Drosophila melanogaster Clock Gene Mutants Exhibit a Circadian Rhythm in Visual Contrast Response

Olivia Nippe

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University of York

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Abstract

Most organisms use a molecular timekeeping mechanism centered on the so-called “clock genes”, known to interact with one another in a 24-hour Transcriptional-Translational Feedback Loop (TTFL) to control circadian rhythms intracellularly. However, the discovery of circadian rhythmicity in the oxidation state of peroxiredoxins has suggested that an alternative metabolic oscillator may govern circadian rhythms independently of gene transcription. Although circadian rhythms have been documented in the morphology of the *Drosophila* visual system, much of the underlying physiology remains unclear. It was previously found that a circadian rhythm in the visual transduction amplitude of *Drosophila* persists in some “clock” gene mutants, indicating that the rhythm may persist independently of the TTFL.

In this study the highly sensitive Steady State Visually Evoked Potential (SSVEP) assay was used to assess the visual function of the TTFL mutants $Clk^{Jrk}st^{1}$ *and* per^{0} in order to determine whether a TTFL oscillator is driving oscillations in the visual contrast response of fruit flies, as well as dissect the contribution of individual neuron orders in the retina to the response. We have found that despite a complete loss of circadian rhythmicity in locomotor activity levels the $Clk^{Jrk}st^{1}$ mutant exhibits robust circadian rhythms in contrast sensitivity, with a recurring peak 4 hours after anticipated light onset in the photoreceptors, lamina, and medullary neurons. We conclude that *Drosophila* possess a circadian rhythm in contrast sensitivity that can operate independently of clock gene transcription, and thus is likely synchronized instead by a metabolic oscillator.

List of Contents

List of Figures

Acknowledgements

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Author's Declaration

I declare that all the work presented in this thesis is my own work, unless otherwise acknowledged in the text and figures.

Raw data for the w^{1118} visual responses was collected prior to start of this degree, however all analyses and collection of all other data was performed de novo for this thesis.

No part of this work has been submitted for a previous degree.

1. Introduction

1. 1. Overview

The visual system of *Drosophila melanogaster* has proven an excellent model of circadian rhythmicity in the past, with almost exclusive links to regulation by the transcriptional-translational feedback loop (TTFL) based molecular timekeeping mechanism. An alternative hypothesis however concerning a TTFL independent metabolic oscillator in conjunction with previous evidence of a circadian rhythm in visual transduction of a TTFL-impaired mutant indicates the need for further study of circadian rhythms in visual electrophysiology. This study aims to investigate whether a TTFL or metabolic oscillator likely controls the circadian rhythm in visual transduction by employing the highly sensitive SSVEP assay to measure rhythms in the visual response of TTFL-impaired mutants.

1. 2. What are circadian rhythms?

The term "circadian rhythm" refers to any process in an organism that undergoes just one complete cycle over the course of a 24-hour period, and that persists in the absence of environmental cues. It is widely believed that the purpose of such rhythms is to allow an organism to better adapt to an environment that is itself cyclical, with a 24-hour cycle of changing light and temperature levels (Sheeba et al., 1999; Yerushalmi and Green, 2009). A better understanding of the cyclic nature in which our physiology changes may prove to be of great importance, for example, in treating sleep disorders, such as those that present as a non-motor symptom of Parkinson's Disease, but that also appear in other contexts, including shift workers or people that are affected by jetlag (Jankovic, 2008; Sack et al., 2007). The organism *Drosophila melanogaster* is an excellent model for studying changes in rhythmicity. In addition to their short generation time, high fecundity and great genetic tractability, there has been extensive documentation of fruit flies exhibiting circadian rhythms (Konopka and Benzer, 1971; Pittendrigh, 1954). Furthermore, many components of the mammalian molecular timekeeping mechanism have homologs in *Drosophila* (Kloss et al., 1998; Panda et al., 2002; Rutila et al., 1998; Takumi et al., 1999).

1. 3. The Transcriptional-Translational Feedback Loop

The conventional hypothesis is that all circadian rhythms are based on the "clock genes", the set of genes that are known to interact with one another in a 24-hour transcriptional-translational feedback loop (TTFL) to control rhythms intracellularly (reviewed in Blau, 2001 and Edery, 2000 and summarised below) (Figure. 1). In this loop (in the case of *Drosophila melanogaster*), a heterodimer comprised of dCLOCK (dCLK) and CYCLE (CYC) activate the transcription of the two clock genes *period* (*per*) and *timeless* (*tim*), as well as other so-called Clock Controlled Genes (CCGs) at approximately midday in what is often referred to as the positive arm of the TTFL. While environmental light levels are high, the protein Cryptochrome (CRY) is activated, and targets TIM for degradation by the proteasome. As light levels decrease, TIM levels accumulate until they are sufficiently high to outcompete the kinase Double-time (DBT), which targets cytoplasmic PER for rapid degradation, for binding of PER. TIM and PER then form a stable heterodimer and translocate to the nucleus close to midnight. Here, one or both components of the PER:TIM complex inhibit dCLK:CYC, thus inhibiting their own transcription, as well as that of the CCGs. This forms the negative arm of the TTFL. PER:TIM is also thought to act indirectly via the nuclear receptor E75 to derepress its inhibition of dCLK:CYC (Kumar et al., 2014). In this way the PER:TIM dimer creates a delayed upswing in dCLK levels. PER and TIM are eventually degraded in the nucleus around dawn, relieving their inhibition of the dCLK:CYC complex, with the result that *per* and *tim* transcription is activated once more, but also that *dClk* expression is downregulated. The loop then recommences. This cycle takes 24 hours and results in circadian expression of its own components and of CCGs downstream of dCLK:CYC.

