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Towards an Action Spectrum for Photoentrainment of the *Chlamydomonas ReinhardtII* Circadian Clock

Christa Gaskill
Western Kentucky University

TOWARDS AN ACTION SPECTRUM FOR PHOTOENTRAINMENT OF THE *CHLAMYDOMONAS REINHARDTII* CIRCADIAN CLOCK

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Christa Gaskill

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TOWARDS AN ACTION SPECTRUM FOR PHOTOENTRAINMENT OF THE

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Date Recommended 12/8/08

Sigrid Jacobshagen
Director of Thesis

Cheryl D. Davis

Louis A. Sherman

Dean, Graduate Studies and Research Date

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Directed by: Sigrid Jacobshagen, Claire A. Rinehart, and Cheryl Davis

Department of Biology Western Kentucky University

Chlamydomonas reinhardtii is an important model organism used to study circadian clock components. The circadian clock is the molecular mechanism by which nearly every organism measures time. Timekeeping is largely based on clock gene feedback loops where protein products repress transcription of their own genes. This central oscillator mechanism is robust and free running but adjusts to external stimuli such as light and temperature through input pathways. The output pathway carries signals that regulate activities for the appropriate time of day. An example of an activity controlled by the circadian clock in *Chlamydomonas* is its phototactic behavior.

A suggestion for the identity of the photoreceptors involved in the input pathways can be obtained from an action spectrum, which plots the strength of entraining ability against the visible spectrum of light. To this end, circadian rhythm data are collected using an automated phototaxis machine and the shift in phase upon a light pulse is analyzed using a new algorithm. In this study, the phototaxis machine and its analysis algorithm were characterized and optimized together with general growth conditions for *Chlamydomonas*. An apparatus for administering light pulses was also characterized.

Once this was accomplished, data were collected for a phase response curve of *Chlamydomonas* to white light. A phase response curve shows the extent of phase shift

versus the time during a circadian cycle the light pulse is given. The phase response curve suggests that the optimal time for an entraining light pulse in a wild-type strain of *Chlamydomonas* grown in cycles of alternating 12 h light and 12 h dark is LD19, or 7 hours into the dark phase. This is the time at which the organism is most sensitive to an entraining white light pulse. It is therefore also the optimal time for giving narrow wavelength light pulses in order to generate an action spectrum.

CHAPTER I

INTRODUCTION

The circadian clock is the molecular mechanism by which nearly every organism measures time (Merrow et al. 2005). Many of the details of molecular timekeeping are not known, but in general it is based on clock gene feedback loops in which protein products repress transcription of their own genes (Van Gelder et al. 2003, Dunlap 1999). This central oscillator mechanism is robust and free running but adjusts to external stimuli such as light and temperature through the input pathway (Harmer et al. 2001). The output pathway carries signals that regulate activities for the appropriate time of day (Devlin 2002).

The central purpose of a circadian clock is to maintain a stable phase relationship to important external time cues, often called zeitgebers. Most species have an endogenous period of 22-25 h, which is then entrained to the 24 h solar cycle (DeCoursey 2001). It was shown to be evolutionarily adaptive that locomotor activity and a myriad of cellular processes can be prepared for and carried out at the appropriate time of day (DeCoursey et al. 1997, Ouyang et al. 1998). A central oscillator is essential for triggering behavioral events for organisms that live in environments where time cues are rare or unavailable (Riccio and Goldman 2000). Also, continuous time consultation is necessary for species that perform celestial navigation and migration (Hoffman 1960). Research shows that laboratory strains of some species may develop internal clocks that free-run with a period outside of the range normally found in the wild or even lose clock function altogether as a result of the removal of environmental pressure (Costa et al. 1992).

Several factors of light in the natural setting could be responsible for entrainment: transitions of dawn and dusk, changes in the spectral quality and light intensity during the day, and the continuous presence of light (Pittendrigh and Minis 1964). Other factors such as temperature and nutrition may also reset the phase of the clock but are generally compensated for in terms of the clock's period (Bunning 1956). Organisms differ in the kinds of time cues that entrain the circadian clock, according to their adaptive needs.

There are two models to explain how circadian clocks are entrained to the environmental light/dark cycles (Aschoff 1960, Bruce 1960). The continuous model stresses the importance of gradual changes in the environment and the idea that light has a continuous action on the clock during entrainment in the wild. It is also based on the idea that the speed of the clock changes proportionally to the intensity of the light. This is a consequence of "Aschoff's rule" that increasing light intensity tends to shorten the period of rhythms in day-active organisms while lengthening the period in nocturnal organisms (Aschoff and Wever 1962). In contrast, the discrete model focuses on the importance of singular events as time-cues, including dusk and dawn. Approaches based on the latter model have had the greatest success for many species when conducting circadian clock experiments, as it has been shown that light pulses as short as 1 s can stably entrain the circadian clock in some organisms (DeCoursey 1989). In general, light stimuli with abrupt transitions do appear to mimic entrainment under natural conditions.

We have based our study of the input pathway photoreceptors in the unicellular green alga *Chlamydomonas reinhardtii* on the discrete model. It is unknown which photoreceptor adjusts the clock in *Chlamydomonas*. We first studied the ability of the organism to adjust the phase of its circadian clock depending on when a light pulse is

given during a circadian cycle. Based on similar studies we expected the cultures to show phase delays when light pulses were given during the early half of subjective night and phase advances during the later half of subjective night (Johnson 1990). Generally, no phase changes are observed when light pulses are given during the subjective day. The time at which a light pulse is given is sometimes expressed in circadian time, or "CT" which is based on the real period of the clock divided into 24 CT units rather than on an exact 24 hr cycle. The start of subjective night is conventionally designated "CT12" while the beginning of subjective day is "CT0." In order to investigate an organism's phase shifting abilities, a very common experimental design (Johnson 1990) —and the one we used in this study—is to entrain the organism to a 24h period of regular light/dark cycles of equal length. The organism is then allowed to "free-run" in darkness (DD) while experimental cultures are given pulses of light at different times. The phase of the experimental group can then be compared to the phase of the control and a "phase shift" value is generated for each light-pulsed time point. The result is a phase response curve (PRC), which is a graph that plots the extent of phase shift versus the time the light pulse was given. A PRC is also an important step in studying the effect of different qualities of light because it shows the time at which a light pulse causes the greatest phase shift. To generate an action spectrum, the full spectrum of light is tested in narrow wavelength increments to determine the intensity and duration needed for each wavelength to induce an arbitrary phase shift (Johnson 1990). Giving light pulses of varying wavelengths during the time of greatest phase shift ensures the highest possible sensitivity of the analysis. A comparison of the action spectrum to the absorption spectra of the various

photoreceptors found in an organism is helpful in narrowing down which ones are likely to be responsible for entraining the clock.

The photoreceptors reported for *Chlamydomonas* are rhodopsins, phototropins, and cryptochromes. Rhodopsins A and B, found in the *Chlamydomonas* eyespot, are responsible for phototaxis mediation (Sineshchekov et al. 2002, Schmidt et al. 2006). Phototaxis is the movement of an organism with respect to the direction of a light source. In *Chlamydomonas*, the extent of phototaxis is controlled by the circadian clock and therefore shows a circadian rhythm (Bruce 1970). Rhodopsin A absorbs maximally at 510 nm (or longer) and saturates at high intensity while rhodopsin B absorbs maximally at 470 nm and saturates at low intensity. *Chlamydomonas* also contains a chlamyrhodopsin, whose function is unknown except that it is not involved in phototaxis (Fuhrmann et al. 2001).

One phototropin gene is found in the *Chlamydomonas* genome and the protein has been localized to the flagella and the membranes (Huang et al. 2002 and Huang et al. 2004). It has many functions in higher plants with the action spectrum generally showing a major peak at 450 nm and two minor peaks at 425 nm and 470 nm (Briggs and Christie 2002). Phototropin is known to be important in the blue-light mediated steps in the sexual life cycle of *Chlamydomonas* (Huang and Beck 2003) but its action in the circadian clock is unknown.

Cryptochromes are blue-light photoreceptors that entrain the circadian clock to the daily rhythms of light and dark in both the fruit fly *Drosophila* and the plant *Arabidopsis* (Somers et al. 1998, Stanewsky et al. 1998). In some cases, cryptochrome is thought to work in cooperation with or as an intermediate of phytochrome, a red light

absorbing photoreceptor in plants (Spalding and Folta 2005). There are many processes that have been reported to be controlled by red light in *Chlamydomonas* (Oldenhof et al. 2006). However, no red light photoreceptor has been found in the genome of *Chlamydomonas*, including phytochrome (Mittag et. al. 2005). In mammals, cryptochromes were demonstrated to be involved in the central oscillator rather than the input pathway (Cashmore 2003).

