) consistent with models of a radical pair mechanism. The larval *Drosophila* assay developed for this work has numerous advantages over current research models used to study light-dependent magnetic compass mechanisms. My research demonstrated that *Drosophila* larvae are capable of learning an arbitrary magnetic direction relative to their media, orient with a high degree of precision, and utilize magnetic cues as a primary mechanism for short distance movement. While previous behavioral assays have been able to demonstrate magnetic compass orientation in a variety of organisms, the experimental power of the *Drosophila* system, and specifically the opportunity to use molecular and genetic techniques makes this model system especially well suited to characterize the biophysical, molecular, and neurophysiological bases of the light-dependent magnetic compass.

Although larval *Drosophila* ultimately proved to produce the strongest magnetic orientation, initial experiments with adult flies provided evidence for a behavioral response similar to that of migratory birds (European Robins and Australian Silvereyes) when exposed to a high intensity of long-wavelength monochromatic light during orientation testing. These findings suggest that the underlying magnetic compass mechanisms mediating these behavioral responses in flies and birds may be more similar than previously suggested.

Evidence for the use of a light dependent magnetic compass by both larval and adult *D. melanogaster* suggest that these two developmental stages use the same type of magnetic compass mechanism. The larval system has a number of advantages, however, including more rapid data acquisition, and stronger orientation than adult *Drosophila*.  Additionally, goal oriented magnetic compass orientation by both developmental forms of *Drosophila* suggests that use of magnetic cues is adaptive for extremely short distance movements, in contrast to the classical view that magnetic cues may only be of use during long distance migration.   Furthermore, larval *Drosophila* possess a less complex nervous system, and a more limited range of goal oriented movement behavioral responses, making them a more suitable system in which to characterize the intrinsic properties of the light-dependent magnetic compass, and the radical pair mechanism. The findings in chapter 3 also indicate the larval response is mediated by a simple detector with little secondary processing, so the behavior of the whole organism can be used to 'visualize' fundamental properties of the biophysical process underlying the light-dependent magnetic compass. Characterization of the larval magnetoreception mechanism is likely to give insight to the design of the system in adult *Drosophila*, other insects and, potentially, in more complex organisms.

Earlier work has pointed to similarities between the behavioral responses of several species of migratory birds and the newt (*Notopthalmus viridescens*). Interestingly, the findings from larval flies indicate that the functional properties of the light-dependent magnetic compass in these insects are remarkably similar to

those in vertebrates, suggesting that this mechanism may have evolved in an invertebrate ancestor predating the divergence of modern vertebrate lineages that occurred over 450 million years ago.  Like amphibians and birds, larval *Drosophila* utilize an inclination magnetic compass that is light-dependent and exhibits an axially symmetrical response, consistent with models of a radical pair mechanism.  Furthermore, in all three groups spectral antagonism appears to be an intrinsic property of the light dependent magnetic compass.  The responses of larvae indicate that antagonistic short- and long-wavelength inputs to the magnetic compass produce inverse or complementary patterns of response, as shown previously in both amphibian and avian species.

Future work using specific manipulations of wavelength, light intensity, and radio frequency fields are likely to yield more information about the biophysical process underlying the light-dependent magnetic compass.  Most notably, migratory birds (*E. rubecula*) exhibit random orientation when exposed to specific alignments of a low-level radio frequency field in the Mhz range (~ 0.1% of the static field intensity), as well as to a magnetic field that is at least 20% stronger or weaker than the standard geomagnetic field.  The ability of migratory birds to eventually orient to their seasonally appropriate migratory direction after exposure to an altered static field intensity for several days indicates that after the initial exposure, they are able to relearn to use magnetic cues.  Tests of migratory birds that have been exposed to a low-level RF field for several days prior to testing are needed to determine whether birds respond to changes in RF.  The ability to orient after prior exposure to conditions that initially

produced disorientation (e.g. migratory birds exposed to changes in magnetic field strength) suggests that the response of the magnetic compass is altered, but not eliminated under these conditions.  The high degree of similarity in the light-dependent magnetic compass responses of adult and larval *Drosophila*, *N. viridescens*, and *E. Rubecula*, points to a common underlying mechanism, however, the complexity of vertebrates and adult insects (i.e., both their behavior, and underlying nervous system) limits the ability to test for fundamental effects of experimental treatments on the pattern produced by a radical pair mechanism.

Tests of larvae exposed to similar radio frequency fields and changes in magnetic intensity may reveal that magnetic information is present in both of these experimental treatments (e.g. RF exposure and modified field intensity). The larval system could be used to determine which portions of the pattern produced by a RPM, if any, are altered under these experimental conditions.

The larval behavioral assay represents the first experimental system in which a behavioral response can be used to reveal fundamental properties of the biophysical mechanism underlying the light-dependent magnetic compass.  The reduced nervous system, limited visual receptors, and behavioral simplicity of *Drosophila* larvae makes them well suited to study aspects of the light dependent magnetic compass that is not possible in more complex organisms. Indeed the behavior of larval *Drosophila* acts as a link between theoretical, artificial, and experimental models of radical pair systems. Furthermore, the use of molecular tools will allow further research with *Drosophila* larvae to systematically "dissect"

their photoreception system to determine which specific receptors are involved in

light dependent magnetoreception.