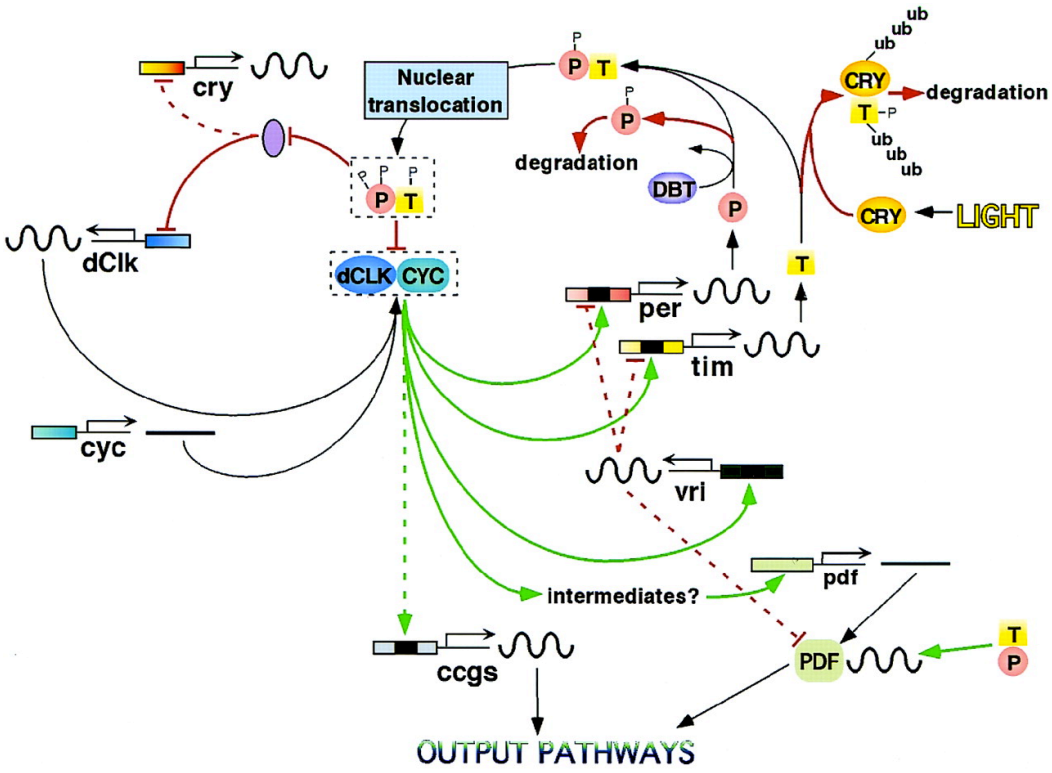

Figure 1: Model of circadian clock in *Drosophila melanogaster* showing photic input pathway (light; CRY) and two downstream effector pathways (ccgs, clock-controlled genes; PDF, pigment-dispersing factor) (Figure from Edery et al., 2000). During the late day/early night, the levels of PER (indicated by large P) and TIM (indicated by T) reach critical concentrations that favor dimerization, an event that stabilizes PER and stimulates the nuclear entry of the PER-TIM complex. The enhanced degradation of monomeric PER in the cytoplasm as a result of DBT-mediated phosphorylation events and the light-induced degradation of TIM (in the photoreceptors), contribute to a delay in the nuclear accumulation of PER and TIM. In the nucleus, PER, TIM, or both 1) interact with dCLK:CYC, blocking its ability to stimulate transcription of per, tim, vri, and possibly ccgs and 2) by a mechanism that is not clear, upregulate expression of dClk and cry. Not shown is the degradation of highly phosphorylated PER and TIM in the nucleus, which relieves the block on dCLK:CYC-mediated transcription and leads to the downregulation of dClk and cry expression. Green lines, pathways leading to upregulation; red lines, pathways leading to downregulation; dashed lines, uncertain pathways. Small black boxes indicate E-box elements; small P, phosphorylation; ub, ubiquitin.

1. 4. Circadian rhythms in the fruit fly visual system

The visual system of fruit flies (Figure. 2) in particular is recognized as possessing a number of processes that are controlled in a circadian manner. That is to say that these processes have been found to be rhythmically controlled by the molecular clock, independently of environmental cues such as light levels to which the organism can become entrained, known as zeitgebers. In the first optic neuropil, or lamina of *Drosophila melanogaster* the cross sectional axon area of the L1 and L2 large monopolar cells swell at the beginning of both the day and night under normal light: dark (LD) cycling, mirroring rhythms in locomotor activity levels, and the cross sectional area of the L1 cells continue to fluctuate significantly under constant conditions (Pyza and Meinertzhagen, 1999). In a congruent fashion, the L2 dendrite length is seen to lengthen at the beginning of the day. This structural plasticity in axon caliber persists in constant darkness and is altered or abolished in cry^{b} and per^{01} clock gene mutants respectively (Weber et al., 2009).

Such temporal changes in morphology and physiology under constant conditions are generally attributed to governance by the molecular clock in specific TTFL-expressing cells. In the visual system, the photoreceptors and lamina glia are thought to possess “peripheral clocks”, where cycling components of the TTFL have been visualized, for instance by staining methods, and so are thought themselves to express the TTFL in order to uphold visual circadian rhythms when in constant darkness (DD) (Cheng and Hardin, 1998; Ewer et al., 1992; Liu et al., 1988). The lamina, in contrast has not been shown to express the TTFL autonomously, as evidenced by work on *Drosophila melanogaster* and close relative *Musca domestica*. It instead receives circadian input from other cell groups, both in the visual system, and from the so called “master pacemaker” (1^{st} to 4^{th} small ventral lateral neurons in the accessory medulla, Figure. 3) in the brain whose arborisations extend into the optic lobe (Bałys and Pyza, 2001; Górska-Andrzejak et al., 2013; Pyza and Meinertzhagen, 2003). In the case of L1 and L2 axon caliber, the morning peak is proposed to be stimulated by paracrine release of the neuropeptide pigment-dispersing factor (PDF) from pacemaker cells onto the medullary terminals of the L1 and L2 cells, and is opposed by the action of the ion transport peptide (ITP) released from the 5^{th} s-LN_v to drive the evening peak (Damulewicz and Pyza, 2011). A bimodal rhythm in the abundance of presynaptic active zone

protein Bruchpilot (BRP) in the lamina possesses a morning peak dependent both on TTFL expression by the pacemaker and on direct photic input from the photoreceptors (Górska-Andrzejak et al., 2013). And so it has been seen that rhythms throughout the fruit fly's visual system rhythms are maintained both by the contribution of the molecular clock and by photic entrainment.

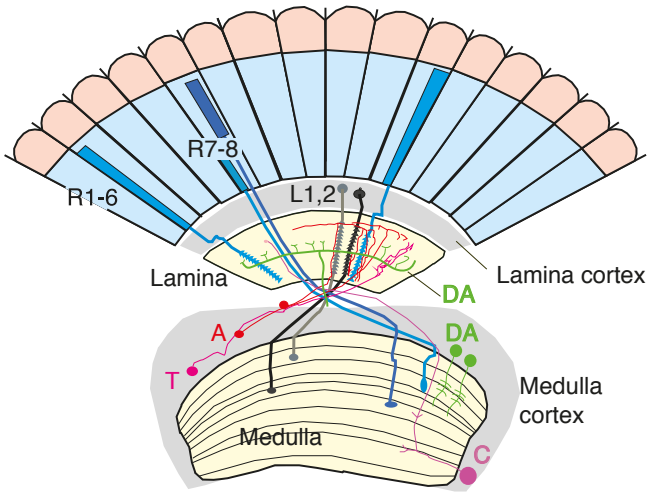

Figure 2: Diagram of the structure of the fly visual system (Afsari et al., 2014)**.** Shown are the photoreceptors (R1-8, of which R1-6 form synaptic connection with the lamina, while R7 and R8 connect to the transmedullary neurons), second order amacrine (A) and the lamina large monopolar cells (LMCs; L1 and L2), and the medulla neurons (C and T) that project to the lamina. Also shown are the dopaminergic neurons (DA) some projecting from the CNS to the lamina and others intrinsic to the medulla itself. For each category of neuron, only one or two representative cells are shown. (Afsari et al., 2014; Pecot et al., 2013).