Interestingly, an analysis of the *Chlamydomonas* genome has revealed the presence of an animal-like cryptochrome (Mittag et al. 2005) in addition to the previously demonstrated plant-like cryptochrome (Small et al. 1995, Stanewsky et al. 1995). This is the first example of an organism with both types, which evolved independently from DNA photolyases (Cashmore et al. 1999). An eventual goal of this study is to establish whether both, one, or none of the cryptochromes are involved in photoentrainment. This information will be valuable in determining aspects of early circadian clock evolution.

Chlamydomonas is an important model organism used to study the evolution of circadian clock components as well as many cellular processes such as chloroplast function, flagella development and function, and metabolism (Mittag et al. 2005, Harris 2001, Werner 2002). It is also a model for some diseases that affect humans. Its easy and inexpensive maintenance, availability of mutant strains, and its haploid genome make this organism an ideal model (Harris 1989). In addition, much progress has been made in sequencing its genome (http://www.biology.duke.edu/chlamy_genome/cgp.html). The entrained phase and period of its clock can be observed by automated measurement of the extent of phototaxis (Bruce 1970). Measurement of locomotor activity like phototaxis is a common way to indirectly assess the central oscillator as most organisms show a

rhythm in activity (Bovet and Oertli 1974, Pittendrigh 1981). It is this property that we made use of in our study on the input pathway photoreceptor(s).

Some effort has already been made to identify the photoreceptor(s) responsible for entrainment of the circadian clock in *Chlamydomonas.* Carl Johnson and Takao Kondo have produced action spectra for resetting the circadian clock of a cell wall-less strain (CW15) in dim light or dark conditions, but there is conflicting evidence as to whether *Chlamydomonas* is entrained by blue light (Kondo et al. 1991, Johnson et al. 1992). The authors reported that light entrainment by cells in constant dim light is mediated through photosynthesis. Cells in constant darkness are only entrained by green and red light and this effect is not mediated through photosynthesis. These results suggest that blue-light photoreceptors like cryptochromes do not play a role in clock entrainment in *Chlamydomonas*. However, when Johnson and Kondo used the wild-type strain CC125 of *Chlamydomonas*, blue light was effective in entraining the circadian clock (personal communication). There were several design flaws in the production of these action spectra, however, which are most likely the reason for the conflicting results. The major flaw in the experimental setup by Johnson and Kondo was most likely that the circadian rhythm of phototaxis, which they used as their indicator of the extent of phase shift, was monitored in dim background light. The cultures therefore went from dark conditions to dim light conditions upon placement into the phototaxis machine. As the authors already demonstrated, this placement by itself can cause a phase shift depending on when it occurs during a circadian cycle. Therefore, what the authors most likely measured was a combination of phase shifts caused by the light pulse and the placement into the phototaxis machine.

The main purpose of this study was to lay the groundwork for a reevaluation of the action spectrum for photoentrainment of the circadian clock in *Chlamydomonas* by improving the experimental design. In particular, optimization experiments using a wild-type strain of *Chlamydomonas* were performed and monitoring of its circadian rhythm of phototaxis was done without background light. In this context, a phototaxis machine was constructed and characterized together with an algorithm for its data analysis. In addition, a light pulse setup was built and characterized and the growth conditions were optimized. In the end, data on the extent of phase shift upon white light pulses at various times during a circadian cycle were obtained and evaluated.

CHAPTER II

MATERIALS AND METHODS

Chlamydomonas strains and growth conditions

Chlamydomonas reinhardtii wild-type strain CC124 from the *Chlamydomonas* Center strain collection was used in this study. Cells were kept axenically on agar slants of YA medium (Sueoka 1960). All experiments were performed with liquid cultures grown photoautotrophically in 0.3 HSM (Sueoka 1960). For experiments designed to test the newly constructed phototaxis machine, cells were inoculated from slants into either 250 ml medium in a 1 L Erlenmeyer flask or into 1 L medium in a 2.8 L Fernbach flask. These cultures were grown on a shaker at 150 rpm under a 12 hour light/12 hour dark cycle with a light intensity of 63.9 μmol photons m^{-2} sec^{-1}. The room temperature was variable but in most cases 20-24^{o}C.

For experiments to determine phase shifts upon light pulses, liquid stock cultures in 0.3 HSM were grown on the shaker under the same conditions as mentioned above and used to inoculate 1L bottles of 0.3 HSM at 10^4 cells/mL. These experimental cultures were grown in a temperature controlled incubator at 18-22°C under a 12 hr light/12 hr dark cycle with aeration from an aquarium pump. The intensity during the light phase was 92.35 μmol photons m^{-2} sec^{-1} from both the front and the behind. A cardboard and aluminum foil construction was initially used to light-proof the incubator. When this arrangement was shown to interfere with the proper temperature control of the incubator, the gasket doors were painted black.

Administration of light pulses

Cultures were grown in the incubator until late log phase (10^6 cells/mL) under strict 12h L/12 h D cycles such that the dark cycle coincided with the workday. Cultures were therefore exposed to at least 4 synchronizing light/dark cycles under these new conditions before they were subjected to light pulses. Just before the start of the dark cycle, 3-3.5 mL cultures were aliquoted into 35mm Petri dishes. Petri dishes were labeled with the time and intensity of the light pulse to be given and deposited into a dark box. Since the cultures must be handled in total darkness from that time on, it was important to develop a regular method to keep track of them. The method used was to line up stacks of 7 cultures along the back left hand side of the dark box, with #7 (the least intense light) on the bottom. After the light pulse was given, each culture was placed back into the dark box along the back right hand side starting with the highest intensity.

Cultures were subjected to light pulses of graded intensities from an Oriel 150 W solar simulator (Spectra-Physics, Stratford) at various time points of their dark phase. During a light pulse, the collimated light beam passes through a dichroic cold mirror (Newport Corporation, Irvine), which transmits infra red and UV light while reflecting light in the visible range (420-630 nm) to the cultures. A series of seven beamsplitters are supposed to reflect 70% of the light beam unto a culture while 30% is transmitted to the next beamsplitter (Melles Griot Optics Group, Rochester). Therefore, light intensity should be reduced by a factor of 3.3 for each successive culture (Figure 1). However, our measurements indicated that the beamsplitters only reduced the light by a factor of 2.6 (Table 3). The light is diffused with 30° diffusers (Edmund Optics, Barrington) before

reaching the culture for even application (Figure 4). The pulses lasted for 30 minutes, after which the cultures were placed back into the dark box. At the end of the dark period all cultures—including the controls that did not receive a light pulse—were placed into the phototaxis machine. This step was also performed in total darkness.

Light Intensity and Evenness of Light Field during Pulse

Photo paper was cut and placed in each slot to assess the effectiveness of the 30^{o} diffuser in spreading the collimated light beam evenly. Some experimentation with neutral density filters and length of exposure was necessary to produce a clear picture for each slot.

The LI-250 light meter (LI-COR, Lincoln) gave a reading of 3, 271 μmol photons m^{-2} sec^{-1} directly before the entrance to the beamsplitter corridor. Because the sensor of the LI-250 light meter is too large to fit into the slot for the petri dish under each diffuser, a different method of measuring the light intensity that directly reached the cultures had to be used. For this purpose, a USB2000 spectrophotometer (Ocean Optics, Dunedin) was attached to a R400-7-SR probe. The probe was modified with a homemade ½ reflector from a small pelican flashlight mounted in Makins clay using part of a 15 W light bulb envelope as a diffuser, glued with epoxy. The excitation "light source" end of the spectrophotometer was covered. The OOIBase 32 operating software allowed us to set the total light or another slot as the 100% reference. Each slot was then measured as a percentage of this reference. The results were put into an excel file and only the results in the range transmitted by the cold mirror were averaged together. Then actual fluence rates were calculated based on the starting fluence rate that entered the beamsplitter

corridor. Because of the sensitivity of this instrument, different neutral density filters and exposure times were needed to adhere to the instructions for using the software.

