PHOTOSENSITIVITY AND MAGNETOSENSITIVITY OF THE DROSOPHILA CIRCADIAN CLOCK

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University of Missouri-Kansas City, 2014

ABSTRACT

The experiments in this thesis addressed whether magnetic fields can enhance entrainment (phase determination) of the circadian clock by light. The experiments were conducted using a species of small fruit flies, *Drosophila melanogaster*. A solenoid was built in order to create the magnetic field.A magnetic field was produced inside a wire coil by passing an electric current through the coil, with the strength of the field proportional to the current. Light pulses were produced by a monochromator in the incubator, while the programmable timer within the constant temperature incubator was used to produce12 hr.: 12 hr. light:dark (LD) cycles or constant illumination with blue light (LL).The effects of these treatments on Drosophila locomotor activity rhythms were measured in activity monitors. For the first part of the study, magnetic field pulses of 4.5mT (160-fold increase of the earth's magnetic field) delivered together with blue light pulses at ZT15 produced an enhanced phase delay in subsequent circadian locomotor behavior in constant darkness, compared with the effect of light alone or magnetic fields alone. The effect was saturable and was observed within blue wavelength range (450nm±0.003nm) but not at red wavelengths (700nm±0.013nm). The second part of the

study showed that 12hr:12hr cycles of magnetic field strength (oscillating between earth's magnetic field and 160 times earth's magnetic field) could drive 24hr cycles of locomotor activity in LL but had no effect on circadian phase of locomotor activity in constant darkness. The effect in constant light did not require the normal CRY photoreceptor protein because it was still observed in cry^b mutant flies, which remained rhythmic in LL and magnetism with the phase of the magnetic field rather than that of the previous LD cycle. The results demonstrate that a strong magnetic field can entrain the circadian clock in a light-dependent but CRY-independent manner.

The undersigned, appointed by the Dean of the College of Arts and Science, have examined a thesis dissertation titled "Photosensitivity and Magnetosensitivity of The Drosophila Circadian Clock", presented by HassanaSamassekou, candidate for the Master of Science degree, and certify that in their opinion it is worthy of acceptance.

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GLOSSARY

Cryptochrome- a class of blue light-sensitive flavoproteins found in plants and animals.

Flavoprotein- a large biological and photoreducible protein containing a nucleic acid derivative of riboflavin.

Photosensitivity- the extent to which a body reacts upon receiving some light particles known as photons.

Photon- a particle that is the quantum of electromagnetic energy and all forms of radiation.

Magnetosensitivity- the aptitude to detect magnetic fields.

Drosophila- the genus of any various small fruit flies.

Circadian rhythm- any biological process that exhibits an endogenously generated oscillation of approximately 24 hours.

Wavelength- the distance between one peak or crest of a wave of light and the next equivalent peak or crest.

Zeitgeber- any exogenous cue that synchronizes an organism's endogenous time-keeping system, known as internal clock, to the earth's 24-hour light/dark cycle or 12 month cycle.

Radical pair- a radical is a molecule with an unpaired electron spin and thus has spin s=1/2.

Spin of a particle- an intrinsic form of angular momentum carried by an elementary particle.

Singlet state- this occurs when the spins of the two electrons are aligned oppositely.

Triplet state- this occurs when the net spin is 1.

Geomagnetic field- Earth's magnetic field which ranges from 25 μT to 65 μT (0.25 G-0.65 G).

Ampere's-Law- the relation between the total amount of magnetic field around some path due to the current that passes through that enclosed path: $\oint \boldsymbol{B}.\, d\boldsymbol{l} = \mu_0 I_{enc}$.

Solenoid- a long thin loop of wire (usually enfolded around a plastic core) that creates a magnetic field when an electric current is passed through it.

Monochromator- an optical instrument that transmits a mechanically selectable narrow band of wavelengths of light or radiation selected from wavelengths of ultraviolet rays to infrared rays which are available at the input.

Femtosecond Pump probe spectroscopy- an experimental technique that is used to measure absorption of a sample in the excited state.

Absorption- the technique by which the energy of a photon is taken up by matter (usually the electrons of an atom), and transformed to another form of energy.

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DEDICATION

I dedicate this thesis to my father Sory Samassekou, and my uncle Oumar Sy for their love, affection, encouragements, supports and valuable contribution for the success in my life.

CHAPTER 1

INTRODUCTION

An overview of the research project is presented in this chapter. It includes a brief description of the background, the problem statement, the research objectives, the approach, and some long range consequences of the study.

1.1 Background

Circadian rhythms are biological cycles that demonstrate a period of about 24 hours. Plants, animals, fungi, and some bacteria displayed rhythms driven by circadian clocks. In 1729, the French astronomer Demarian became the first one to point out that rhythms might be regulated inside an organism rather than controlled by a periodic milieu.[1]According to Sehgal et al., Demarian monitored the movements of a Mimosa plant's leaf in constant darkness; he concluded that the the 24-hour periodicity of the movement was maintained in constant darkness. Two centuries later, Kalmus and Bunning proposed an endogenous means of relating the periodicity of the rhythm to the variation of the temperature fluctuations; this revealed an effect independent of Earth's rotation.In the1960s, Hamneret.al. conducted an experiment at the South Pole with several organisms that were placed on a rotating turntable. The rotation mimicked that of Earth in terms of periodicity but in the opposite direction. They noticed that the circadian rhythm of eclosion in Drosophila was recurrent, demonstrating that it was not dependent on the earth's rotation.[1] However, not all rhythmic functions are maintained by an

endogenous system. There are also diurnal rhythms- those driven directly by the day-night cycle.

Circadian rhythms distinguish themselves by some particular characteristics. One of the features is a periodicity of approximately 24 hours. Another quality is environmental/ecological cues' abilities to synchronize or reset the cycle. Light is the most important factor in synchronizing/resetting periods. The term zeitgeber was first coined by Jurgen Aschoff. Zeitgeber("timegiver" in German) is any exogenous cue that synchronizes an organism's endogenous time-keeping system. The course of that synchronization is referred to as entrainment. Zeitgeber time, most often denoted ZT, corresponds to a particular time in the 24-hour cycle consisting of 12hr: 12hr of light and darkness (LD). ZT0 refers to the time at which lights come on while ZT12 refers to the time at which lights are turned off. Intuitively, one can notice that ZT24 and ZT0 correspond to the same point because the daytime hours are between 0 and 12 while the night time ones are between 12 and 24. A phase is defined as the time at which a specific activity or a rest takes place. A phase shift in the circadian rhythm is caused by a change in the phase of the rhythm (typically the result of a change in the light cycle timing). This process is similar to the most general concept of jet lag. Finally, oscillations of circadian rhythms are temperature compensated.[1] Studies done by Colin Pittendrigh in the 1950s confirmed that the periodicity is relatively constant over a broad range of temperature.[1]

The molecular mechanism of circadian rhythms has been investigated with genetic analyses in model organisms-the most prominent of which is Drosophila. Kalmus and Bunning introduced Drosophila as a test subject in their work on circadian rhythm in

Drosophila eclosion. Biologists interested in the examination of circadian rhythms started using Drosophila for experimental means. That was the case for Colin Pittendrigh in this investigation of the properties of the phase-resetting and in his implementation of the notion of temperature compensation in the middle of the twentieth century. In addition, in the early 1970s, Ronald Konopka and Seymour Benzer introduced studies of the clock mechanism after exploring the single gene mutants in Drosophila.[2]Due to similarities between mammalian clock and Drosophila clock, the mechanism introduced for Drosophila has helped the studies of the mammalian clock.[1]

The Drosophila mechanism is an interesting biological concept. The process starts at the stage of gene transcription. The *period* gene (*per)* and the *timeless* gene (*tim)* are activated by transcription of the two proteins, CLOCK (CLK) and CYCLE (CYC), which bind as CYC/CLK heterodimer to their promoters. At night, PER and TIM proteins build up to from a heterodimer; this makes PER more stable and not subject to degradation by DOUBLETIME protein (DBT) phosphorylation. PER/TIM interacts with CYC/CLK inside the nucleus to remove it from the promoters or *per* and *tim*; this causes transcription of the *per* and *tim* genes to be repressed. As the sun rises, TIM is degraded by a light-dependent interaction with CRY, causing PER to degrade as well. Consequently, PER/TIM no longer represses, resulting in the beginning of the transcription of *per* and *tim*. The cycle continues.[1]

The entrainment of the Drosophila circadian clock to light involves both the intracellular photoreceptor CRY and the visual photoreceptors, which provide neural input to the circadian cells of the brain and are themselves a circadian cell type.

Collaboration between a group of biologists *from The Scripps Research Institute and NSF Center for Biological Timing* and *Brandeis University*, explored the importance of CRY in Drosophila's circadian rhythm by analyzing the cry^b mutant, which does not make functional CRY protein. Activities in LD cycles were monitored for cry^b flies, which are deficient for CRY. Adults were entrained for 5 days (12 hr: 12 hr LD) by means of a 640-lux white light; then they were subject to a phase shift. On the sixth day, the lights were put on 4 hr later, and the light in the new condition was switched to blue light at 0.16-lux (cry^b). Flies remained in the second condition for 5 days, and then all the activity data for each individual were monitored by means of actograms. They confirmed that light destroys TIM and, cry^b is a nonfunctional protein. Stanewsky et al. concluded that cry^b is an apparent null mutation in a gene encoding *Drosophila*'s cryptochrome. Cry^b exhibits poor synchronization to light–dark cycles in genetic conditions that are responsible for external blindness or the need of several hours of daily rhythm resets; no response is demonstrated to brief light pulses. Cry^b flies are rhythmic in DD, associated with strong PER and TIM cycling in certain pacemaker neurons.[3] However, in most circadian cells of the cry^b mutant, TIM protein does not oscillate, presumably because it is not degraded in response to light. Prior work had shown the light-dependent degradation of TIM is part of the entrainment pathway of Drosophila.[4-7]

A biology research group from Brandeis University investigated the photosensitivity of CRY. Emery et al., in their paper entitled *CRY, a Drosophila Clock and Light-Regulated Cryptochrome, Is a Major Contributor to Circadian Rhythm Resetting and Photosensitivity,* revealed some further understanding of the gene responsible for the

regulation by light of Drosophila's inner clock. Wild-type Canton-S and *y w* flies were exploited for molecular and behavioral studies. They also recognized and analyzed *cry*, the novel *Drosophila* cryptochrome gene. The transcription of *cry* was revealed to be under circadian control and influenced by the *Drosophila* clock genes *period*, *timeless*, *clock*, and cycle. In addition, they revealed a noticeable effect of light exposure to CRY protein levels. Significantly, circadian photosensitivity is increased in a *cry*-overexpressing strain.[8]Subsequent work from the Kay lab identified the molecular basis of CRY's photoreceptive properties. It forms a light dependent interaction with TIM, thereby triggering degradation of TIM.[9] This notion is described for the mechanism of Drosophila's clock which was discussed earlier.