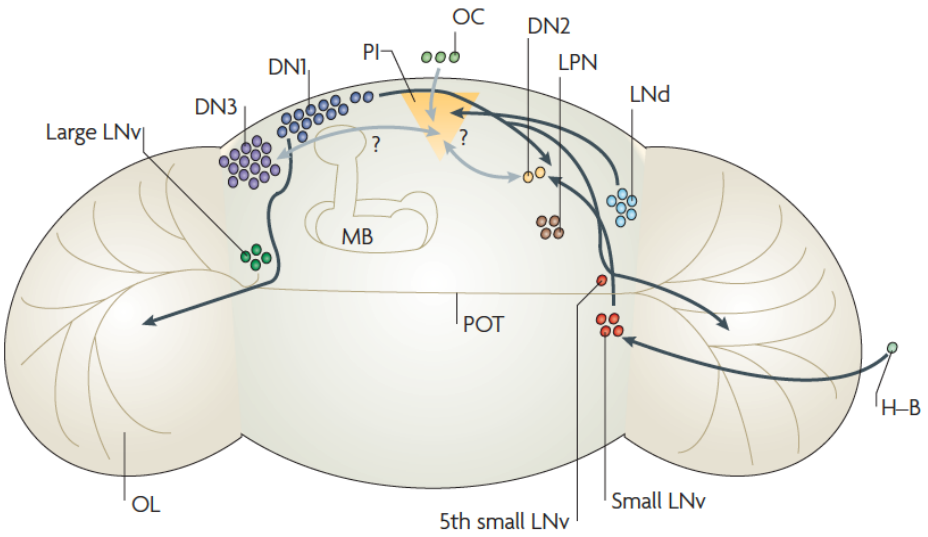

Figure 3: Anatomical circadian pathways in flies (Figure from Gerstner and Yin, 2010). In fruit flies (*Drosophila melanogaster*), various light-receiving cells are involved in functional neuroanatomical connections, such as those in the Hofbauer–Buchner (H–B) eyelets and ocelli (OC), or from the optic lobes (OL). These project to circadian pacemaker cells, the lateral neurons (LN), via the posterior optic tract (POT). LN subtypes include the large, small, and 5th small ventral LN (LNv), as well as the dorsal LN (LNd). Little is known about the functional connectivity between these pacemaker cells and other clock cells, such as the dorsal neurons (DN1, DN2 and DN3 subtypes) the lateral posterior neurons (LPN) or cells that are involved in sleep and memory formation, such as the pars intercerebralis (PI) and mushroom bodies (MB). DNs and LNs comprise the ~150 cells of the clock network in the fly brain (Gerstner and Yin, 2010).

1. 5. Visual electrophysiology of clock mutants: an unexpected rhythm

The electrophysiology of neurons in the fly visual system that underlie the morphological changes have been largely overlooked in the past. The few studies that have examined visual electrophysiology in *Drosophila* have employed electroretinography (ERG) in order to record the visual response amplitude from a trace generated by the pooled depolarization of retinal neurons (Belušic, 2011). A study by Stark describes a circadian rhythm in the sensitivity of the wild type *Drosophila* ERG which seemingly inexplicably persists in the previously termed "arrhythmic" *period* gene mutant strain per^{01}, and persists, or is at most only subtly altered in the short and long period mutants per^{S} and per^{L} respectively (Chen et al., 1992). This raises questions concerning the degree of regulation on certain circadian rhythms such as visual transduction by the molecular clock, indicating control instead by an oscillating factor outside of the TTFL.

The discovery of circadian rhythmicity in the oxidation state of peroxiredoxins both in red blood cells and *Drosophila* whole head homogenates has suggested that an alternative oscillator may govern circadian rhythms independently of gene transcription (O'Neill and Reddy, 2011). This hypothesis proposes that a more ubiquitous process such as metabolism may control certain rhythms as opposed to, or in addition to the clock genes that have previously been associated with circadian output (Figure. 4).

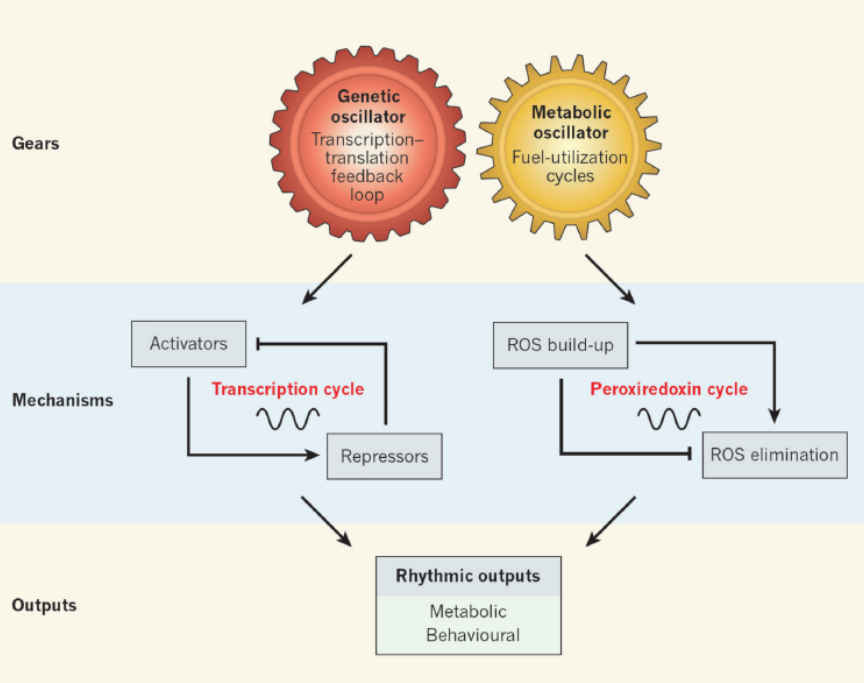

Figure 4: Coupling of genetic and metabolic clocks (Figure from Bass and Takahashi, 2011). Two types of circadian oscillator maintain synchrony between the light–dark environment and internal biochemical processes. These are genetic oscillators, which consist of a transcription–translation feedback loop, and - as two new studies show (O'Neill and Reddy, 2011; O'Neill et al., 2011)- metabolic oscillators, which are involved in fuel-utilization cycles and consist of the cycle of oxidation and reduction of peroxiredoxin enzymes. The two oscillator types are coupled, both driving rhythmic outputs (such as photosynthesis reaction cycles in plants and the feeding–fasting cycle in animals) in synchrony with Earth's rotation.