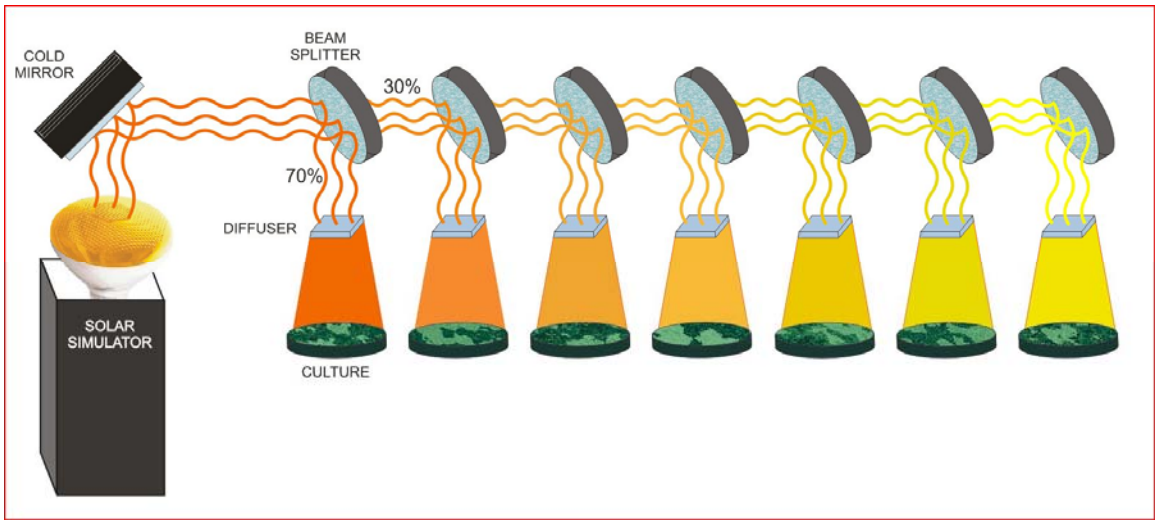


Figure 1. Light pulse set-up. The visible portion of a collimated light beam from a solar simulator is reflected by a cold mirror. Seventy percent of the light transmitted to each successive beamsplitter is reflected onto a culture through a 30^{o} diffuser. Thus, the light intensity decreases by a factor of 3.3 for each successive culture.

Phototaxis data collection

The recently constructed phototaxis machine measures relative phototaxis in hourly cycles by passing a 3mm diameter light beam through each culture and detecting light transmittance with a photosensor (TSL257, TAOS, Plano). The test light bulb is an LED with a maximum at 507 nm and a full width at half maximum of 30 nm (RL5-A7032, Super Bright LEDs, Inc., St. Louis). This range has been shown to be specific for eliciting phototaxis mainly through excitation of the Rhodopsin A and B photoreceptors (Sineshchekov et al. 2002). As the individual cells swim into the light path, less light is transmitted to the photosensor.

In our experiments, cultures were always shaken for 2 minutes before each test light cycle. White background light from (RL5-W45-360,Super Bright LEDs, Inc., St. Louis) LEDs was used for the remaining 45 minutes in preliminary experiments to characterize the phototaxis machine but not in the final light pulse experiments. There is one test LED under each of 60 cultures while the background lights are at the intersections above cultures. Each culture placement is referred to as a "channel." For the light pulse experiments, one or two 0.15 neutral density light filters are present in channels where needed to bring the 3 mm test light beam into the range detectable by the photosensor located above the culture.

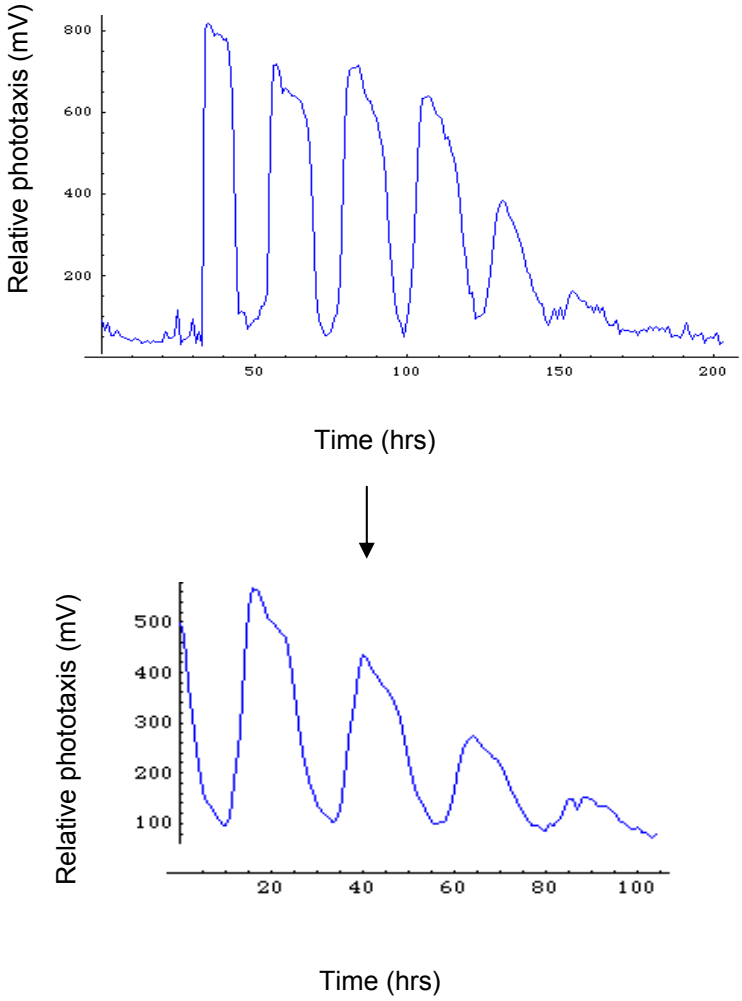
The system uses a custom software package designed in LabView specifically for this study. It allows the user to specify the total cycle time, time under the test light ("Data Sampling Time" or "minutes" when averaged together), time between measurements during the test light, and whether or not to use background light during the remainder of the cycle. The test light cycle for our experiments was 15 minutes though

any length of test light cycle can be chosen. The software also allows the user to choose the duration of shaking and to decide whether the cultures will be shaken immediately before the test light, immediately after the test light, or both. The system holds a total of sixty cultures in 35 mm petri dishes separated into two light-proof boxes stacked on top of a variable speed shaker (Eberbach Co., Ann Arbor). The shaker speed can be manually adjusted to minimize spillage of the cultures. A water bath (Fischer, St. Louis) was connected to both boxes to keep the internal temperature close to 18°C. Software created in LabView recorded the temperature every minute separately from phototaxis data.

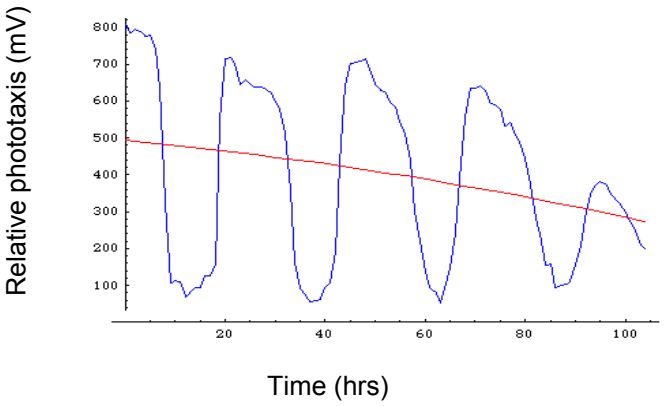
Data Analysis

Phase and Period data were generated using an algorithm developed by Dr. Bruce Kessler. The algorithm produces a model of the raw data through these steps:

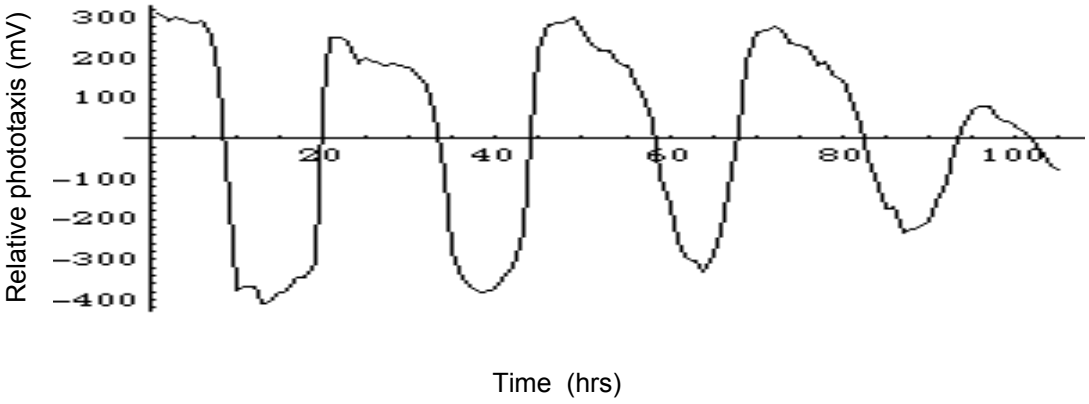
1. Experimenter determines valid data range.