Emery et al. subsequently showed that CRY mediates the effects of constant light on the clock. Their work revealed that the cry^b mutation in Drosophila cryptochrome (dCRY) stops an important photoresponse of circadian rhythms; this causes arrhythmicity under constant light (LL) conditions in wild type flies.[10] According to their studies, cry^b flies were rhythmic in intense LL conditions, as opposed to wild-type flies. Thus, the cry^b mutation impaired the circadian photoreception pathway so deeply that the fly became insensitive of LL conditions. Since this mutant also had a poor response to short light pulses in the previous study[8], they concluded that circadian photoreceptor must be exclusive to these responses in *Drosophila*.

How then can cry^b flies entrain to different 24-h light–dark cycles?[10] Entrainment of activity rhythms to light persists in cry^b mutants because the visual photoreceptors provide redundant entrainment of the central brain neurons controlling the activity

rhythms. In the absence of both visual photoreceptors and the CRY protein, fly activity rhythms are not entrained by light.[11]

Several studies have been done on magnetic field detection by Drosophila. This work revealed that magnetosensitivity of Drosophila exhibits both light and CRY dependency. Some of these investigations assessed the capacity of flies to orient when subjected to magnetic fields, while another study assessed the lengthening of circadian period by light. By contrast, my current research studies are based on the examination of the enhancement of entrainment (setting of circadian phase) of flies (Drosophila) by light due to magnetic field exposure.

Gegear et al. (2008) investigated the effects of magnetic field on Drosophila.[12]Flies were placed into the preparation tube with or without sucrose reinforcement and a magnetic field. Wavelength dependence was examined using long-wavelength pass filters.The assay determined whether flies were able to use a magnetic field, which they had been trained to associate with food, to find the food source. They determined that the ultraviolet-A/blue-light photoreceptor cryptochrome is essential for these light-dependent magnetosensitive responses in Drosophila. Wild-type flies demonstrated major naive and trained responses to a magnetic field under full-spectrum light (~300–700 nm); however, no response was shown when wavelengths less than 420 nm were blocked. Importantly, CRY-deficient cry^{o} and cry^{b} flies did not show any responses to a magnetic field under full-spectrum light. Furthermore, CRY-dependent magnetosensitivity did not necessitate a functioning circadian clock. They believe that their studies revealed the first genetic evidence for a CRY-based magnetosensitive structure in any animal.[12]

Yoshii et al. explored the magnetosensitivity of Drosophila's circadian clock.[13]The activity of flies was recorded automatically and individually with infrared light beams at a constant temperature of 20.8°C. They showed that Drosophila's clock is magnetosensitive and CRY dependent. When earlier works on magnetosensitivity of the circadian clock were done, CRY had not been detected yet and the influence of the magnetic field on the circadian clock was obscure. Present research and results on CRY's mediation of light inputs have been discussed earlier. The magnetic field effect produced a lengthening of the period in constant light (compared with constant light alone), comparable to that produced byan increased intensity of blue light. This was mainly shown in CRY-overexpressing flies. No responses were shown in red light. The results strongly support a radical-pair model proposing light-activated flavin-based photoreceptors as sensors for magnetic fields.[13]

The two main models for magnetoreception are well described by the theoretical and computational biophysics group of the University of Illinois Urbana-Champaign. They based their models on studies of birds, which can use magnetic fields for navigation during migration.

1. Magnetite-based model

It proposes that the avian compass has its origin in small particles of magnetite located in the head of the bird. "In the proper geometry, iron oxide clusters can behave like a compass needle and magnetic bacteria use such a compass needle mechanism to find up and down. An alternative possibility is to use short-lived, specialized

photochemical reactions, for which thermal noise does not have time to effectively mask effects of magnetic fields."[14]

2. Radical-pair based model

It proposes that the avian compass may be formed in a chemical reaction in the eye of the bird, involving the creation of a radical pair. In Drosophila, cryptochromes appear to be a particularly fascinating candidate for photo-magnetoreceptors. In animals such as Drosophila, cryptochromes can be implicated directly as light inputs into the circadian clock. Cryptochromes are present in quite a few organisms that revealed magnetic field effects. In addition, as described above, the photochemical properties of cryptochromes demonstrated some abilities to detect weak magnetic fields. Cryptochromes are triggered by means of an intraprotein electron transfer system that yields radical pairs. The radical that is generated triggers the protein in order to stimulate biological activity. Any feature that enhances the existence of the radical will essentially augment the cryptochrome signal at a given photo fluence (light intensity). Similarly, anything that diminishes the lifetime of the radical will automatically decrease cryptochrome signal at a specific light intensity.[14]

There are also structures of Drosophila CRY.[15-17] Processes involved with cryptochrome signaling (such as hypocotyl growth inhibition) are enhanced under a magnetic field of 5 G (as compared with an Earth-strength 0.5 G magnetic field).[18]

1.2 Problem Statement

It has been shown that flies can detect magnetic fields in a light-dependent manner and orient towards them, and that magnetic fields enhance the effects of light-dependent

period-lengthening of circadian rhythms. However, the capacity of magnetic fields to set the phase of circadian rhythms as a zeitgeber has not been assessed. The work presented in this thesis analyzes whether magnetic fields can serve as a zeitgber with two types of assays. One examines whether magnetic field pulses can enhance the phase-resetting of rhythms by light pulses; in these assays, the phase of the activity rhythm is measured in constant darkness in flies that have received prior pulses and compared with flies that have not received the pulses. The other assay determines whether 12hr:12 hr oscillations of magnetic fields (on:off) can set the phase of an activity rhythm, and if so whether the effect of the magnetic field requires light or can be detected in constant darkness. If light is required for these effects, I will determine the optimal wavelength for the magnetosensitivity in Drosophila (blue or red) and whether the CRY protein is required. Finally, I will determine the threshold strength for the magnetic field at the optimal wavelength. The partnership between Dr. Price's group, from the Department of Molecular Biology and Biochemistry at the UMKC, and our group has helped answer some of the questions raised.

Cryptochromes, which are photoreceptors located in the birds' eyes, are thought to be involved in magnetic orientation during migration. In Drosophila, cryptochromes are responsible for the light-dependent capability to detect the geomagnetic field. The existence of a mechanism by which the circadian clocks are entrained by magnetic field changes has remained uncertain. By finding the range of the magnetic field strength and an optimal wavelength for any effects of magnetic fields on entrainment, this study will be able to advance our understanding of the interaction of magnetosensitivity and the

circadian system. This research is very valuable in the domain of science because its success could inform investigations of magnetosensitivity of human beings. Moreover, Physics will once again assert its incredible value in describing the beauty of natural phenomena.

However, there are some constraints in this study. The main limitations will be the control of the constant temperature, affecting the temperature between the coils of the solenoid. Any small temperature fluctuations will lead to a small effect on the magnetic field being applied to the Drosophila melanogaster. However, the main issue is that temperature fluctuations can also entrain the circadian clock independent of any magnetic field effect. Nonetheless, the magnetic/light pulses in the first type of experiment (phase resetting) will be limited to short time intervals that will reduce the amount of heat generated in the fly's body.

After completion of this research, we anticipate follow-up studies to investigate the effects of magnetic fields on human beings. For instance, can blind people who cannot entrain to light cycles effectively use the magnetic fields to enhance circadian entrainment? This study is expected to lead to the answers of such questions. If carried out successfully, the results of this research should confirm the hypothesis that Drosophila's circadian clock is linked to their magnetosentivity at the blue wavelength range.

CHAPTER 2

EXPERIMENTAL METHODS

The experimental methods of the research are presented in this chapter. The preparation of cuvettes, the experimental devices, and data analysis are described.

2.1 Drosophila

Drosophila are small flies that have light yellow to dark brown pigment with red eyes. They are very small in size, as shown in Fig. 1. Therefore, a microscope is needed in order to examine them. Drosophila, which tends to be abundant in tropical regions, can be found around the world due to their attraction to fruits like bananas, grapes, berries, swamps, rainforests, deserts, and so on. They have been brought to the United States through the transport of fruits and especially bananas. The wild type strain of flies used in this study was isolated in Canon Ohio and therefore is known as the Canton S strain. Several *Drosophila* species, including *Drosophila melanogaster* (current sample), are very close to human beings genetically. In addition, it is easy to breed large numbers of Drosophila, making them very suitable candidates for studies related to genetics.

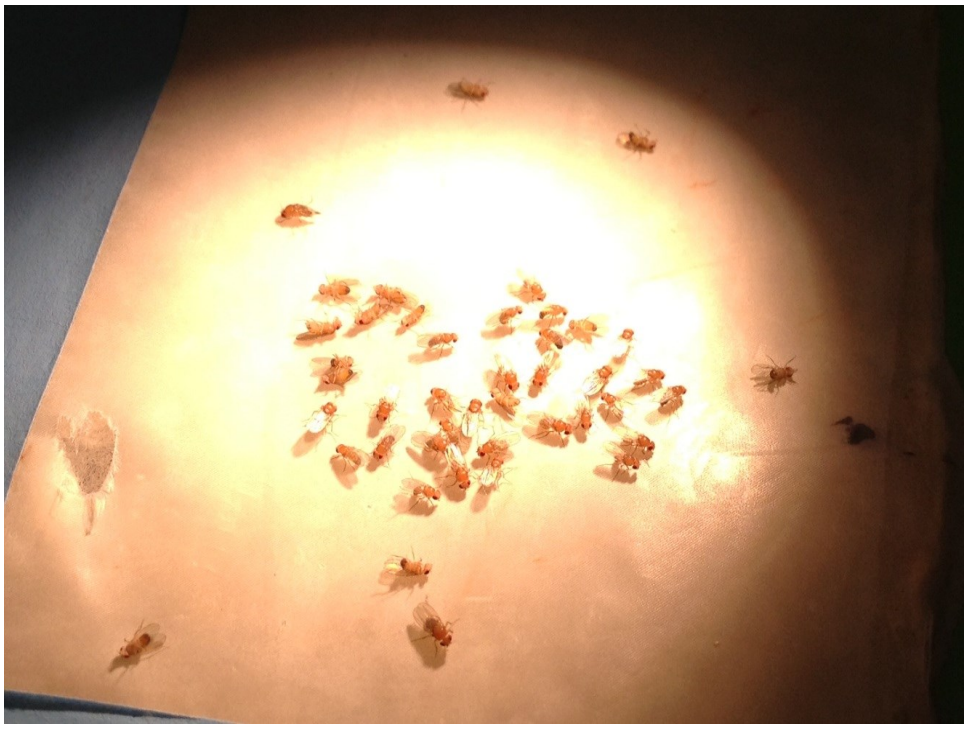


Figure 1. View of Drosophila from a microscope.

Due to their large reproduction, the females are not used for this studied. They could lay eggs while the experiment is in progress, making it impossible to monitor the activities of the flies in an accurate manner. Therefore, in our study, only males are used for the assays. The distinction between the males and females is done by means of a microscope. The males have a solid black posterior, while the female have a stripped one. Fig. 2 illustrates the difference between the two sexes.