1. 6. What is controlling circadian rhythmicity in visual response amplitude?

In this study the highly sensitive Steady State Visually Evoked Potential (SSVEP) assay was used to measure the visual function of the *Drosophila* TTFL mutants $Clk^{Jrk}st^{1}$ *and* per^{01}. This technique has been shown to have a higher signal to noise ratio than the traditional flash electroretinogram approach due to the elimination of out of band noise prior to analysis. This assay also allows dissection of the contribution of individual neuron orders in the retina (the photoreceptors, lamina, and medulla) to the response, and has demonstrated clear functional homology between the visual responses of *Drosophila* and vertebrates (Afsari et al., 2014). The $Clk^{Jrk}st^{1}$ mutant is nocturnal under diurnal conditions and demonstrates abolished locomotor rhythmicity under constant conditions as a result of a premature stop codon in the C-terminal activation domain which prevents activation of d*Clk* expression by *Drosophila* C-terminal binding protein (dCtBP) (Allada et al., 1998). The per^{01} fly strain is null for the *period* gene with a lack of light anticipatory locomotor behavior under LD and completely abolished locomotor rhythms under DD (Allada et al., 1998; Konopka and Benzer, 1971). Both *dClk* and *per* are key components of the transcription-translation feedback loop (Blau, 2001), and the *per* gene has been shown both to regulate certain visual circadian rhythms such as in lamina dendrite morphology (Weber et al., 2009), and in other cases to be independent of visual circadian rhythms, such as in ERG sensitivity (Chen et al., 1992). The aim of this study was therefore to determine whether a TTFL oscillator is driving oscillations in the visual contrast response and response amplitude of fruit flies.

2. Materials and Methods

2. 1. Drosophila stocks

Stock vials of *Drosophila melanogaster* were raised and maintained on a yeast-sucrose-agar food medium (Carpenter, 1950). The per^{01} fly strain was kindly provided by Prof. Ralf Stanewsky (University College London). Clk^{Jrk} st^{1} flies (#24515) were obtained from the Bloomington Stock Centre (Indiana University). Clk^{Jrk} st^{1} carries a secondary mutation, st^{1}, which causes bright red eye colour due to being null for the brown eye pigment xanthommatin (Have et al., 1995). Due to the visual nature of the assays used in this study, response amplitude could have varied due to eye pigmentation, and as such, a control with identical eye color was required for each clock gene mutant strain. The st^{1} scarlet-eyed fly line (#605, Bloomington Stock Centre, Indiana University) was therefore used as the Clk^{Jrk} st^{1} control. *Canton-S* (*CS*) wild type (from laboratory stock) was crossed with iso^{4147}, with isogenic chromosomes 2A + 3A (Sharma et al., 2005) and was used as a control for the per^{01} strain. All flies were kept in 25°C room with a 12hr: 12hr light: dark schedule, and were allowed to lay eggs on the food. After 2 days, adult flies were removed from the vials. Male flies were collected within ~18 hours of eclosion.

2. 2. Photoentrainment for visual response analysis

Once collected, flies were photoentrained in 12hr: 12hr light:dark (LD) cycles for 6 days in a constant temperature room (25°C). LD6 measurements were taken on the 6th day of photoentrainment to show any diurnal rhythms. Circadian rhythms were determined by measuring the flies' responses on the 1st or 2nd day of constant conditions following photoentrainment (termed DD1 or DD2 respectively). Following 6 days of photoentrainment flies were transferred to constant darkness (DD) and constant temperature (again 25°C) for 16-24 hours (DD1 readings) or 40-48 hours (DD2 readings) before being prepared for visual response analysis. Constant conditions were maintained in order to prove that a rhythm was truly circadian; as such a rhythm should persist in the absence of environmental cues or zeitgebers.

2. 3. Preparation for SSVEP and ERG

Flies were trapped in a shortened Gilson pipette tip using a pooter, so that only the head and fore legs were exposed (Fig. 5), and then secured with a small amount of nail polish (Creative Nail Design), avoiding the eyes and without flooding the tip. In the case of flies that were currently experiencing subjective night (ZT12, 16, and 20) or were being kept under constant conditions for circadian time (CT) readings, this preparation process was performed under a red filtered light in order to minimize interference with the flies' current light cycle (Chiu et al., 2010). Each fly was allowed to recover in the dark for a period of ~20 minutes prior to visual response measurement.

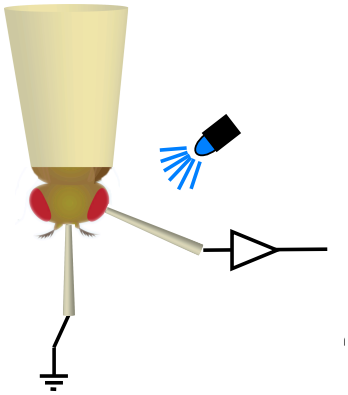

Figure 5: Trapping *Drosophila* and recording a visual response (Figure from Afsari et al., 2014). The fly is trapped in a shortened Gilson pipette tip and exposed to a blue LED flash. Glass recording and reference electrodes are rested on the eye and mouthparts of the fly, respectively, and the output from the recording electrode is amplified and digitized (see text).

2. 4. SSVEP and ERG

Visual responses of the flies were obtained via the SSVEP assay (steady state visual evoked potential), the full details of which, including those of SSVEP analysis and the stimuli used, are described in (Afsari et al., 2014). Essentially, upon having been given time to recover from being secured in a pipette tip, each fly was placed (in its pipette) in a ring chamber, and a micromanipulator was used to place a glass drawn contact reference electrode filled with simple *Drosophila* saline (Heisenberg, 1971) on the mouthparts of the fly to prevent any feeding movements during recording, while a second saline filled recording electrode was placed on the surface of the eye, gently so as not to damage it. Again, in the case of flies that were currently experiencing subjective night or were under constant conditions, the electrode placement was performed using a dissection microscope with a red filtered light. The output from the second electrode was amplified as described in (Hindle et al., 2013), and recorded using the DasyLAB program (Measurement Computing Corporation, 2012). DasyLAB was also used to confirm the quality and stability of each fly's photic responses by examining the response upon manually toggling the stimulation LED. Flies were then exposed to a randomized sequence of flickering blue LED light, in which a either a single square wave with mean flicker illumination of 12 Hz, known as the "probe", or a wave formed by the sum of two square waves of mean frequencies 12 and 15 Hz, the "mask", were delivered. The resultant responses were then analyzed using a Fourier transform (Bracewell, 1978) to extract the response amplitude of the individual frequency components. Flies that were unable to produce a robust photic response as determined by ERG trace analysis were omitted from the data set.