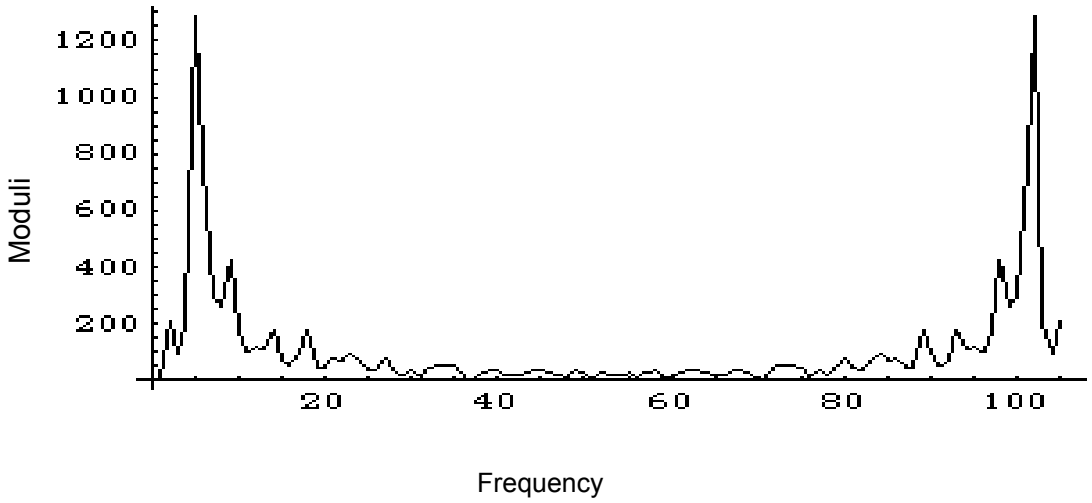
2. The algorithm finds a least squares quadratic fit to the data-i.e. of the form: $f(x) = ax^2 + bx + c$.



3. The algorithm subtracts this quadratic model from the data so that the data oscillate about the horizontal axis.



4. The algorithm takes the discrete Fourier transform of the adjusted data to determine the dominant frequency. This provides an "acceptable" window of periods for a least square fit to a sinusoid.



5. The algorithm solves for k, n, p, and r to minimize

$\sum$(adjusted $data_{(t)}$- g (t))^2 in order to find a least squares fit of the data to the model:

$$g(t) = ke^{-n} Cos\left[\frac{2\pi}{p}(t-n)\right]$$

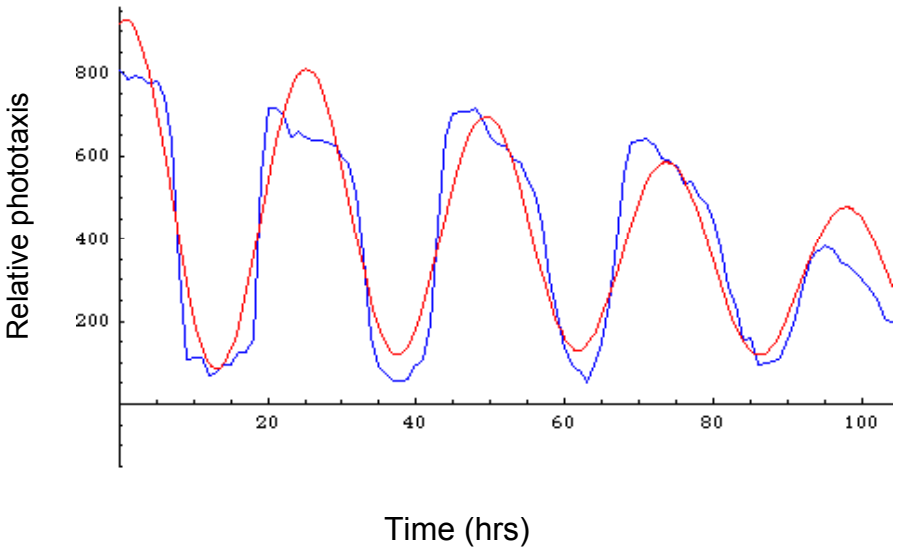
6. The algorithm minimizes a second time, solving for a, b, c, k, r, n, and p within the constraints set in step 5:

$$h(t) = at^2 + bt + c + kE^{-rt} Cos\left[\frac{2\pi}{p}(t - n)\right]$$

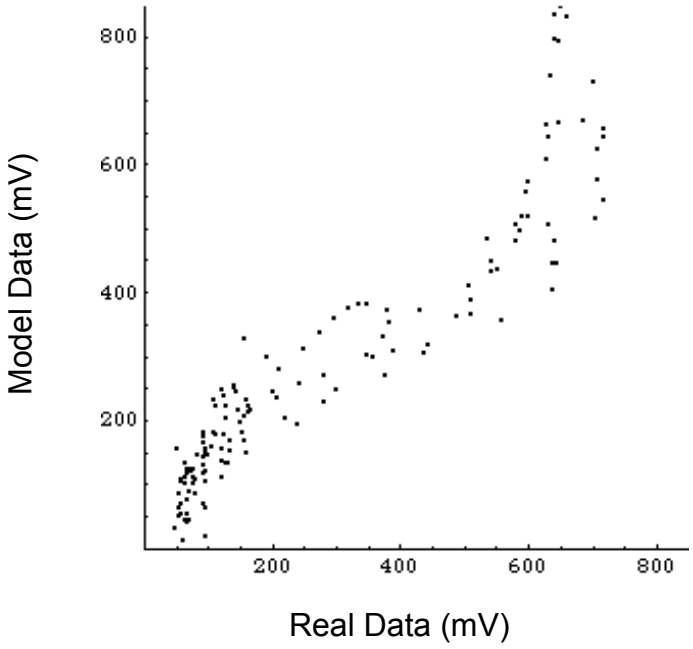
where:

- p is the period
- Phase is pn/2π

7. The algorithm adds the least squares quadratic fit back in and generates a graph of the best fit (red) to the raw data (blue), along with a correlation coefficient graph and value. Although the algorithm produces a correlation coefficient value, these graphs allow the experimenter to visually inspect how well the model fits the raw data.



Correlation Coefficient



In order to be consistent in the analysis of phase shifts upon light pulses depicted in Figure 6, the exact middle of the first trough of the control cultures was always used as the start and the end of the last clear peak as the end of the valid data range. These boundaries were then applied to all data from a particular experiment.

Parallel Analysis

Dr. Claire Rinehart modified and parallelized the MATHEMATICA algorithm to be compatible with the data format collected by the phototaxis machine. The original algorithm was designed for only one data point per hour, which was calculated by subtracting the final transmittance (after 15 minutes) from the initial transmittance detected during the test light cycle. The software on the machine is capable of collecting

data points at any time intervals but for the purposes of this project we limited the collection to every minute during the hourly 15-minute test light cycle. Each data point corresponds to a light intensity detected by the photosensor, which is expressed as voltage. The modifications allowed us to execute the algorithm for each culture at as many minute points as we liked at once (Table 1). For example, we could choose to look at the differences between phase and period data when taking minute 11 as our data point rather than minute 15. The parallel version of this program also allowed processing of all or a range of time values in order to derive average and variance data (Table 2) and was only available when using the Bioinformatics Center cluster. Otherwise, without parallel processing, only single time samples were chosen for analysis at a time. Minute 12 was always used in the analysis of the light pulse experiments.

Table 1. Verbose data generated by running the algorithm on the cluster. The data sampling time 6-16 for this particular experiment refers to the light transmission data collected at 5-15 minutes into the 15 minute test light cycle. Period, phase and correlation coefficient results are given for each sampling minute. The table shows only two of the sixty channels as an example. Each channel represents the data collected from an individual culture.