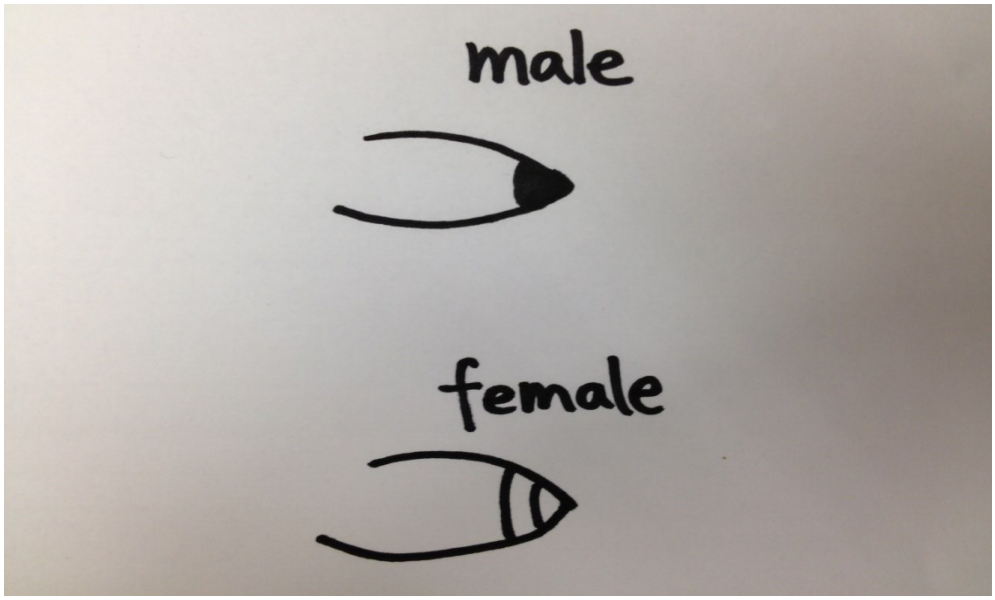


Figure 2. Distinction between male and female Drosophila.

2.2 Cuvette Preparation

Cuvettes are small tubes, with a tolerance of wavelengths ranging from 380 nm to 780 nm, and will contain Drosophila during the locomotor activity assay experiments. They will be sealed at one end with a piece of parafilm and fly food, and at the other end with some cotton, as shown in Fig. 3.

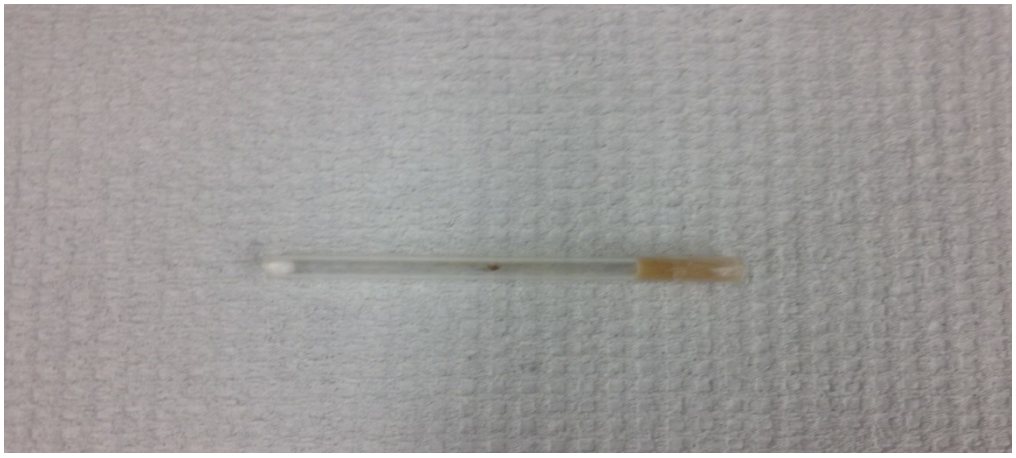


Figure 3.A cuvette enclosing a Drosophila.

The cuvette preparation is followed by the loading of flies. A microscope, as shown in Fig. 4, is used for the distinction of the genders. Carbon dioxide is used to anesthetize the flies during the loading process. The gray tank in Fig. 5 is fitted with a regulator to deliver a steady stream of carbon dioxide from a porous plate under the flies.

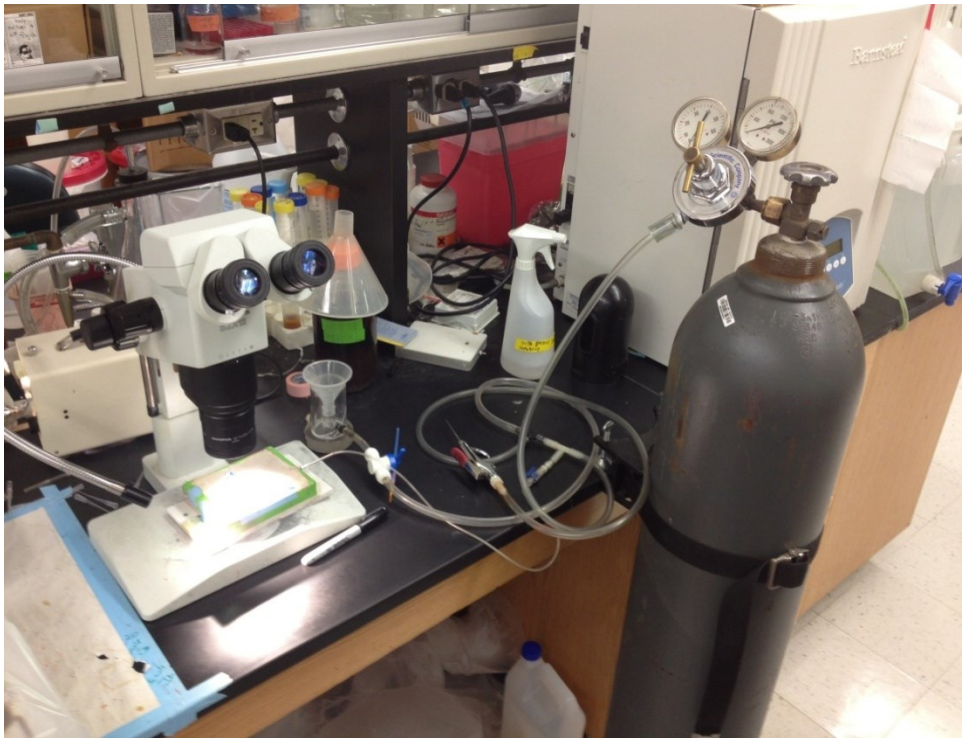


Figure 4. Set-up used to load the Drosophilas. The microscope(left) is used for magnifying of the flies while the tank(right) delivers CO_2which immobilizes the flies.

2.3 Experimental Devices

This experiment will be conducted using *Drosophila melanogaster*. The fruit flies, provided by Dr. Price's group, are kept in the research laboratory at a constant temperature. A solenoid, as shown in Fig. 5, is built in order to create the magnetic field. The strength of the magnetic field is regulated by means of a power supply whose current

can be controlled. A magnetic field was produced inside a wire coil (on the left) by passing an electric current through the coil, with the strength of the field proportional to the current. Light was produced by a monochromator in the incubator, which controlled the timing and wavelength of the lights to produce pulses of light.

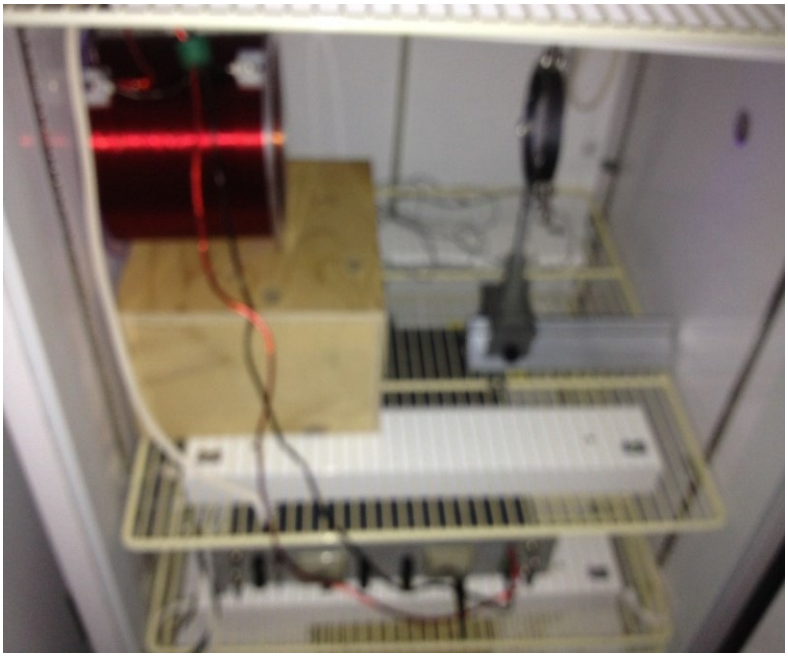


Figure 5. The solenoid (red) enclosed the Drosophilas that are subject to a visible blue wavelength.

The tubes containing the Drosophila are aligned in a box, as shown in Fig. 6. The box is placed inside the solenoid so that the flies can be subject to magnetism and light, or just light if the current is turned off.

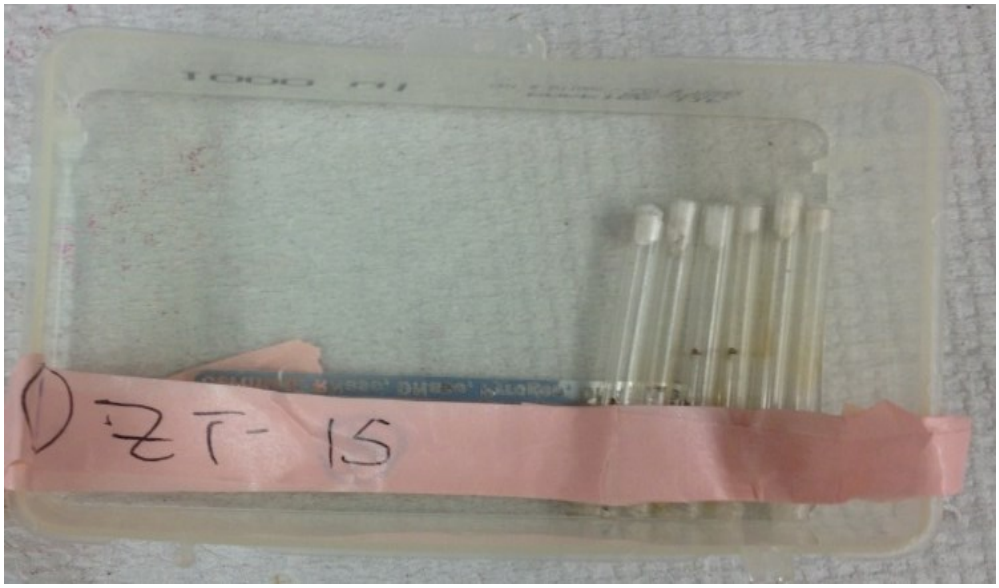


Figure 6. Illustration of Drosophila aligned before being subject to magnetism and light.

A monochromator 74125, as illustrated in Fig. 7, is used in this experiment. This device is very efficient in scanning from the ultraviolet through the far infrared.