2. 5. SSVEP statistical analysis

Flies that produced a robust ERG trace and high quality contact with both electrodes in the ERG assay were further analysed by SSVEP. Changes in the sensitivity of the visual response were calculated from the estimated R_{max} parameter (Figure. 6). Statistical significance of the effect of Zeitgeber/circadian time on R_{max} was determined by a univariate ANOVA ($p<0.05$) of the data acquired from the SSVEP assay and were Bonferroni corrected. Levels of significance are denoted in APA style by letters above data points, where all points denoted with a lower case “a” are found to be significantly different from the point denoted upper case “A”, likewise with “b” and “B” and so on. For clarity between upper and lower case, the letter C has been omitted, and D used instead.

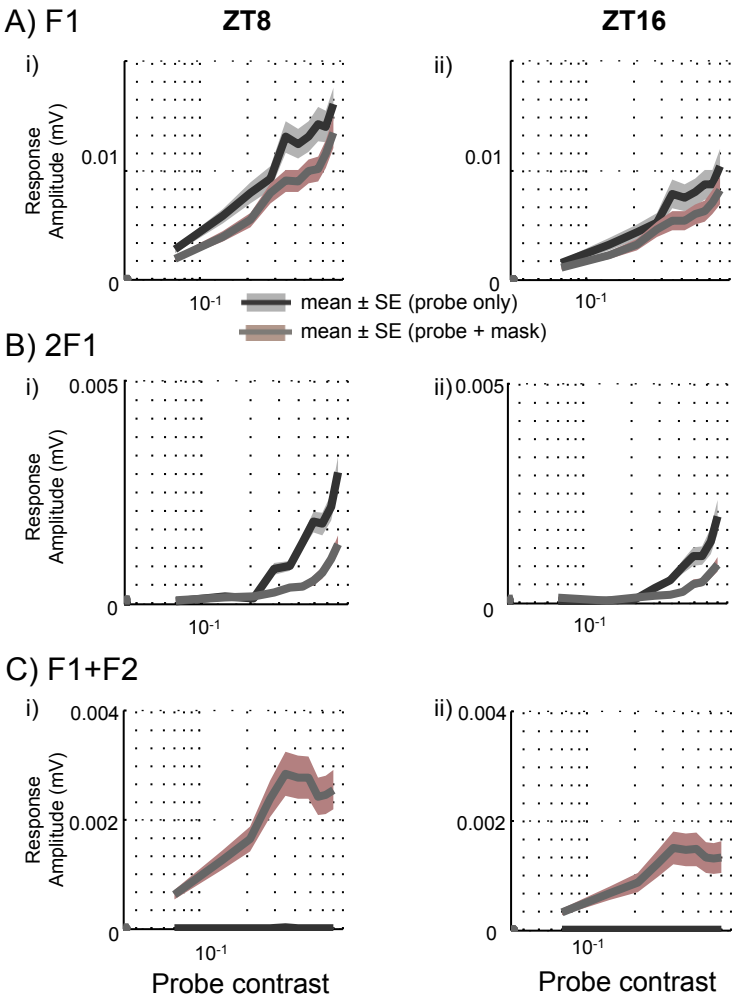

Figure 6: Flies measured at different times of day may present with a variable visual response phenotype at 3 different orders of neurons when assessed by the SSVEP assay. Male *st*[1] flies that had been photoentrained for 6 days in 12:12 light: dark cycles were exposed to a pre-programmed, randomized sequence of flickering blue light at ZT4 and ZT16 (n = 19). The separated photoreceptor response (A), represented by the first harmonic (F1) frequency, lamina response (B), represented by the second harmonic (2F1), and medulla response (C), represented by the intermodular term (F1+ F2), are here plotted versus probe contrast. The dark line indicates the mean response (grey shaded area as ± 1 standard error) to the presentation of a single frequency of flicker ("probe"). The solid grey line indicates the mean response, (pink shaded area as ± 1 standard error) to presentation of the probe plus a 30% mask stimulus as the second frequency. The results demonstrate that *Drosophila* may present with a different visual response phenotype at different times of day, and validates the use of the SSVEP assay to visualize the temporal effects at multiple neuron orders in the visual system. In this study both the masked and unmasked maximum response amplitude, or $R_{max,}$ for each component is determined from these contrast response function curves generated by the SSVEP assay and are used to represent the strength of visual transduction.

2. 6. Assaying circadian rhythms in locomotor activity

Male flies were collected within ~18 hours of eclosion and anaesthetized with CO_2. Males were used rather than females, whose egg laying activity can affect an accurate measurement of rhythms in locomotor activity (Chiu et al., 2010). All males were transferred with a fine paintbrush to individual 5mm diameter glass tubes plugged at one end with 5% sucrose set agar (Fluka Analytical, 1%). A small amount of cotton wool was placed into the other end using a pair of forceps. The set agar end was finally covered with a plastic tube cap perforated with small holes to allow ventilation (Trikinetics, Waltham, MA, USA). Tubes were set on their sides until all flies had awoken and then loaded into DAM2 activity monitors (Trikinetics, Waltham, MA, USA). The activity monitors measured the frequency with which each fly tripped a beam of infrared light that crossed the center of the tube. The DAM monitors were kept in a light and temperature controlled incubator (25°C) and flies were photoentrained in 12hr: 12hr lights on: lights off (LD) cycles for ~3.5 days, and then kept in constant darkness (DD) for a minimum of 7 more days. Locomotor activity was collected in bins of 2 minutes.

The data collected by the DAM software was used to generate actograms for each individual fly using the ImageJ program (Abramoff et al., 2004) with the ActogramJ plugin (Schmid et al., 2011). A Lomb-Scargle periodogram analysis was performed using the ActogramJ plugin in order to determine which flies exhibited true circadian rhythmicity and the length of their freerunning period (Refinetti et al., 2007; Schmid et al., 2011). Flies were defined as rhythmic if the results of Lomb-Scargle analysis met the following criteria; (1) Exactly one distinct peak was deemed significant with a probability of $p>0.05$, and (2) The peak was in the range of 21-27 hours. Representative group profiles of locomotor activity rhythm for each genotype, both after 3 days of 12:12 hour light: dark cycles and after 3 days of constant darkness, were generated by averaging the activity levels of all flies in bins of 30 minutes.