Channel	Data Sampling Time	Period (hrs)	Phase (CT units)	Corr Coeff
1	6	25.16020473	15.11274068	0.937306594
1	7	25.16158114	15.12896289	0.920411168
1	8	25.16319087	15.13367941	0.908323237
1	9	25.2044829	15.07056611	0.902404049
1	10	25.18492218	15.08482399	0.891007655
1	11	25.17441174	15.06591541	0.886744937
1	12	25.18458343	15.02089786	0.890855236
1	13	25.18053123	15.00498071	0.890734816
1	14	25.19930587	14.97320069	0.898603888
1	15	25.23205268	14.93945704	0.900898559
1	16	25.23961756	14.90622139	0.912025933
2	6	25.64575556	15.64157049	0.940094332
2	7	25.66346707	15.60262666	0.930298129
2	8	25.68111288	15.58561555	0.926644066
2	9	25.71350877	15.50760883	0.923258165
2	10	25.68965634	15.49787746	0.921238154
2	11	25.70987981	15.48047611	0.920171331
2	12	25.72266427	15.458679	0.922159595
2	13	25.71707568	15.48001612	0.922109745
2	14	25.73447251	15.47251307	0.926659194
2	15	25.79472538	15.42658328	0.929617448
2	16	25.77276493	15.44153334	0.932748022

Table 2. Summary data generated by the cluster. The cluster automatically averages the results from all the data sampling points for each channel and generates an overall standard deviation value for phase and period. The summary data in this table were generated from the individual data shown in Table 1.

Channel	Period Mean (hrs)	Period SD (hrs)	Phase Mean (CT units)	Phase SD (CT units)	Corr Coeff Mean
1	25.18953494	0.02706695	15.04013147	0.077236859	0.903574188
2	25.71318938	0.044009776	15.50864545	0.07014024	0.926818017

CHAPTER III

RESULTS

The purpose of this thesis was to lay the groundwork for a reevaluation of the action spectrum for photoentrainment of the circadian clock in *Chlamydomonas* by improving the experimental design. In order to accomplish this, we first needed to characterize the algorithm for data analysis. We then optimized the growing conditions of the algae, the application of light pulses, and the conditions in the phototaxis machine. Once this was done we were able to test the extent of phase shift to a white light pulse at various times during a circadian cycle in order to determine the optimal time for light pulses of narrow wavelength ranges.

Preventing Light Leakage Into the Growth Incubator

Since cultures for light pulse experiments were grown in an incubator with the dark phase occurring during the workday, it was important to ensure that complete darkness was maintained in the incubator while room lights were on. Only in this way could proper synchronization of the culture by the 12 h light/ 12 h dark cycle be achieved. In addition, complete darkness was necessary in the dark box that housed the aliquoted samples and in the room anytime the cultures were being transferred.
X-ray film was used to test for light leakage. Films were exposed in the dark box and in the incubator for three hours while the room lights were on. The first test showed significant light leakage into the incubator (results not shown). A subsequent test after application of a cardboard construction showed light clearly coming from underneath the incubator (results not shown). After aluminum foil was applied to the bottom gasket of

the door, tests showed only a small amount of light contamination (Figure 2). Phototaxis rhythm quality increased dramatically after these efforts were made to block outside light from entering the incubator. No light contamination was observed in the dark box (Figure 2).

Due to overheating of the incubator, the cardboard construction had to be removed. The gaskets on the incubator were painted black and judging from the x-ray film experiment this was sufficient to prevent light leakage (Figure 3).

Figure 2. X-ray film test for light leakage #1. Top: Control film that was not exposed. Middle: Film taken from the incubator with cardboard construction to prevent light leakage. Bottom: Film from the dark box. Exposure time was about 3 hours in each container with room lights on.

Figure 3. X-ray film test for light leakage #2. Top: Unexposed control film. Bottom: Film exposed in the incubator after gaskets were painted blank. Exposure time was 4 hours with room lights on.

Light Intensity and Evenness of Light Field during Pulse

Photo paper was cut and placed in each slot to assess the effectiveness of the 30^{o} diffuser in spreading the collimated light beam evenly. Some experimentation with neutral density filters and length of exposure was necessary to bring the light intensity into the range appropriate for the photo paper for each slot. Light application in general was quite even with a slight intensity increase in the center for the first 3 or 4 slots (Figure 4). Preliminary light meter measurements during the construction of the solar simulator set-up suggested that the 30^{o} diffusers were the best option (not shown).

Overall, each slot received on average 2.6 times less light than the previous slot (Table 3). This number was lower than the expected factor of 3.3 for each beamsplitter. In general, the variability in the measurements was approximately 3% (not shown). This number was taken from simply setting the transmittance of a slot as the reference and then taking the transmittance under that slot again to see how close the result was to 100%. The observed results were between 97% and 103%.

Figure 4. Evenness of the light field applied as pulses to the cultures. Photo paper in a 35mm diameter petri dish was exposed in each slot for a few seconds. The numbers refer to slots #1 through #7 in order of decreasing light intensity.

Table 3. Estimated fluence rate under each slot in the light pulse set-up. The fluence rate for slot 1 was calculated as a percentage of the total (3,271 µmol photons m^{-2} sec^{-1}), which was set as the 100% reference rate. Other slot fluence rates were then calculated by setting one slot as the 100% reference and taking the percentage of transmittance in subsequent slots. *Average of two independent measurements. The average decrease in light intensity was by a factor of 2.6 for each successive slot.

Total: 3,271 µmol photons m^{-2} sec^{-1}	Average Calculated Fluence Rate (µmol photons m^{-2} sec^{-1})	Standard Deviation	Factor Decrease
Slot 1	632.61		5.2
Slot 2	228.8*	14.63	2.7
Slot 3	74.97*	10.73	3.1
Slot 4	31.28		2.3
Slot 5	13.13*	0.48	2.4
Slot 6	5.02		2.6
Slot 7	2.18		2.3

Algorithm Robustness

Step one of the algorithm consists of choosing a range of data for analysis. Since the subjectivity of the experimenter in this decision might have an impact on the results obtained for period and phase, the impact of this decision was determined by comparing the analysis results of one data set when different ranges of data were chosen. In Table 4, differences in these results are shown when the starting time was varied by 10 hours and when the ending time was varied by 25 hours. These differences are representative of the flexibility in the range a user might choose even when adhering to the general guidelines of choosing the first trough as the start and the last clear trough as the end. There was no significant difference in either period or phase shift results.

Table 4. Algorithm robustness. Average phase shift and period values from the analysis of a set of data using three separate ranges. The basic data range is 25-110 hrs. The range shortened at the start includes hours 35-110 while the range shortened at the end includes hours 25-85. Treatment indicates the start time of a 30 minute light pulse. These values are from the highest light intensity given at that time point. The ranges are not statistically significantly different (analysis of variance with range and treatment factors, $\alpha = 0.05$)**.**

Range:	35-110		25-110		25-85	
Treatment	Period (hrs)	Phase Shift (hrs)	Period (hrs)	Phase Shift (hrs)	Period (hrs)	Phase Shift (hrs)
LD16	25.4475	1.6081	25.6845	1.393	25.5905	1.5063
LD17	25.4628	1.9242	25.7795	1.4815	25.7074	1.5865
LD18	25.7225	1.6618	25.9159	1.3931	25.7422	1.5873
LD19	25.6031	1.5797	25.58	1.6934	25.644	1.6694
LD20	25.2088	0.3672	25.6137	-0.0112	25.4939	0.2504
LD21	24.2243	-3.9304	24.2164	-3.3603	24.1099	-3.3015
LD22	24.531	-3.7233	24.642	-3.5542	24.4609	-3.3993

Channel Quality and Optimal Data Collection in the Phototaxis Machine

One phenomenon in the phototaxis machine was the tendency for some of the channels to exhibit a "cut-off" for the peak in the rhythm (Figure 5). This was attributed to the fact that light intensity was beyond the capacity of the photosensor, which has an upper limit of 5 mV. About 13 channels were consistently cut-off with several others only occasionally cut-off. Neutral density filters were placed in these channels to bring the transmitted light beam into the range of the photosensor. During the calibration of the channels, the maximum light received was standardized to 5 mV. However, since the light intensity that reaches the photosensor also depends on the cell density of the culture, "cut-offs" cannot always be avoided. Therefore, it was important to understand how these "cut-offs" affected the algorithm's ability to model the data and consequently the period and phase values that it produced. Analysis of variance and a Tukey multiple range test showed that while the period was unaffected by peak cut-off, there was a significant difference between the phases of cut-off and normal channels. Generally, the average correlation coefficients were lower in the cut-off channels indicating a reduced fit to the model.