Figure 7. Monochromator 74125.[19]

The standard features include a built-in electronic shutter, filter wheel control, and a family of interchangeable gratings and slits. The CS-260 uses an asymmetrical in-plane Czerny-Turner optical configuration. Throughput is high and stray light is very low. Optically flat black paints and baffles and high efficiency spherical mirrors and gratings are used to minimize surface reflections, and thus stray light.[19]The optical mechanism by which the device performs is portrayed in Fig. 8.

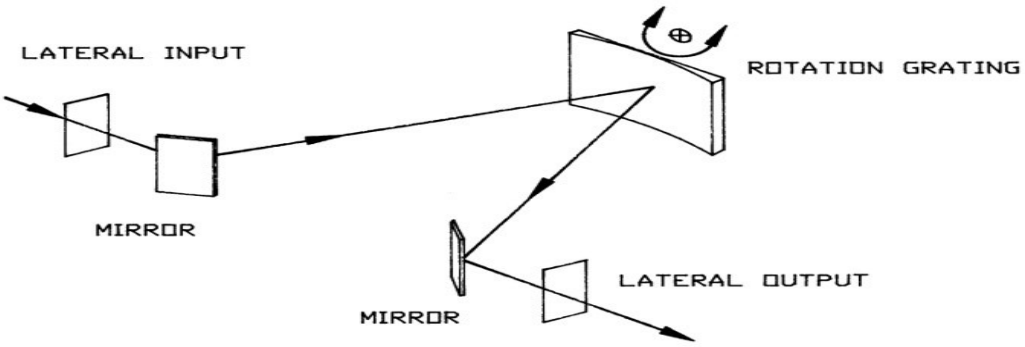


Figure 8. Optical mechanism of the monochromator.[19]

An incubator, shown in Fig. 9, is used in order to entrain the flies. The incubator can be programmed to produce a12 hr. on: 12hr off cycles of light. The incubator encloses several vials which are separated based on the age of the Drosophila.

Figure 9. Incubator.

2.4 Locomotor Activity Assays

For over a half-century, circadian activity rhythms have been investigated in Drosophila. The assays are performed as shown in Figure 10.

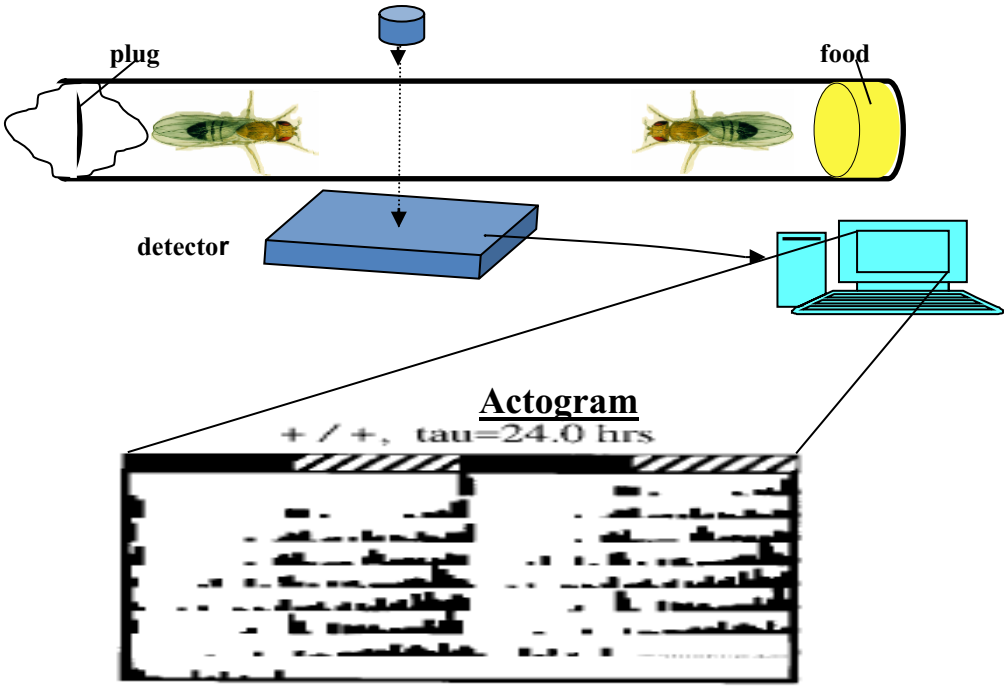
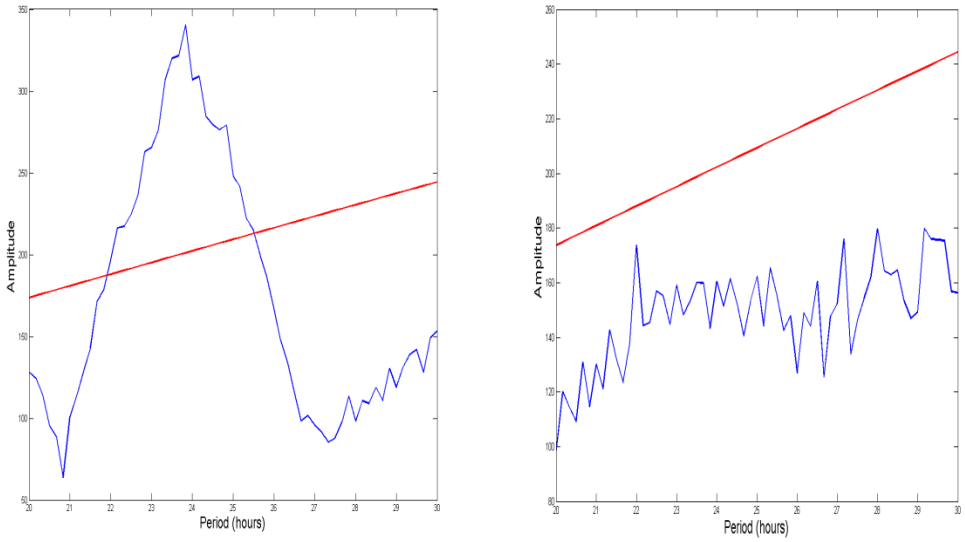


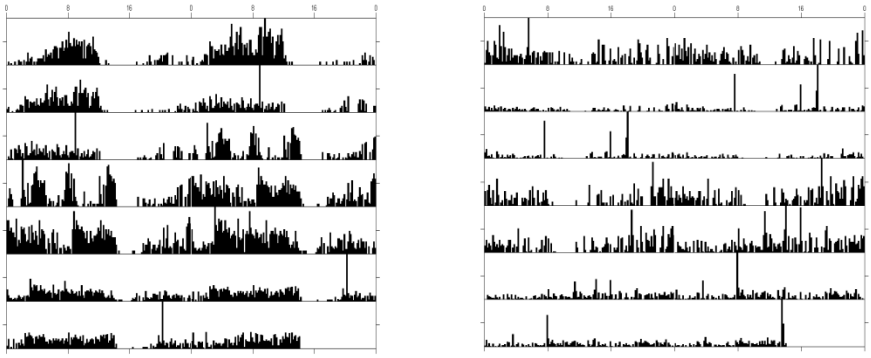
Figure 10. Activity Assays.The activity of each fly was monitored in activity monitors which detect deflections of an infrared beam and record the numbers in 10 minute bins. The 7-10-day record of the flies is plotted as an Actogram. Here, the assay was performed in constant darkness, but the phase of the rhythm is set by the time of the previous light:dark cycle, which is plotted above the record.

A computerized program is used to record activity. ClockLab (a Matlab-based circadian analysis software package from Actimetrics, Inc) is used to get the periodograms and actograms of the assays. In actograms, the amount of activity in consecutive bins periods is plotted along a line with a length of 48hours. The amount of activity is indicated by the height of the black bar. Data are double-plotted so that the day on the right of the line is repotted on the left of the line immediately below. Circadian behavior is visualized by scanning from left to right. Figure 11 is an illustration of periodograms and actograms for Canton S flies in12hr: 12hr light and

dark (LD) cycle; it demonstrates rhythmiticity (left) and arrhythmicity (right). For each condition in my experiments, the activity records of approximately 16 flies were obtained for a given condition (genotype, light, magnetism). In addition, the 7-10 day-record of each fly was averaged to produce an activity profile for the 23.5-24.5 hour circadian cycle of the Canton S flies. Activity offsets (evening activity phase) were determined as the time at which the average activity crossed the mean activity level during the falling phase of the activity profile. The average phase and synchrony of each experimental and control (unpulsed) group were determined by vector addition. Alternatively, the average phase of an experimental or control group was determined from a 23.5-24.5 hr activity profile for an averaged activity record for the entire group. The magnitude of any phase shift was determined by subtracting the evening phase of the experimental group from the evening phase of one of the two unpulsed control groups. The second method used for data analysis is known as the average waveform method.



(A). The periodogram on the left shows rhythmicity in DD while the one on the right display arrhythmicity in LL.



(B). The actogram on the left shows rhythmicity in DD while the one on the right display arrhythmicity in LL.
Figure 11. Illustration of periodograms and actograms.

2.5 Experimental Strategy

The first part of the experiment consists of using the monochromator in order to illuminate the Drosophila at different wavelengths during the dark phase of an LD cycle.

The current through the coils will be produced by a power supply activated manually for short pulses of light and magnetism, followed by activity assays in constant darkness to determine the effect of magnetism on the light-dependent phase shift of the circadian rhythm, and the magnetic field will be generated at the same time lights areon (except in the controls where magnetic field pulses were given without light or light was given without a magnetic field pulse). Fly activity will be monitored in constant darkness (DD) with no magnetic field in Trikinetics fly activity monitors (on the left in figure 12).

Alternatively, for the experiments involving application of a magnetic field to flies in constant light (LL) or constant dark (DD), the monitors can be placed inside the coil for activity monitoring during diurnal magnetic field cycles or placed outside the coil for monitoring of activity without magnetic field oscillations. The temperature is kept constant by the incubator. The current is also kept constant during the pulse in order to have a constant applied magnetic field.

These experiments will employ constant illumination (LL) or darkness (DD), with or without a 12hr on: 12hr off cycle of magnetic fields of varying strengths produced by changing the current. This is analogous to the experimental set-up shown in Fig. 12. Illumination was produced by blue fluorescent bulbs initially programmed to be on for 12 hrs and off for 12 hrs before a switch to LL or DD. The magnetic field was either activated in phase with the previous light period or dark period of the LD cycle, as indicated in the description of the experiments.

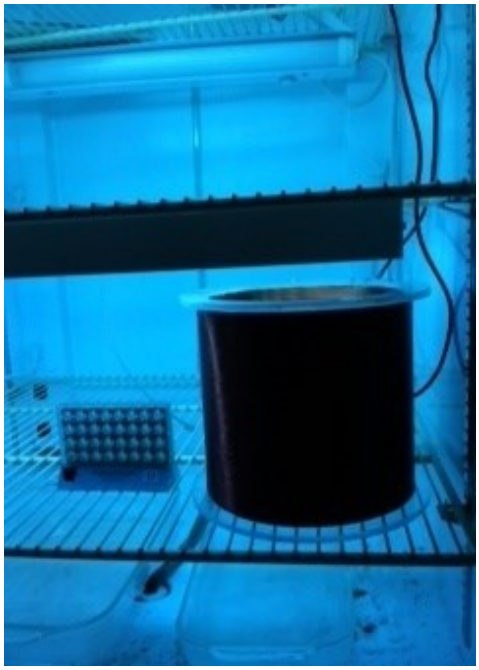


Figure 12. Experimental set-up for cycles of magnetism. A magnetic field was produced inside a solenoid by passing an electric current through the coil, with the strength of the field being proportional to the current. A DC power supply on a timer (12hr on:12 hr off cycles) was used to generate current through the solenoid. Light was produced by high intensity blue fluorescent bulbs in the incubator with wavelengths ranging between 450 nm and 470nm.