3. Results

3. 1. Locomotor activity rhythms of TTFL mutants

The purpose of the locomotor experiment was to confirm the previously documented behavioural phenotypes of both WT and TTFL mutant *Drosophila* (the latter of which are associated with arrhythmicity under constant DD conditions (Allada et al., 1998; Konopka and Benzer, 1971)). To this end, flies were photoentrained in activity monitors in 12:12 light: dark cycles for 3 days under constant temperature (25°C), before undergoing 7 further days of constant darkness (DD) also at constant temperature. The locomotor activity levels of both control and mutant flies were measured using an activity monitor and were averaged into 30-minute bins (Figure. 7). A Lomb-Scargle periodogram analysis was performed on the resultant actogram plots of all flies assayed, and was used to determine power of rhythmicity and free-running period length (for full details of this assay and criteria for determinable locomotor circadian rhythmicity see Materials and Methods).

The scarlet-eyed control flies st^1 exhibit 2 clear peaks in locomotor activity levels under LD conditions, which center around light on- and offset or ZT0 and ZT12 (Figure. 7A). There is a strong appearance of anticipation of the morning "M" peak towards the end of the dark period, evidenced by a gradual increase in average activity, however there is no obvious indication of similar anticipation of the lights-off transition. 68.6% of the st^1 flies were found to be DD rhythmic by Lomb-Scargle analysis, and those that were rhythmic had an average free-running period length of 24.4 hours (Figure. 7B). The definition of the M and E peaks is diminished under DD conditions, however decreasing activity levels at CT12 and low activity levels throughout the subjective night visually demonstrate some retention of the circadian rhythm.
The homozygous molecular clock mutant $Clk^{Jrk}st^1$ has no M or E peaks in locomotor activity, but does have a strong nocturnal rhythm under LD conditions (Figure. 7C). It has relatively constant activity levels during the day, which then increase by approximately 60% 30 minutes after light offset and remain fairly constant until ZT0. The sharp differences in activity that occur at

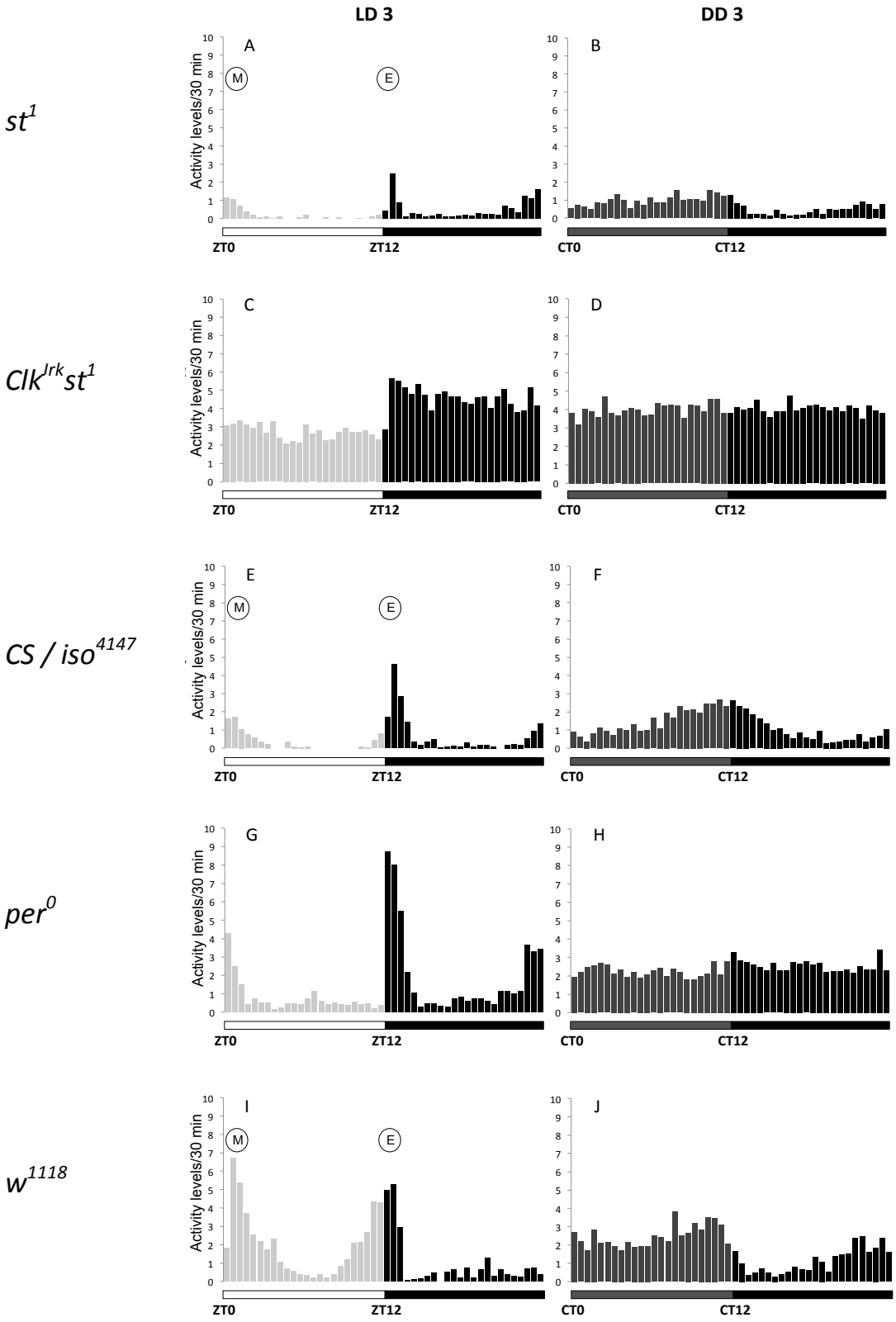

Figure 7: Daily rhythms in locomotor activity of control (*st*^{1}, *CS / iso*^{4147}, and *w*^{1118}) flies (A and B, E and F, I and J respectively) and of clock gene mutant (*Clk*^{Jrk} *st*^{1} *and per*^{0}) flies (C and D, G and H respectively). Male flies of each genotype (n≥18) were photoentrained for 3 days in a 12:12 hour LD (light: dark) cycle before being subject to 3 days in DD (constant darkness). Graphs in the left column

show the average activity levels on LD3 while graphs in the right column show average activity levels on DD3. All vertical bars represent the average activity levels (in arbitrary units) recorded in 30-minute bins during the light or anticipated light period (light and dark grey) and the dark or anticipated dark period (black). The horizontal bars below LD graphs represent when the lights were on or off (white or black, respectively). ZT0 and ZT12 represent the Zeitgeber time in hours, or the start and end of the defined photoperiod respectively. For DD graphs; CT0 and CT12 represent the circadian time in hours, or the start and end of the anticipated light period in constant dark conditions (denoted by the grey bar). In panels A and E the letters M and E denote the morning and evening peaks in activity respectively.

the two light transitions indicate a lack of light anticipatory behavior in the $Clk^{Jrk}st^{1}$ mutant. It is worth noting that the lower levels of activity observed in the mutant between ZT0 and ZT12 are only relatively so, as even then the mutant is more active than the control fly. Under constant darkness, only 16.6% of $Clk^{Jrk}st^{1}$ flies were found to be rhythmic and of these the average DD period length was slightly lengthened in comparison to the control, at 25.2 hours (Figure. 7D).