Another important aspect of the phototaxis machine was determining the optimal time point(s) during the test light cycle used for measurement of phototaxis. The parallel processor was capable of producing a phase and period value plus standard deviation for each channel by averaging together the phase and period from a range of minutes during each test light cycle (Table 1 and Table 2). In other words, the algorithm was run for each minute separately and then the results were averaged together. The period and phase indicated in Table 5 are these values for all channels averaged together but separated

based on whether or not there was cut-off in the peaks. Average cut-off and normal period and phase results from minutes 12-16 were also compared to minutes 6-16 and no significant difference was found.

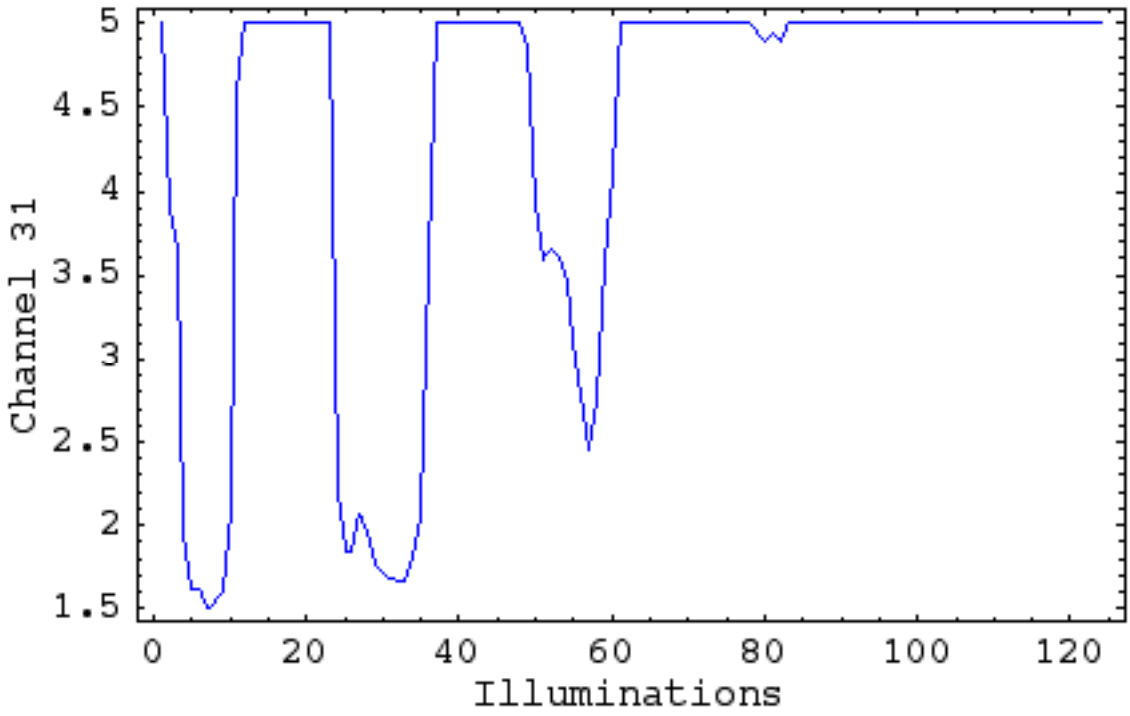


Figure 5. Peak "cut-off." A representative graph of peak cut-off. Channels like this required an extra neutral density light filter to bring the light intensity within a measurable range for the photosensor.

Table 5. Peak cut-off effect on the algorithm's calculations. Average period, phase, and correlation coefficient and their standard deviations were determined when running the algorithm to average the test light time points 12-16 for normal and cut-off channels. The number in parentheses indicates the number of channels that fell into each category. Results from one of three independent experiments with similar results are shown. All three experiments were used to determine that there was a significant difference in phase between normal and cutoff channels. There was no significant difference in period. This was done using analysis of variance and Tukey multiple range test (α =0.05).

	Period (hrs)	Phase (hrs)	Correlation Coefficient
Sample minutes 12-16			
Cut-off (13)	25.37±.98	6.13±.30	.93±.02
Normal (46)	24.72±.31	6.08±.55	.96±.02

The Impact of the Timing and Intensity of Light Pulses During Entrainment

A phase response curve is a graph in which the extent of phase shift is plotted versus the time a light pulse was given. In this graph, the time when the light pulse was applied is given in LD times. LD refers to the 12 h light/12 h dark cycle the cultures were exposed to. LD0 refers to the time the lights came on and LD12 to the time the lights went off. The phase response curves that were generated from all 7 intensities are depicted in Figure 6, with standard deviations in Table 6. These phase response curves follow the usual pattern of phase delays upon light pulses given during the first half of the dark period and phase advances during the second half. The transition between phase delays and phase advances in Figure 6 occurs between LD20 and LD21.5. At this region of the graph, the curves do not show a smooth pattern. Table 7 confirms the statistical difference between time points before and after the transition range, with some uncertainty during the transition phase between LD20.5 and LD21.

We initially assumed that this anomaly might be explained by differences in growth conditions. Most of the experiments (6 out of 8) were performed on cultures that were grown in the incubator with a cardboard construction to prevent light leakage (Figure 7) while the last few (2 out of 8) used cultures grown in the incubator without cardboard but with gaskets painted black (Figure 8). The unusually high standard deviation for LD20.5 (Table 6) seemed to support this assumption, since the two measurements the standard deviation is based upon are derived from experiments that differ in this parameter. However, statistical analysis shows no significant difference between experiments based on incubator conditions. It should be noted that the data available after painting were very limited.

When comparing the impact of varying light intensity on phase shifts, a pattern emerged in which increasing light intensity correlated with decreasing phase shift mean (Table 8). When considering how the light pulse timing and intensities impacted the period values the majority of these treatments gave no significant differences. Statistical differences were only observed between some of the extreme values in the timing experiments (Table 9) and the intensity experiments (Table 10) but because other extreme values showed no statistical difference these differences were deemed irrelevant.

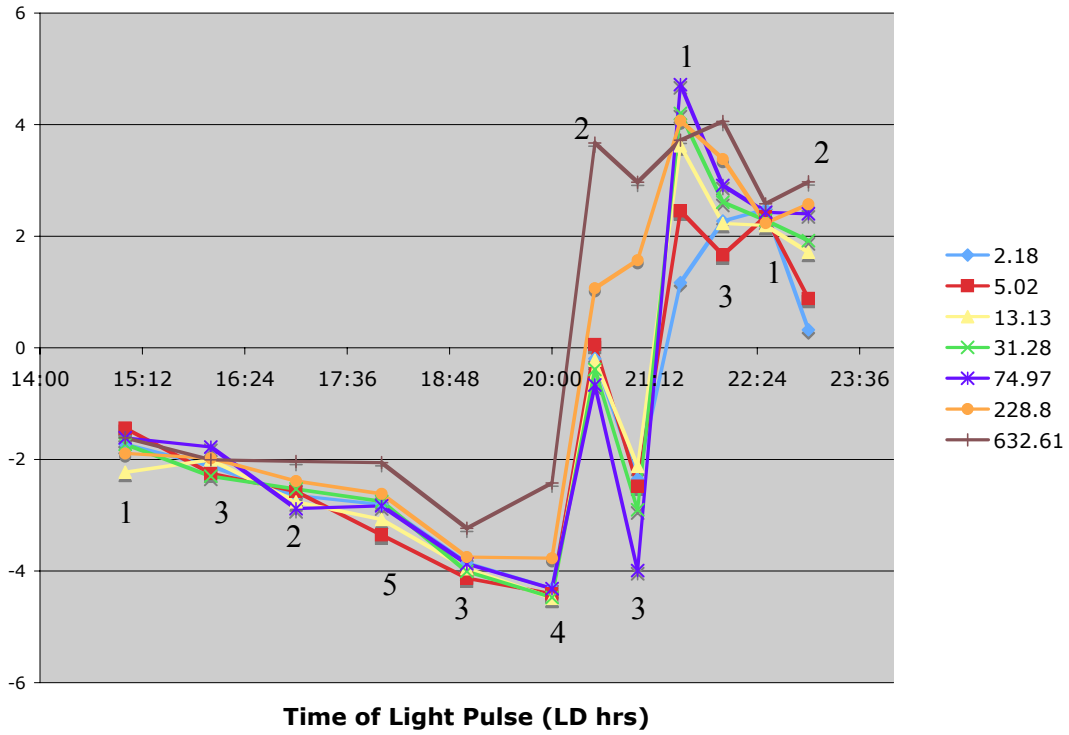


Figure 6. Phase response curve. This graph combined the phase shift results from all experiments before and after the incubator door gaskets were painted. Light pulse intensities corresponding to the various curves are shown on the right in units of µmol photons m^{-2} sec^{-1}. Numbers above and below indicate the number of independent experiments at that time point.