2.6 Specific Pulse Conditions

All pulses were done at wavelength of 450.013nm (blue) except one. The last pulse is done at a wavelength of 700.013nm (red). The light bulb was a 75 Watt Xenon light. The room and incubator temperature was kept at 25^{o}C with negligible fluctuations less than 2^{o}C. The number of turns of the solenoid was about 343. The current produced by the power supply in the assays involving magnetism of 160X the earth's field is about 1.9A. The voltage was around 12.5V. By using Biot-Savart Law, the magnetic field was calculated to be 4.48.mT. A magnetometer was used to ensure the uniformity of the field inside the solenoid. A small variation was recorded (3.92mT to 5.34mT).

For experiments involving wild type Canton S and cry^b mutants, flies with the different genotypes were loaded in sequential positions in the monitors, so there was no consistent difference in their position that could have produced a difference in the average magnetic field to which each genotype was exposed.

For the first part of the experiment, the assays were performed using the following setups: control group (no pulse), light only (blue), magnetism only, light (blue) plus magnetism simultaneously, and light (red) plus magnetism simultaneously. 10 s and 1 min pulses were applied. Two unpulsed control groups were typically analyzed, in order to determine the extent of experimental variability in phase determination.

CHAPTER 3

RESULTS & DISCUSSION

This chapter demonstrates the methods used for data analysis, the results, some illustrations of circular plots and waveforms, and a discussion section.

3.1 Experimental Strategy and Data Analysis for Phase-Resetting Experiments

Theactivities of the flies weremonitored and analyzed byactogram, chi-square (χ^2)periodogram and waveform analysis by the means of ClockLab (a Matlab-based application). This led to theexamination of the rhythmicity of the flies with and without exposure to the magnetic field.Strong phase delays areproduced by light pulses delivered at ZT-15 (in DD, 3 hours after the end of a 12hrlight phase), while strong phase advances produced by light pulses delivered at ZT-21 (in DD, 9 hours after the end of the 12hr light phase). In my experiments, the flieswere subjected topulses of light (red or blue) and/or magnetic field 3 hours after the end of a 12hr:12hr LD cycle (i.e., at ZT15). There were two control groups considered as unpulsed. For each condition, the activity records of approximately 16 flies were obtained. In addition, the 7-10day-record of each fly was averaged to produce an activity profile for the 23.5-24.5 hour circadian cycle of the Canton S flies. Activity offsets (evening activity phase) were determined as the time at which the average activity crossed the mean activity level during the falling phase. The average phase and synchrony of each experimental and control (unpulsed) group were determined by vector addition. Alternatively, the average phase of an experimental or

control group was determined from a 23.5-24.5hr activity profile for an averaged activity record for the entire group. The magnitude of any phase shift was determined by subtracting the evening phase of the experimental group from the average evening phase of the two unpulsed control groups.The second method used for data analysis is known as the average waveform method.

3.2 Results for Phase-resetting Experiments

Three assays were performed with light pulses and/or magnetic pulses at ZT 15. These experiments are tabulated separately below, and a summary table reports the average phase shifts produced (Tables 1-7). Representative circular plots and activity profiles are shown in figures 16 and 18respectively.In all three experiments the largest phase shifts were produced with a combination of blue light and magnetism. These larger phase shifts were calculated with both the vector addition method and the average waveform method. The effect of magnetism was produced when coupled with light in the blue spectrum (wavelength of 450.013nm) and not with light in thered spectrum (wavelength of 700.013nm), which was notconsistently effective in producing phase shifts. The results showed saturation at pulses equal to or longer than a minute, since enhancement of phase-shifting by magnetism was most consistently seen with 10-sec pulses and was not consistently enhanced by longer light and magnetism pulses, which did not further shift the phase in comparison with the phase shifts produced by 10 sec pulses of blue light and magnetism. Magnetism pulses alone did not consistently produce phase shifts. The strength of these conclusions is compromised by the high variability of the phase shifts

and the relatively small increase in the phase-shift produced by the magnetism coupled with blue light, relative to the control conditions.

- First assay

Table 1.Vector addition method (1st assay).

Conditions	Offsets(hr) with 10s pulse	Length of vector (number of rhythmic flies)	Phase shift(hr) with 10s pulse
	ZT-15		
Unpulsed	12.92	0.97* (6)	____
Light only	13.28	0.93* (7)	-0.36
Light + Magnetism	13.69	0.95* (15)	-0.77
Magnetism	12.91	0.92* (13)	0.01
Light + Magnetism (Red)	13.19	0.97* (9)	-0.27

For these tables and all others for the vector addition method, the offset times are those determined by the phase of the resultant vector calculated from the phase of the individual fly phase vectors plotted on a 24 hour circle, with time 0=ZT0. The length of the resultant vector is a measure of phase coherence; * indicates the length of the vector produces a Z value (number of flies * (length of the vector)^2) that is greater than that for rejection of the null hypothesis by the Rayleigh test (null hypothesis: no phasing of the group, $p<0.05$). Phase shifts are calculated as the offset phase of the unpulsed controls (or the average phase of these, if there are two unpulsed groups) minus the offset phase of the pulsed group.

Table 2. Average waveform method (1st assay).

Conditions	ZT-15	
	Offsets(hr) with 10s pulse	**Phase shift(rad) with 10s pulse**
Unpulsed	13.13	____
Light only	13.29	-0.16
Light + Magnetism	13.29	-0.16
Magnetism	13.13	0
Light + Magnetism (Red)	13.29	-0.16

Offsets for these groups are calculated as the time at which the averaged activity profile for the rhythmic flies in the group falls below the mean activity level for the group. Phase shifts are calculated as the average offset phase of the unpulsed control groups minus the offset phase of the pulsed group.

- Second assay

Table 3.Vector addition method (2nd assay).

Conditions	Offsets(hr) with 10s pulse	Phase shift(hr) with 10s pulse	Length of vector (number of rhythmic flies)	Offsets(hr) with 1min pulse	Phase shift(hr) with 1min pulse	Length of vector (number of rhythmic flies)
	ZT-15					
Unpulsed	11.52	-0.22	0.89 (4)	11.96	0.22	0.42 (5)
Light only	13.19	-1.45	0.94* (7)	13.69	-1.95	0.93* (12)
Light + Magnetism	14.50	-2.76	0.94* (9)	13.63	-1.89	0.93* (11)
Magnetism	12.51	-0.77	0.93* (8)	13.27	-1.53	0.83* (10)
Light + Magnetism (Red)	13.75	-1.01	0.92* (9)	13.97	-0.23	0.95* (14)

Table 4. Average waveform method (2nd assay).

Conditions	ZT-15			
	10s pulse		1min pulse	
	Offsets(hr)	Phase shift(hr)	Offsets(hr)	Phase shift(hr)
Unpulsed	12.55	0.08	12.71	-0.08
Light only	13.47	-0.84	13.62	-0.99
Light + Magnetism	14.85	-2.22	14.08	-1.45
Magnetism	12.40	0.23	13.62	-0.99
Light + Magnetism (Red)	13.17	-0.54	12.40	0.23

- Third assay

Table 5.Vector addition method (3rd assay).

Conditions	Offsets(hr) with 10s pulse	Phase shift(hr) with 10s pulse	Length of vector (number of rhythmic flies)	Offsets(hr) with 1min pulse	Phase shift(hr) with 1min pulse	Length of vector (number of rhythmic flies)
	ZT-15					
Unpulsed	10.03	0.51	0.69 (4)	11.05	-0.51	0.95* (9)
Light only	11.81	-1.27	0.91* (6)	13.36	-2.82	0.96* (6)
Light + Magnetism	12.31	-1.77	0.95* (9)	13.16	-2.62	0.93*(9)
Magnetism	10.81	-0.28	0.82* (9)	11.73	-1.19	0.93* (8)
Light + Magnetism (Red)	12.26	-1.72	0.96* (7)	11.47	-0.93	0.93* (11)

Table 6. Average waveform method (3rd assay).

Conditions	ZT-15			
	10s pulse		1min pulse	
	Offsets(hr)	Phase shift(hr)	Offsets(hr)	Phase shift(hr)
Unpulsed	12.99	-0.50	11.99	0.50
Light only	12.63	-0.19	12.71	-0.27
Light + Magnetism	13.01	-0.57	13.62	-1.18
Magnetism	11.48	0.96	12.40	0.04
Light + Magnetism (Red)	13.24	-0.80	12.40	0.04

Table 7.Summary Table.

Conditions	ZT-15							
	10s pulse				1min pulse			
	Vector Addition		Average Waveform		Vector Addition		Average Waveform	
	Average Phase shift(hr)	Standard Deviation	Average Phase shift(hr)	Standard Deviation	Average Phase shift(hr)	Standard Deviation	Average Phase shift(hr)	Standard Deviation
Unpulsed	0.00	0.45	0.00	0.41	-	-	-	-
Blue Light only	-1.03	0.58	-0.40	0.38	-2.39	0.62	-0.63	0.51
Blue Light plus Magnetism	-1.77	1.00	-0.98	1.09	-2.26	0.52	-1.32	1.09
Magnetism only	-0.35	0.32	0.40	0.50	-1.36	0.24	-0.48	0.73
Red Light plus Magnetism	-1.00	0.73	-0.50	0.32	-0.35	0.82	0.14	0.13

These are the average phase shifts and standard deviations calculated from those of the previous 6 tables.

The phase resetting experiments showed that pulses of blue light and magnetic field had stronger phase delays than with blue light alone or with red light and magnetism when using either the vector addition method or the average waveform method. In addition, the effect of magnetism was more consistent with10sec pulses than with 1min ones, most likely due to saturation of the phase delay response with the 1 min pulses of light. Magnetism alone did not produce consistent phase delays or advancing, suggestingthat light is required for the magnetosensitivity. However, the phase shifts were highly variable with large standard deviations, precluding definitive results with this approach.