The isogenic crossed wild type fly, *CS / iso*^{4147}, exhibits a similar rhythm in activity levels to that of the scarlet-eyed control (Figure. 7E). There is a slight increase in activity levels around peak times (ZT0 and ZT12) in comparison to st^{1}, which reveals that in addition to anticipation of the M peak, there is some anticipation of the E peak prior to the lights-off transition. This is also true of the wild type fly's behavior under constant darkness, where the evening peak is still distinguishable from the otherwise dampened rhythm, and some anticipation of light onset is revealed at the end of the subjective night (Figure. 7F). The power of free running rhythmicity is stronger than that of the scarlet-eyed control, with 92.3% of the *CS x iso*^{4147} flies found to be DD rhythmic by Lomb-Scargle analysis. Those that were rhythmic had an averaged DD period length of 23.7 hours.

The second TTFL mutant, per^{0}, retains a strong ability to photoentrain, with both M and E peaks under LD conditions in spite of disruption to the molecular clock. Anticipation of the evening peak however is lost, with a very sharp increase in activity immediately following the lights-off transition (Figure. 7G). There appears to be some anticipation of the morning peak. Generally, activity levels remain low, but at peak times, the mutant's activity levels are seen to be almost 50% higher than those detected in the wild type control. Under constant darkness the per^{0} fly is mostly arrhythmic, with only 18.8% of the per^{0} flies possessing a detectable rhythm and of these the average DD period length was a shortened 22.2 hours (Figure. 7H).

Another eye colour defective wild type fly, the white-eyed w^{1118}, was also assayed. This strain possessed clear circadian locomotor rhythmicity. Under LD conditions there were clear M and E peaks in activity levels at each light transition, with obvious anticipation of said transitions on both occasions (Figure 7I). The white-eyed fly appeared to take a shorter or even indeterminable "siesta" in the middle of the day. This siesta is a behavior

usually typical of WT *Drosophila* (Hall, 2003) and is observed in the two other control flies assayed here, but in neither of the TTFL mutants. Under constant DD conditions, w^{1118} exhibited strong free-running rhythmicity, analogous to those of the WT and scarlet-eyed control, with diminished distinctiveness of M and E peaks, but retained anticipation of light transitions (Figure. 7J). 100% of the w^{1118} flies were found to be DD rhythmic by Lomb-Scargle analysis with an averaged DD period length of 24.03 hours.

This experiment was successful in confirming the expected behavioural phenotypes of each fly strain. All wild type flies were found to exhibit biphasic rhythms in locomotor activity levels under LD conditions, with peaks at ZT0 and ZT12 and some anticipation of light transitions, although the length of the typical midday siesta was variable, as were the overall levels of activity (lower, in particular, in the case of st^1). All WT fly lines had a majority of flies deemed to retain a truly circadian rhythm, and had an average period length of approximately 24 hours.

The results of the $Clk^{Jrk}st^1$ mutant mirror those previously described (Allada et al., 1998; Kim et al., 2002). The homozygous mutants used in this study had no anticipation of light transitions and nocturnal preferences under LD conditions, with complete abolition of rhythmicity under DD conditions in all but 16.6% of those assayed.

The second TTFL mutant, per^0, also demonstrates its expected locomotor phenotype. The mutant has little evidence of anticipatory behavior of light transitions under LD, with an otherwise normal biphasic diurnal rhythm and is completely arrhythmic in all but 18.8% of flies assayed when under DD conditions.

From these results we can conclude that the WT and control flies used in this study are capable of demonstrating typical circadian rhythmicity, and that the TTFL mutants are representative of their respective phenotypes also, with little to no indication of functioning circadian rhythmicity under constant conditions as determined by the locomotor assay.

3. 2. Circadian rhythmicity in the visual contrast sensitivity of $Clk^{Jrk}st^{1}$

We know that *Drosophila* with disrupted expression of key molecular clock genes such as *dClk* and *per* can demonstrate arrhythmicity under constant conditions, as evidenced by the results of the locomotor assay, and therefore that the molecular clock drives certain manifestations of circadian function. It has also been shown that the same mutant strains can hold certain circadian rhythms under free-running conditions (Edgar et al., 2012), indicating that some circadian output is not governed exclusively by the molecular clock. A rhythm once found in the visual sensitivity of a *period* mutant fly under constant conditions, measured using the less sensitive ERG assay (Chen et al., 1992) now suggests, in light of the notion of alternative oscillators, that a rhythm in the contrast sensitivity of *Drosophila melanogaster* is another circadian rhythm that can function independently of the molecular clock. The hypothesis for this study therefore is that *Drosophila* possess a circadian rhythm in contrast sensitivity under DD free-running conditions that can persist independently of a functional molecular clock. The visual contrast sensitivity of control and TTFL mutant flies was measured by way of the SSVEP assay, in which each fly was exposed to a pre-programmed and randomized sequence of flickering blue LED light. This was performed on flies that had been photoentrained in 12:12 LD for ~5/6 days immediately following eclosion (LD6 readings), and on those that were also kept for a further 24 or 48 hours under constant conditions (DD1 or DD2 readings).