Table 6. Standard deviation of phase shifts in response to treatments varying in the timing and intensity of light pulses. This table demonstrates the variability in values illustrated in Figure 6. Slot number is listed across the top from highest to lowest light intensity. The actual light intensities for each slot can be found in Table 3. The numbers in parentheses refer to the number of measurements (n). Intensities are listed across the top in units of μmol photons m^{-2} sec^{-1}.

	Standard Deviations in Phase shift (hr)						
Pulse Intensity Time of Light Pulse	632.61	228.8	74.97	31.28	13.13	5.02	2.18
LD16 SD (3)	0.06	0.08	0.26	0.46	0.50	0.54	0.65
LD17 SD (2)	0.08	0.48	0.33	0.04	0.57	0.49	0.18
LD18 SD (5)	0.34	0.54	0.59	0.66	0.57	0.33	0.81
LD19 SD (3)	1.31	1.10	0.83	0.72	0.39	0.31	0.75
LD20 SD (4)	2.88	1.60	1.24	1.04	0.76	0.64	0.72
LD20.5 SD (2)	0.45	3.25	4.84	5.46	4.94	4.18	4.25
LD21 SD (3)	0.67	1.82	2.34	1.18	0.86	0.92	0.58
LD22 SD (3)	2.34	1.28	1.97	1.23	1.54	1.71	0.45
LD23 SD (2)	2.79	3.11	2.09	1.56	1.05	0.08	0.55

Table 7. Changes in phase shift means at varying light pulse times. LD time of light pulse is listed across the top from highest resulting phase shift mean to lowest. Statistical groupings based on the Tukey multiple range test are indicated horizontally where LD times with like letters are not significantly different from each other. This illustrates the transition break between LD21 and LD20.5.

20	19	18	17	16	15	21	20.5	22	23	22.5	21.5
a	a										
	b	b	b								
		c	c	c							
			d	d	d	d					
							e	e	e	e	
								f	f	f	f

Table 8. Changes in phase shift means at varying pulse intensities. Intensities (μmol photons m^{-2} sec^{-1}) are listed across the top from highest to lowest phase shift mean. Intensities with the same letter are not significantly different from each other.

2.18	5.02	31.28	13.13	74.97	228.8	632.61
a	a	a	a	a		
			b	b	b	
					c	c

Table 9. Changes in period means at varying times for light pulse treatments. LD times of light pulse are listed across the top from highest resulting period mean to lowest. Statistical groupings based on the Tukey multiple range test are indicated horizontally where LD times with like letters are not significantly different from each other.

15	21	22.5	16	21.5	20	18	23	19	17	22	20.5
a	a	a	a	a	a	a	a	a	a		
	b	b	b	b	b	b	b	b	b	b	b

Table 10. Statistical test for period means at varying pulse intensities. Intensities (μmol photons m^{-2} sec^{-1}) are listed across the top from highest to lowest period means. Intensities with the same letter are not significantly different from each other.

228.8	632.61	13.13	74.97	5.02	31.28	2.18
a	a	a	a	a	a	
	b	b	b	b	b	b

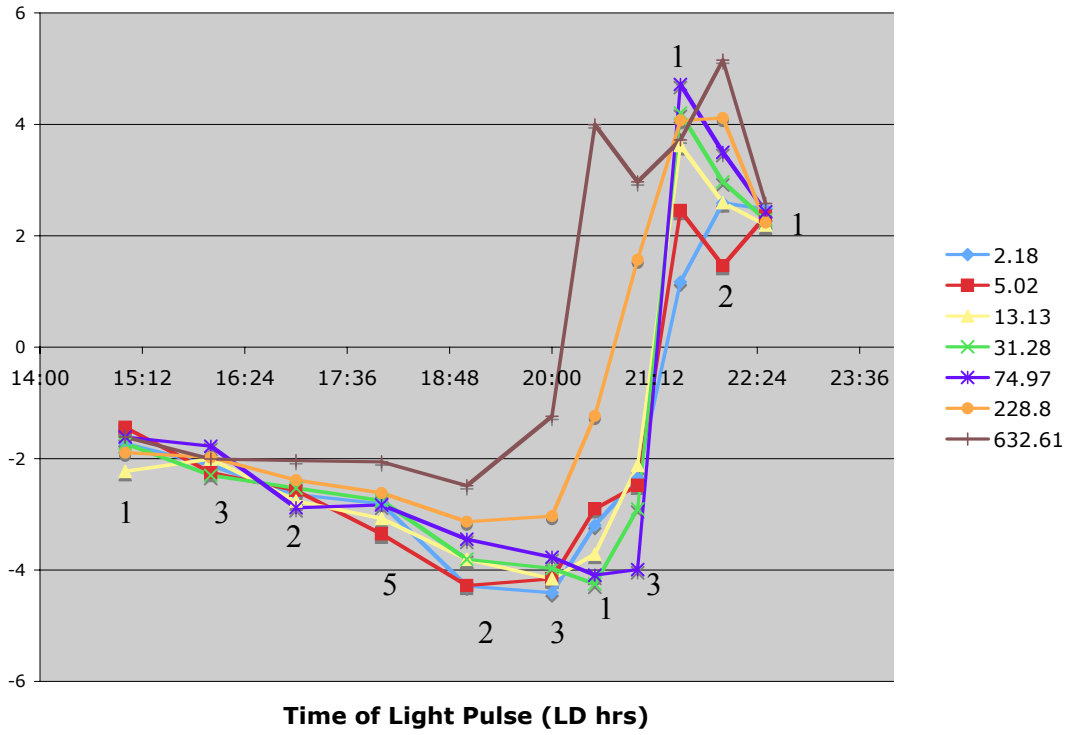


Figure 7. Phase response curve before painting. This graph includes all the data collected while the incubator was covered in cardboard and excludes the data collected after the incubator door gaskets were spray-painted black. Light pulse intensities corresponding to the various curves are shown on the right in units of µmol photons m^{-2} sec^{-1}. Numbers above and below indicate the number of measurements at that time point.

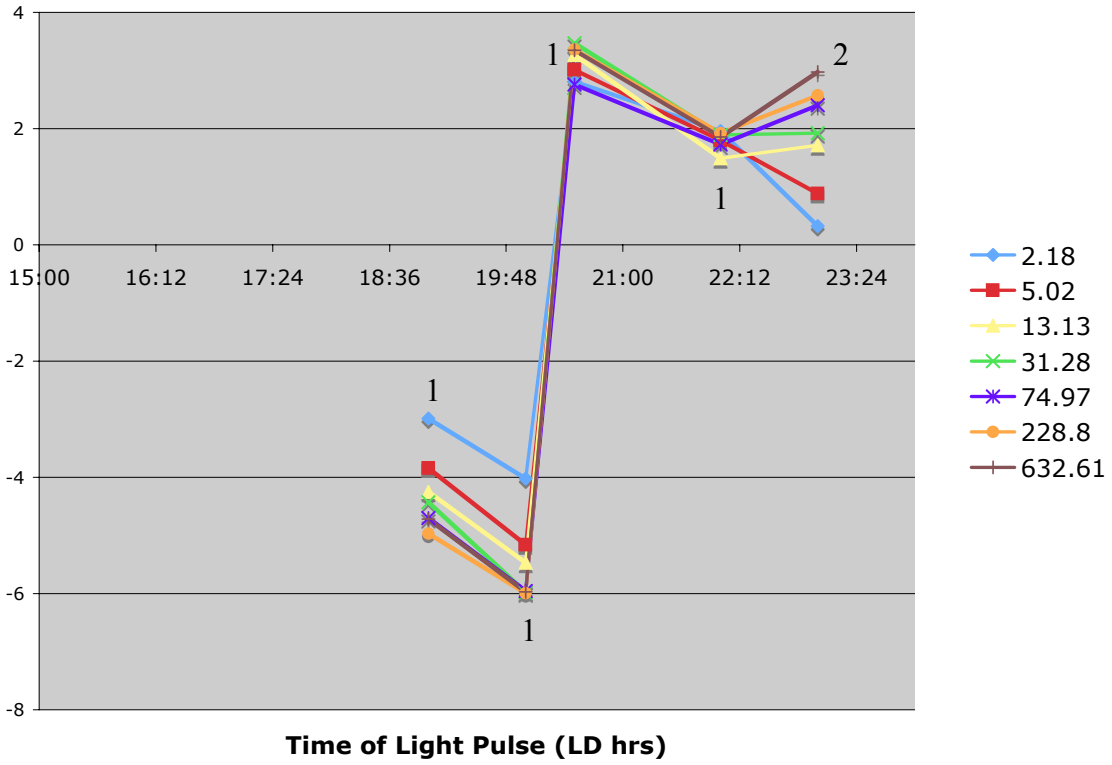


Figure 8. Phase response curve after incubator door painting. This graph represents the data collected after the incubator door gaskets were painted black. Light pulse intensities corresponding to the various curves are shown on the right in units of µmol photons m^{-2} sec^{-1}. Numbers above and below indicate the number of measurements at that time point.