3.3 Experimental Strategy, Data Analysis, and Results for Activity Rhythms in 12hr:12hr On/Off Oscillations of Magnetic Fields

The two general entrainment protocols were used (LD to LL, with magnetism in phase with LD, and LD to DD to LL, with magnetism out of phase with LD). Figures 13 and 14 are illustrations of the experimental designs. Representative circular plots are in figure 16. The average activity profiles of Canton S and cry^b flies are illustrated by figure 18. In DD, the capacity of the magnetism to shift the phase and maintain 24 hour periodicity was examined.In LL, the ability of the magnetic field to maintain rhythmicity rather than arrhythmicity for Canton S flies and to shift the phase was investigated whereas for cry^bflies,only the capacity to shift phase was inspected. This is due to the fact that cry^bfliesare reported to be rhythmic in LL (ref. 5, 6, 18). Representative actograms of a Canton S fly and cry^b fly in the DD to LL regime are displayed by figure 19. The

percent rhythmicity, the period length, the phase coherence and phase are all things that were measured. The percent rhythmicity was calculated by finding the ratio of the rhythmic flies to the total number of living flies and multiplying the quotient by 100. The period length was measured by means of ClockLab (a Matlab application). The phase coherence was done by means of the directional (circular) statistics; the closer the length of the average vector*r* to 1, the more coherent the phase is. Similarly, the farther the length of the average vector to 1 (or closer to 0), the more dispersive the phase is. The phase analysis was done by both the vector addition method and the average waveform method. Rayleigh z test was used to conduct the statistical analysis.Table 8reveals the results of the LD to LL assay. Table 9demonstrates the results of the LD to DD to LL assay.The LD, DD, and LL experiments showed that a strong magnetic (160 times the earth's magnetic field) can be sensed in constant light. A 12hr: 12hr on:off oscillation of this field can restore rhythmicity under LL in wild type flies and even shift the phase of the rhythm from that previously entrained by LD. In addition, re-entrainment of the circadian clock of cry^b mutant flies was observed, demonstrating that normal wild type CRY is not required for detection of the magnetic field. The oscillating magnetic field did not re-entrain either wild type or cry^b flies in DD, demonstrating that LL is required for the effect.

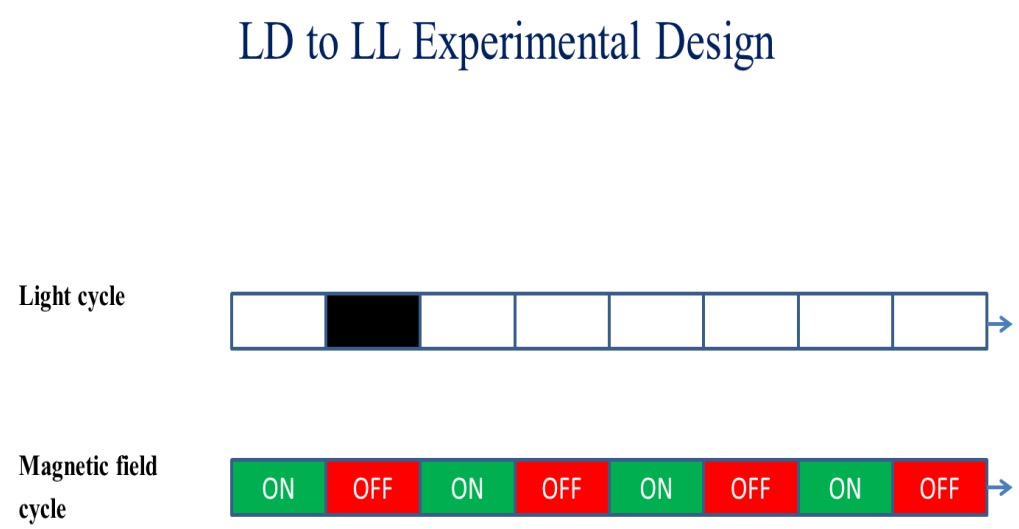


Figure 13.LD to LL experimentaldesign.Canton S and *cry*^{b}flies were entrained in LD for three days with magnetism tuned on during the day. An LL cycle followedwith 12hr: 12hrOn:Offoscillations of magnetic field for at least five days with magnetism turned on during the subjective day.

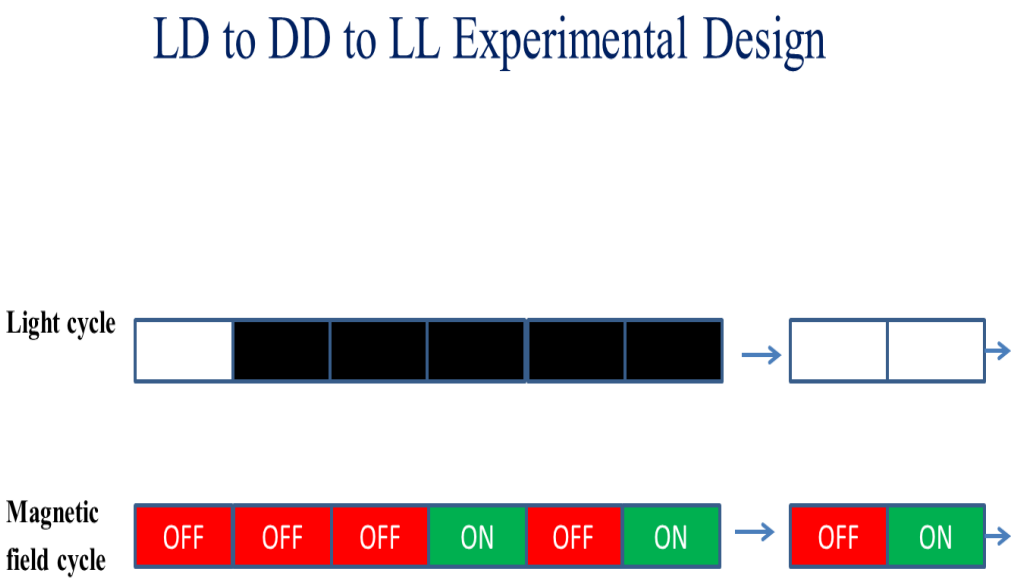


Figure 14.LD to DD to LL experimentaldesign.Canton S and *cry*^{b}flies were entrained in LD for three days without magnetism. A DD regime followed with 12hr: 12hrOn:Offoscillations of magnetic field for at least five days.Then, an LL regime of at least five days was conducted, with12hr: 12hr oscillations of magnetic field opposed to the LD cycle (magnetism turned on during the subjective night).

Table 8.LD to LL.

Conditions	Average period (hr)	% Rhythmicity (total number of flies)	Average phase of offsetby waveform method (hr)	Average phase of offset by vector addition (hr)	Length of average vector
No Magnetism	-	0 (30)	-	-	-
160B	23.43±0.43	96.88[*] (31)	16.06	14.33	0.97[#]
No Magnetism	-	0 (14)	-	-	-
160B	24.06±0.40	45.83[**] (24)	13.85	11.26	0.69[#]
No Magnetism	-	0 (30)	-	-	-
80B	23.17±0.54	15.63 (32)	13.01	12.33	0.89
No Magnetism	-	0 (32)	-	-	-
47B	23.5	3.45 (29)	13.19	13.19	1
No Magnetism	-	0 (32)	-	-	
40B	23.88±0.44	13.33 (30)	22.39	1.04	0.77

Flies were entrained, monitored, and analyzed as described in the text. Kruskal-Wallis Anova nonparametric analysis showed a significant effect of group on percent rhythmicity ($H\ \{5, N= 285\} = 174.9208$ $p < 0.001$). [*] differs from all other groups with $p < 0.02$ (all LL groups pooled together). [**] differs from all LL groups without magnetism with $p < 0.02$. The Rayleigh z-test indicated statistically significant phasing for the indicated groups[(#)].

Table 9.DD to LL.

Conditions	**Average period (hr)**		**% Rhythmicity (total of number of flies)**		**Average phaseby waveform method (hr)**		**Average phase by vector addition (hr)**		**Length of average vector**	
Type of flies	*Cry*^{b}	CS	*Cry*^{b}	CS	*Cry*^{b}	CS	*Cry*^{b}	CS	*Cry*^{b}	CS
DD with no Magnetism	24.91 ±0.3	24.32± 0.48	92.31 (14)	100 (16)	17.98	15.77	16.96	15.32	0.92$^{\#}$	0.95$^{\#}$
DD with Magnetism (160B)	24.82 ±0.49	24.62± 0.50	100 (14)	100 (14)	18.60	17.15	17.53	16.45	0.89$^{\#}$	0.88$^{\#}$
LL with no Magnetism	24.77 ±0.28	24.42± 0.29	57.14 (14)	25 (16)	20.36	17.46	19.03	15.56	0.45	0.67
LL with magnetism (160B)	24.81 ±0.22	24.62± 0.36	85.71 (14)	78.57 (14)	2.63	0.64	2.62	3.21	0.72$^{\#}$	0.70$^{\#}$
LL with no Magnetism-2 weeks	24.33 ±0.53	-	50 (14)	0* (16)	16.66	-	15.54	-	0.70$^{\#}$	-
LL with (160B) 14 days	24.64 ±0.52	24.02± 0.35	46.15 (13)	76.92 (13)	4.66	3.06	4.77	2.33	0.77$^{\#}$	0.75$^{\#}$

Flies were entrained, monitored, and analyzed as described in the text. Kruskal-Wallis Anova nonparametric analysis showed a significant effect of group on percent rhythmicity ($H\ \{11, N= 171\} =74.75004\ p < 0.0001$). * differs from all other groups with magnetism with $p < 0.02$. The Rayleigh z-test indicated statistically significant phasing for the indicated groups (#).

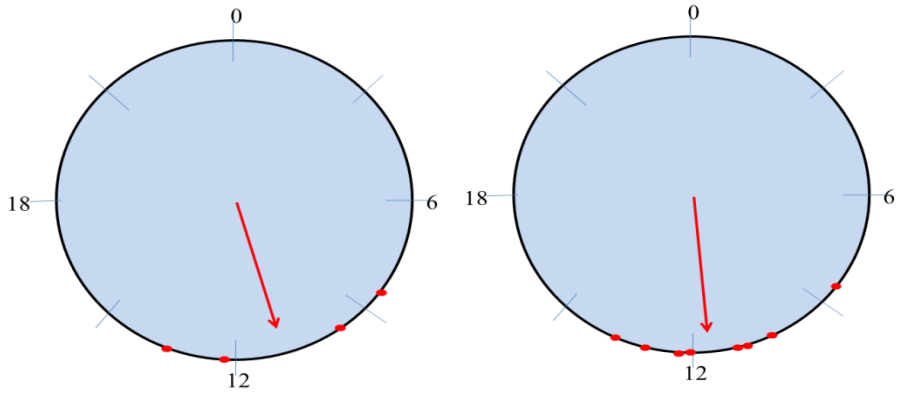
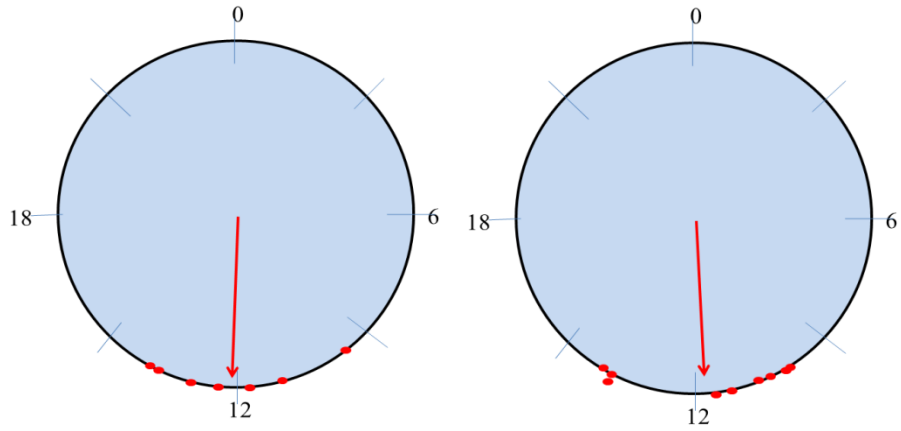
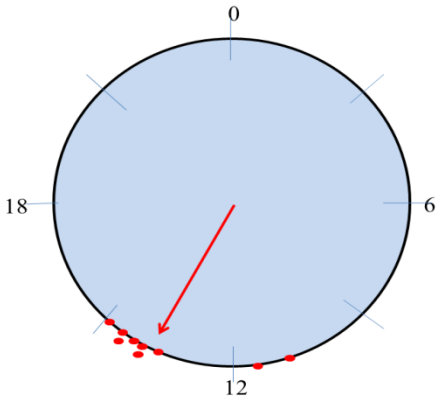


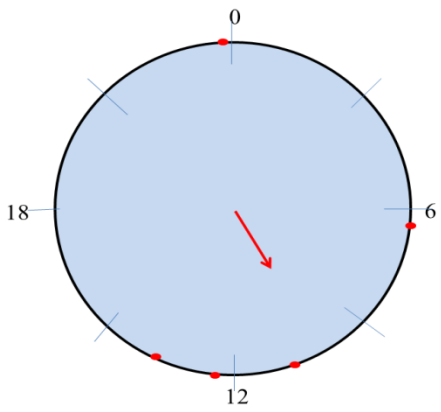
Figure 15. (A). Canton S unpulsed.**(D).**Magnetism only (10s pulse).