CHAPTER IV

DISCUSSION

This study was designed to characterize a phototaxis machine and a data analysis algorithm in preparation for the production of an entrainment action spectrum. Because there were so many new components to our experimental tools, it was important to test and optimize every step from the gathering of data to its analysis.

The algorithm for analyzing phototaxis data was developed with the goal of taking all data points in the circadian rhythm into account when calculating period and phase. The results of the study demonstrate that any influence the experimenter may have when choosing the data range is probably insignificant (Table 4). The original program was successfully modified for use with the increased data collection capabilities of the phototaxis machine and for use on the Bioinformatics Center's parallel processing hardware when more minute sample processing is needed (Table 1).

In the phototaxis machine, the light filter in each channel that controls the amount of light reaching the photosensor was optimized to reduce the frequency of peak cut-off observations. For future experiments, we also defined the increase in variability in the period and phase when cut-off occurs (Table 5). We know that this phenomenon can cause significant differences in phase results.

The initial light pulse experiments were done using cultures grown in an incubator with a cardboard and aluminum foil coverings to prevent light leakage. While X-ray paper tests and subsequent experimental results indicated much improvement, looking in hindsight at the cardboard results, there is reason to suspect that some light leakage may

have occurred and affected the cultures (Figure 2). Painting the door gaskets black proved to be an even better method for preventing light leakage (Figure 3). However, there is currently no statistical basis for making any distinction between results obtained under these different growing conditions. Although precise sensitivity is unknown in algae, plants are extremely sensitive to light. Some of the very low fluence responses (VLFR) mediated by phytochrome require as little as 10^{-4}-10^{-6} μmol m^{-2} (Mandoli and Briggs 1984). It is difficult to estimate how the sensitivity of the X-ray film compares to photoreceptor sensitivity in *Chlamydomonas*, though the X-ray film is probably less sensitive.

The phase response curve graph (Figure 6) along with statistical analysis (Table 7) suggests that an optimal time for light pulse administration using different wavelengths is LD19. At this point, the largest phase delay was produced without any of the intensities leading to a cross over into phase advance. It is important to choose a time for light pulse administration that will reliably cause either a phase advance or delay. If a light pulse is given during the culture's transition point from delay to advance or "singularity," the rhythm becomes chaotic and the experimental results are difficult to interpret.

The amount of phase shift increases with statistical significance as the light intensity decreases. This is somewhat counter-intuitive. However, the higher intensity light pulse also resulted in an earlier singularity point, suggesting that the lower phase shift at the high intensity might be due to effects from the earlier singularity. It is known that as light pulse intensity is increased, the time until saturation is shorter but the size of the response at saturation is essentially independent of intensity (Johnson 1990). It is

possible that some effect of sensitization was caused by a small amount of dim light contamination in the incubator during the dark phase, which might cause an earlier singularity because of saturation. Light contamination also could have affected the overall synchronization status of the culture.

A few more experiments that vary the duration of the light pulse will be necessary in order to fully characterize the behavior of the cultures. Such experiments could also determine whether light saturation causes an earlier singularity. In addition, some control experiments are still needed such as testing whether placing the cultures into the phototaxis machine has a phase shifting effect. Once this is accomplished, using narrow ranges of visible light to elicit a phase shift can generate an action spectrum. This information will be crucial in answering the question of which photoreceptors are responsible for entraining the circadian clock in *Chlamydomonas*.

There is already some indication of which photoreceptors are involved, at least in some conditions. In their studies on CW15 in dim light, Johnson and coworkers used an experimental design in which the cultures were synchronized by a single 12 h dark period, followed by dim light. Both light pulses and phototaxis measurement were conducted during the dim light period with the dim light turned off during the actual light pulse application and test light cycle (Johnson et al. 1992). They found that the cultures required 6-hour light pulses to generate substantial phase shifts. The action spectrum shows peaks in blue light (450-480 nm) and red light (650-670 nm). Some effectiveness may also be seen at 600 nm. Comparison of absorption spectra suggested that the photosynthetic pigment chlorophyll is probably responsible for entrainment in this case.

Further experiments confirmed the ability of photosynthetic blockers to completely abolish the phase shifts caused by blue and red light.

In the studies on CW15 in darkness, cultures were grown in constant bright light and entrained by one 24 hour dark phase during which light pulses were given, followed by phototaxis measurement under constant dim light (Kondo et al. 1991). Subsequently, fluence response curves at CT18 were measured and an action spectrum was generated showing peaks in the red (660 nm) and green (520 nm (half the efficiency of 660 nm)). The authors report that no photoreceptor with absorption peaks at both of these wavelengths is known in *Chlamydomonas*. Both wavelengths caused identical PRCs as far as breakpoint and direction of the shift. They suggest that there could be two photoreceptors whose resetting pathways converge before acting on the central oscillator of the clock. The red absorbing receptor may be phytochrome, though far-red light failed to reverse the effect of red light in this experiment. Far-red reversibility is a hallmark of many of phytochrome's actions. Blocking photosynthesis had no effect on entrainment. The authors conclude that there may be two distinct and ecologically relevant systems at work-one for cells in darkness and one for cells in light.

There are many reasons why we believe that the action spectrum by Johnson and Kondo for cells in darkness must be repeated. The main problem in their experimental design is that they measured the rhythm of phototaxis with dim background light between test light cycles instead of darkness. In a preliminary experiment, the authors had tested different lengths of dark period before monitoring the circadian rhythm of phototaxis. They found that when the cultures were kept in the dark for more than 24 hours, a phase shift of up to 9 hours occurred simply due to placing the cultures into the phototaxis

machine. For the action spectrum that they generated, they put all cultures--including controls--into the phototaxis machine at 24 hours, which is just before the critical time when the dim light begins to have an effect because hour 24 is the beginning of subjective night for the controls.

Johnson and co-workers attribute the additional phase shift upon the light pulse (at CT18) to the light pulse creating a sensitization to the introduction of the dim light. However, since a light pulse given at CT18 causes a phase shift, the internal clock might already be well into its subjective night when the cultures are placed into the dim light of the phototaxis machine. Therefore, the investigators most likely measured a combination of the effect of the light pulse and the placement into the phototaxis machine. Unfortunately, the use of dim background light is necessary to ensure that the CW15 strain shows a good circadian rhythm of phototaxis. Our experimental conditions will preclude this necessity because we are using the wild-type strain CC124, which shows a good phototactic rhythm with no background light.

In addition to the problems caused by the dim background light during monitoring of phototaxis, the test light that the investigators used to elicit phototaxis was not ideal. They used a white penlight bulb for this purpose, which emits a beam with broad spectral quality. Our improved design features a LED test light in the range that is specific for phototaxis and is thought to have little effect on other photoreceptors of *Chlamydomonas*. Lastly, their use of a cell wall-less strain of *Chlamydomonas* also might have made a difference if the photoreceptors involved are associated with the plasma membrane and dependent on a properly formed cell wall. This is another potential problem that can be avoided by using a wild-type strain. The authors did find that blue light had an effect in

the wild type strain CC125 but it is unknown whether they measured phototaxis in dim light or darkness. Because the results are unpublished, it is unknown whether the strain or testing conditions might be responsible for the finding.

The authors acknowledged that their conditions were not ideal even though these conditions produced larger phase shifts. Though it is advantageous to use experimental conditions that produce the greatest phase shift when attaining an action spectrum, the sensitivity of the organism to light and the relatively little that is known about how it responds means that careful experimental design is necessary. An action spectrum generated under our improved conditions will help settle the question of whether blue light really entrains the circadian clock.

If we can reproduce clock entrainment upon blue light, then the testing of cryptochrome as a possible input pathway photoreceptor will be appropriate. In the future, we plan to use RNA interference strains for both cryptochromes to test the involvement of the photoreceptors in the input pathway of the circadian clock in this organism. Even if neither type of cryptochrome is involved in entrainment, one or both may still be part of the central oscillator.

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