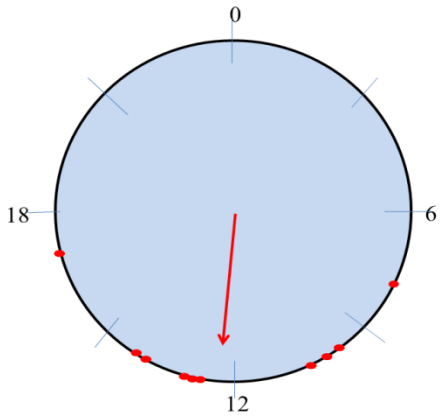


(B). Light only (10s pulse). **(E).** Red Light &Magnestim (10s pulse).

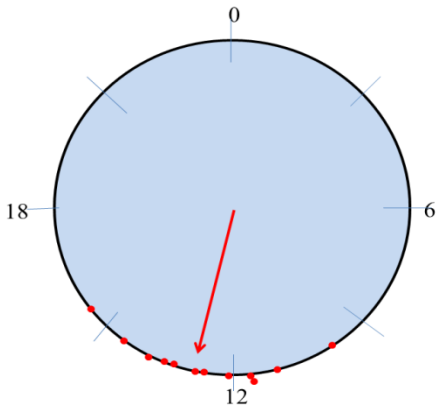


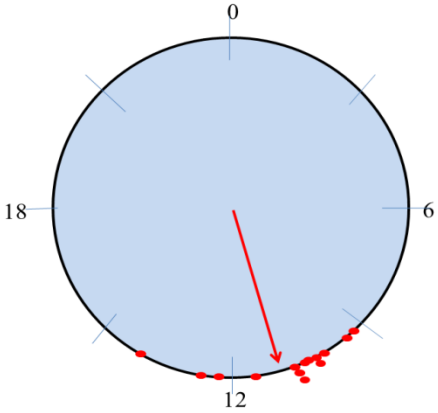
(C). Light & Magnetism (10s pulse).





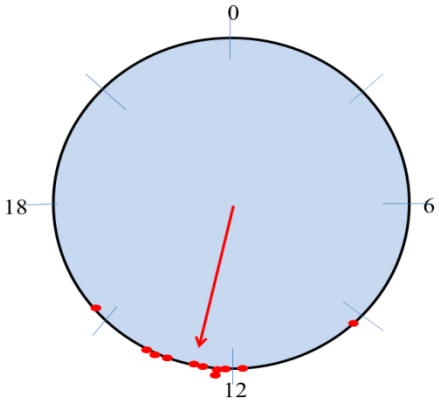
(F). Canton S unpulsed.**(I).**Magnetism only (1min pulse).





(G). Light only (1min pulse).

(J). Red Light &Magnestim (1min pulse).



(H). Light & Magnetism (1min pulse).

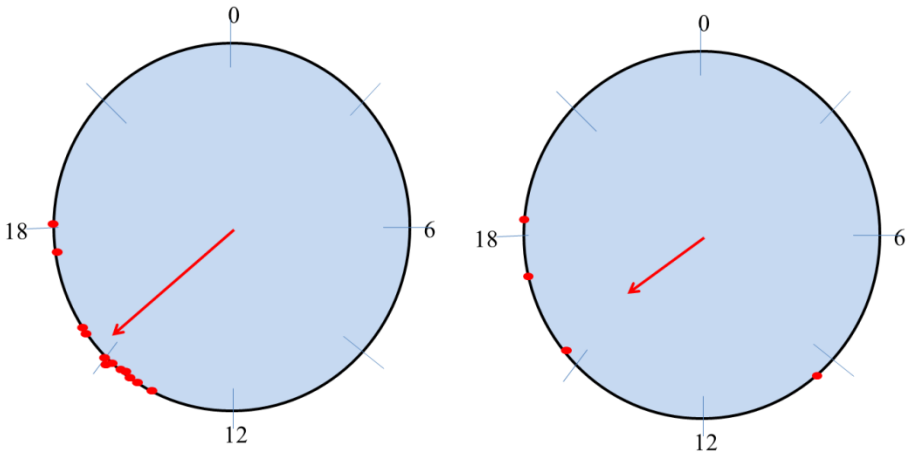
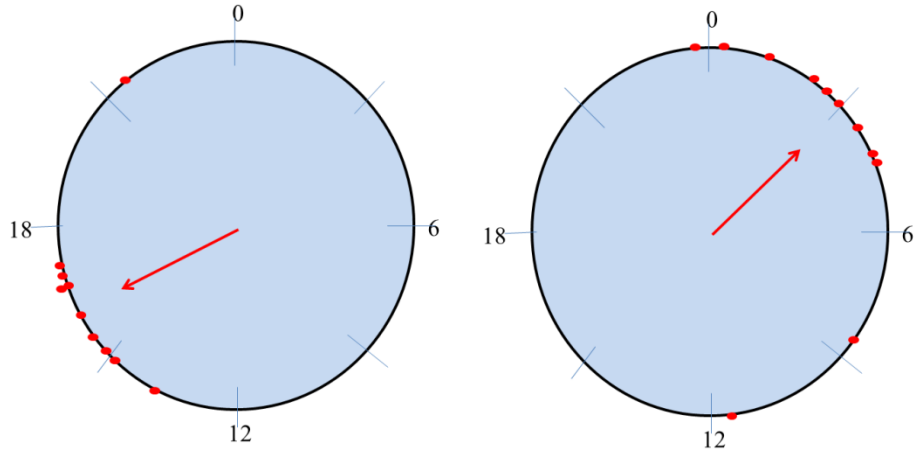
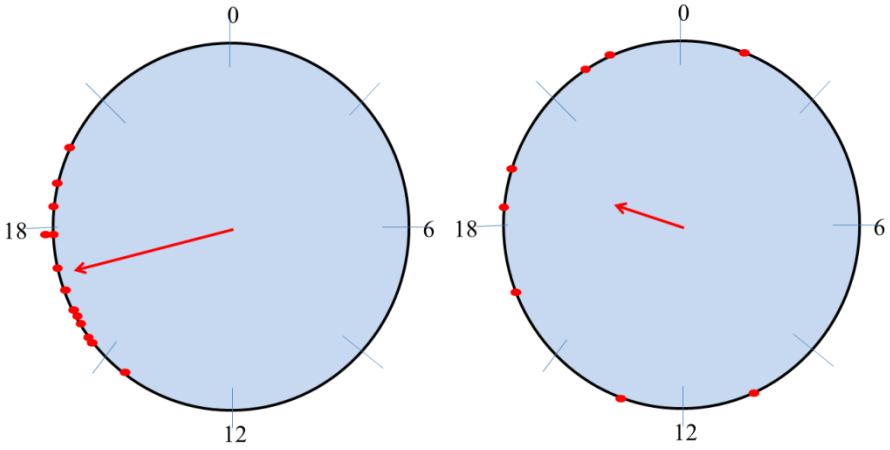


Figure 16. (A). CS in DD without magnetism.**(B).**CS in LL without magnetism.

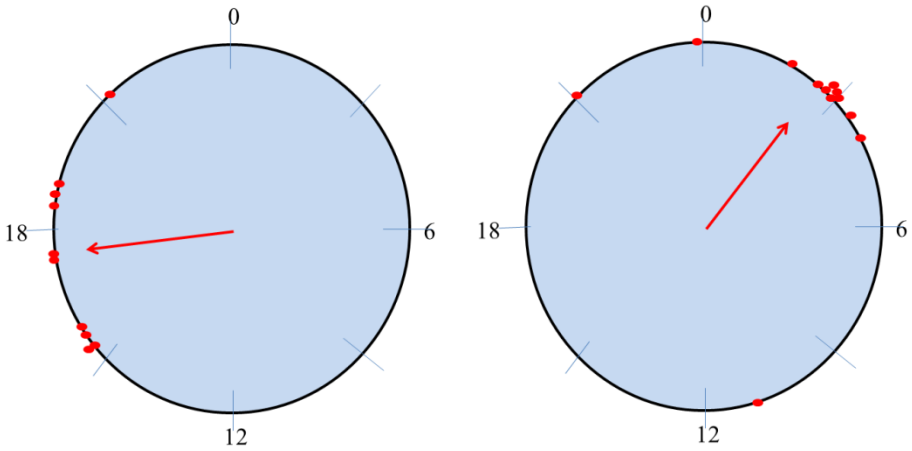


(C). CS in DD at 4.5mT~160B.

(D). CS in LL at 4.5mT~160B



(E).*Cry*^{b} in DD without magnetism.**(F).***Cry*^{b} in LL without magnetism.



(G).*Cry*^{b} in DD at 4.5mT~160B.

(H). *Cry*^{b} in LL at 4.5mT~160B

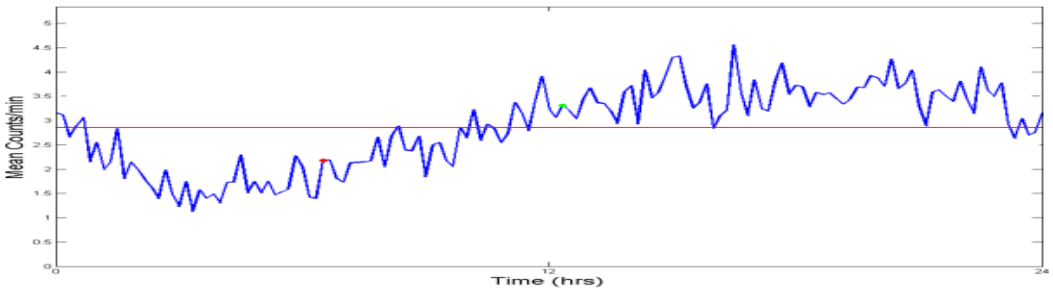
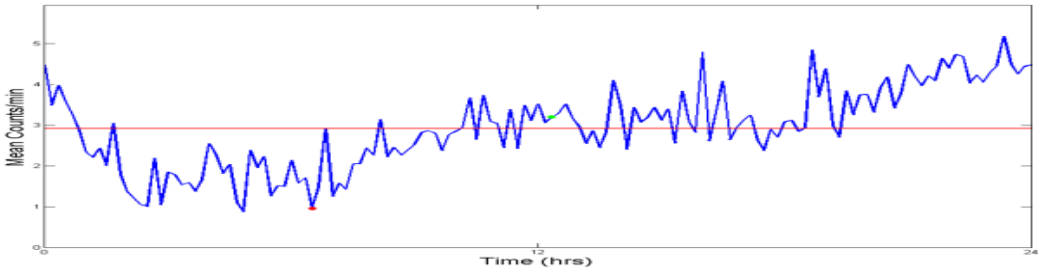
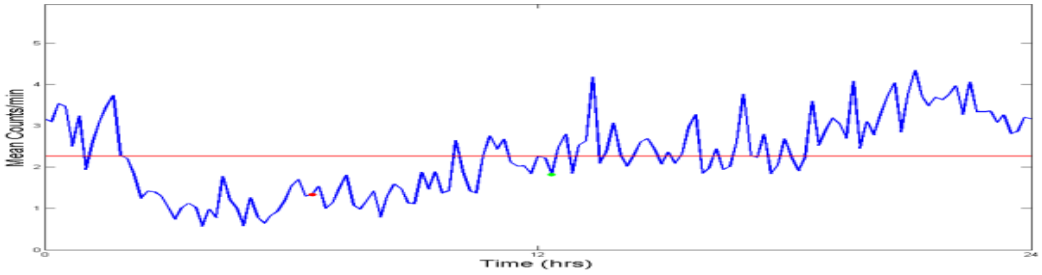


Figure 17.(A.) Unpulsed Canton S flies.



(B.) Canton S flies with 10s pulse of blue light applied at ZT 15.



(C.) Canton S flies with 10s pulse of blue light and magnetism applied at ZT 15.

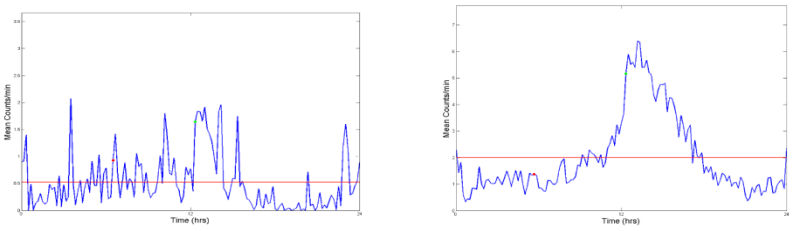
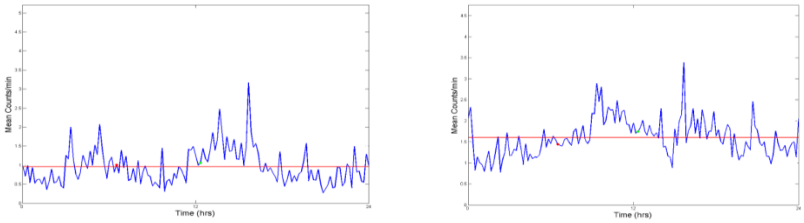
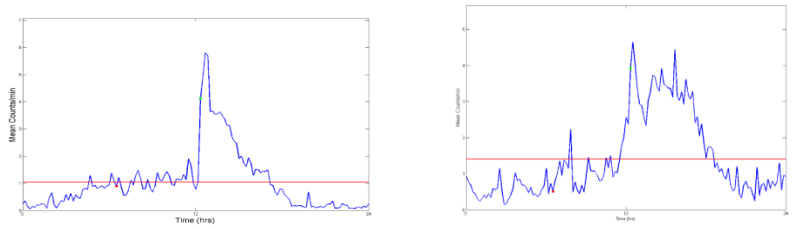


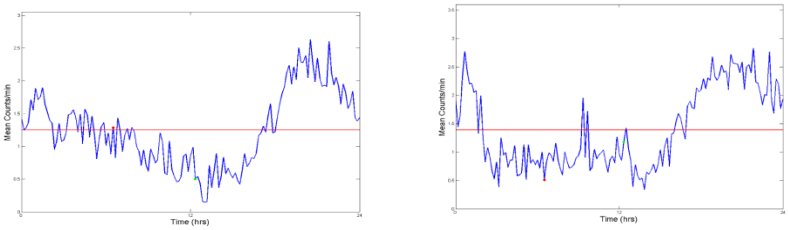
Figure 18.(A). Canton S flies group averages (DD without magnetism).(DD without magnetism). **(E).***Cry*^{b}flies group averages



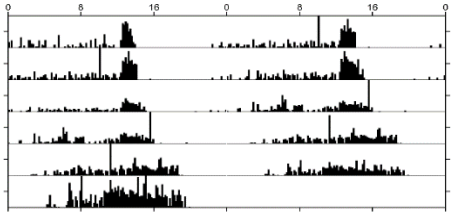
(B). Canton S flies group averages(LL without magnetism).(LL without magnetism). **(F).***Cry*^{b}flies group averages



(C). Canton S flies group averages (DD with 4.5mT~160B).(DD with 4.5mT~160B). **(G).***Cry*^{b}flies group averages



(D). Canton S flies group averages (LL with 4.5mT~160B).(LL with 4.5mT~160B). **(H).** *Cry*^{b}flies group averages



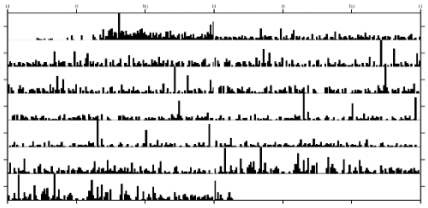
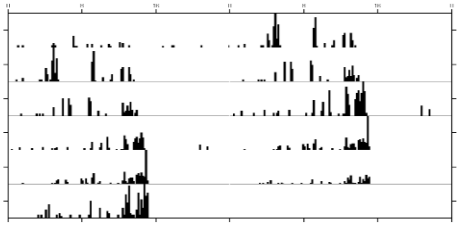
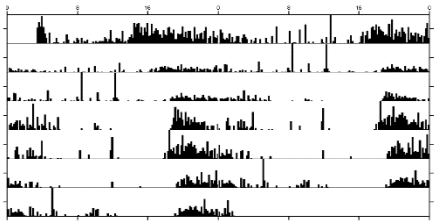
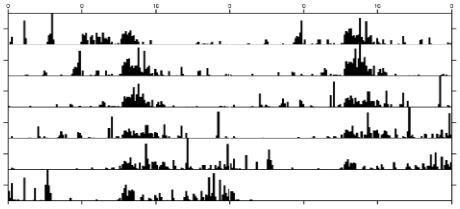


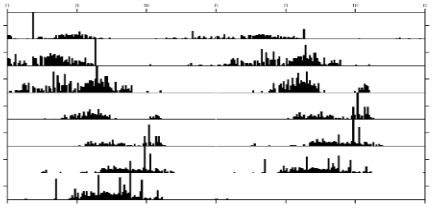
Figure 19.(A). Canton S fly **(B).** Canton S fly
(DD without magnetism).(LL without magnetism).



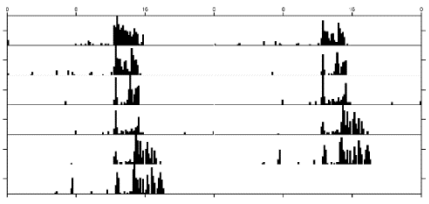


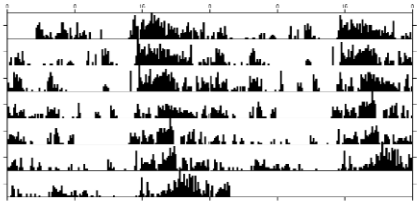
(C). Canton S fly **(D).** Canton S fly
(DD with 4.5mT~160B).(LL with 4.5mT~160B).





(E).*Cry*^{b}fly**(F).** *Cry*^{b}fly
(DD without magnetism).(LL without magnetism).





(G).*Cry*^{b}fly**(H).***Cry*^{b}fly
(DD with 4.5mT~160B).(LL with 4.5mT~160B).

3.5 Discussion

The phase resetting assays suggest that magnetism can enhance the phase-resetting of blue light but has no effect without light or in the presence of red light. The results showed saturation at pulses equal to or longer than a minute.In the presence or constant light (LL), 12hr:12hr cycles of magnetism can drive rhythms of locomotor activity, with activity peaking when the magnetic field is turned off. The effect requires field strengths greater than 80 times that of the earth's magnetic field strength. This effect was seen both in wild type and cry^b flies; the latter remain substantially rhythmic in LL but exhibit phase shifts in the presence of the magnetic field oscillations, thereby suggesting that they can entrain to magnetic fields like wild type flies. Neither genotype demonstrates entrainment in DD to magnetic fields. These results suggest the existenceof a CRY-independent but light-dependent magnetosensitivity that can entrain circadian rhythms at magnetic field strengths much higher than those produced by the earth's magnetism. The mechanism is unlikely to involve magnetism induced heating of the flies, because heating should be produced in both DD and LL. This mechanism would differ from those previously demonstrated for detection of low strength magnetic fields, because these required functional CRY protein (i.e., they were not observed in the cry^b mutant). The existence of the magnetosensor that detects these higher strength magnetic fields is not known but would obviously be of great interest. It is possible that the cry^b protein, which cannot bind flavin because of a missense mutation affecting flavin binding.[3]Nevertheless, retains some capacity to detect light. This hypothesis could be

tested in the *cry*^{o}mutants' flies, in which the CRY gene is completely knocked out. Alternatively, some other protein (perhaps an iron binding protein) is involved.

It is possible that the magnetic field induces a temperature increase in the fly body that serves as a Zeitgeber. However, temperatures effects areunlikelyto be a Zeitgeber during the experiments for several reason. First, the results of the LD, DD, and LL experiments, shown in table 9, argue that magnetism-induced temperature changes as a possible zeitgeber are unlikely because the effects of magnetism require light, while conversion of the magnetic field energy to temperature should not. In addition, theoretically, the maximum energy that could become thermal energy is calculated to be in order of microjoules. That small value shows that temperature is unlikely to be a cue for entrainment during the experiments.

Another study would be to have the actual photoreceptor protein as a sample instead of *Drosophila melanogaster*. The effect on the fly's circadian rhythm would be not assayed. Therefore, that project could be deduced as complementary to ours. An ultrafast laser would be better in this research instead of a monochromator. A Femtosecond pump-probe technique could be used to investigate the vibrational motions joined to the electronic transitions. The absorption could be then studied. This could allow one to calculate magnetic field effects on photoreceptor protein activation and deactivation. In addition, the time of the reaction could be precisely deduced in order to investigate the amount of light absorbed by the protein.

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VITA

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