The scarlet-eyed wild type fly st^{1} demonstrated a highly significant relationship between time-of-day and mean R_{max} in the photoreceptors and medullary neurons ($p>0.05$) (Figure. 8A and 8C). Under LD conditions a multiple comparison of means found there to be a significant increase in contrast sensitivity in the photoreceptors and medulla between ZT0 and ZT8 ($p>0.05$). By ZT8 mean R_{max} increased by 100% in the photoreceptors, and by 200% in the medulla relative to the level of contrast sensitivity at light onset. Statistically speaking, no significant comparisons were found under DD conditions or indeed at any time in the lamina (Figure. 8A-C), however in all 3 orders of neurons the graphs appear to show a rhythm that repeats approximately 16 hours, with less distinct peaks occurring at ZT20, CT16 on DD1, and CT8 on DD2. The results of a Fast Fourier Transform (FFT, not shown) support this observation by indicating that while not sufficiently significant to be highlighted

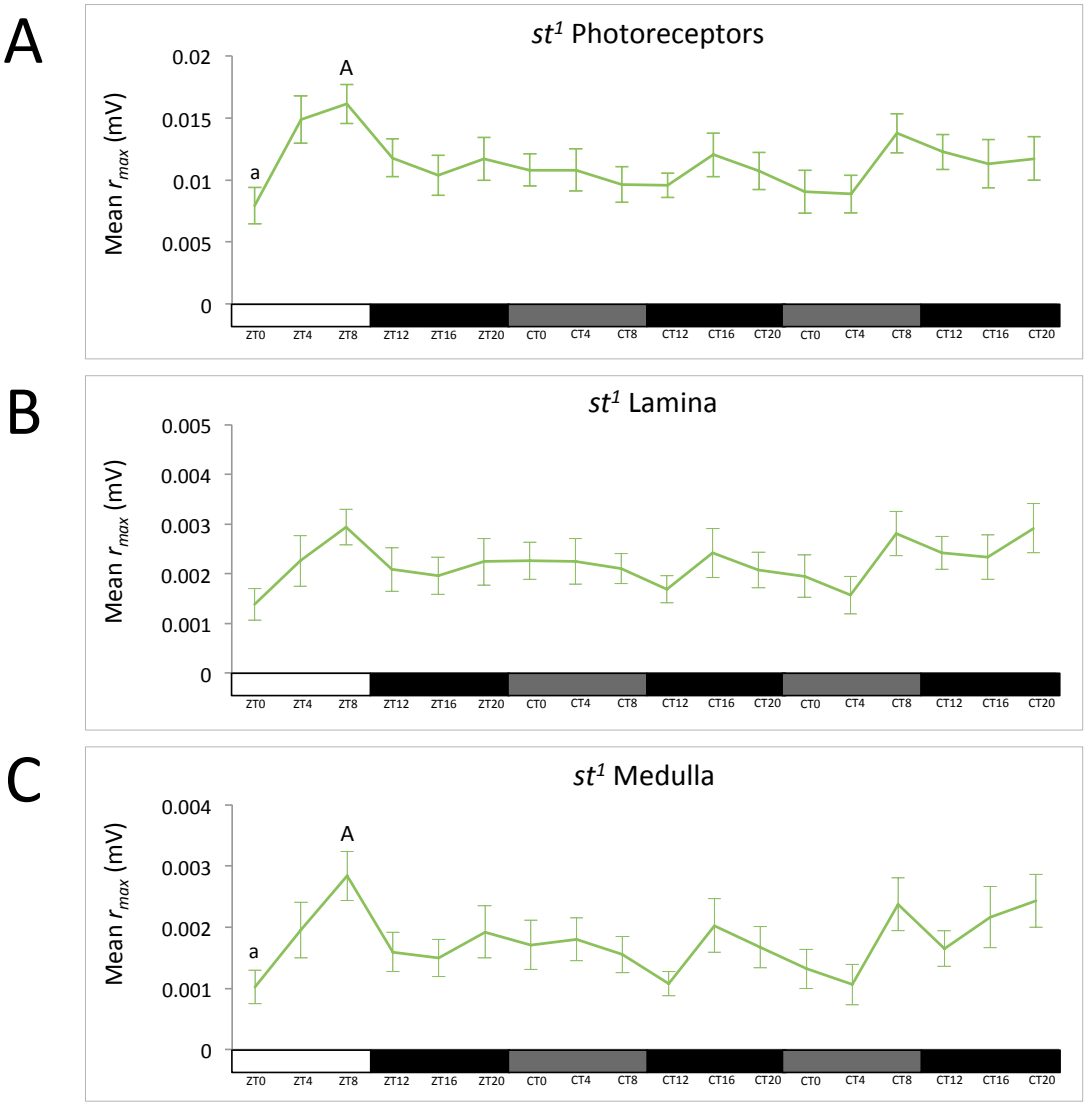

Figure 8: Scarlet-eyed control (*st*^{1}) *Drosophila melanogaster* exhibit a rhythm in mean visual contrast sensitivity in the photoreceptors and medullary neurons under LD conditions. Male flies (n ≥15 for each time point) were photoentrained in 12:12 hour LD cycles for 6 days and had visual responses recorded via the SSVEP assay on LD6, DD1 or DD2. New flies were used for every reading and readings were taken at intervals of 4 hours over 3 days. The mean R_{max} in contrast sensitivity is plotted versus time in Zeitgeber or circadian time. Shown are the mean responses in the photoreceptors, lamina, and the medulla.

by ANOVA, there are most likely 3 cycles occurring over the 3-day time course, or only slightly less likely, 4 cycles over 3 days.

The TTFL mutant *Clk^{Jrk}st^{1}* exhibits a highly significant relationship between time-of-day and the R_{max} of its visual contrast sensitivity in all three orders of neurons assayed ($p>0.005$) (Figure. 9). In the photoreceptor response there was found to be a significant difference between the contrast sensitivity at CT4 on DD1 (peak) and both CT16 on DD1 and CT20 on DD2 (troughs) (Figure. 9A). There was a 59% decrease in contrast sensitivity between DD1 CT4 and DD1 CT16. In the lamina, many more significant comparisons of means were identified, with peaks levels of sensitivity highlighted at ZT4, ZT8 and DD1 CT4, and lowest levels occurring from ZT12-ZT20 (dark phase), DD1 CT8-CT16 and DD2 CT20 (subjective night) (Figure. 9B). These results indicate that in stark contrast to its activity rhythm the *Clk^{Jrk}st^{1}* mutant possesses a unimodal rhythm in contrast sensitivity with a morning peak at ZT/CT4 and lowest values during the subjective night and decreases in sensitivity of 31-70% at these times. It would also appear that this rhythm repeats with an approximate period of 24 hours and persists under constant DD conditions, suggesting it could be defined as circadian. Responses in the medulla also support this conclusion, with a significant decrease (67%, $p>0.005$) between peak values at ZT4 and DD1 CT4 and the trough at DD1 CT16 (Figure. 9C). The FFT results (not shown) state that the *Clk^{Jrk}st^{1}* fly most likely undergoes 3 complete cycles over the 3-day time course, consistent with circadian rhythmicity.

In comparison to the results of the scarlet-eyed control, while the *Clk^{Jrk}st^{1}* fly seems not to experience a shortened period under free-running conditions, the two data sets otherwise follow a similar trend, with a unimodal rhythm peaking during the light/anticipated light period.

While the results of the control fly in this experiment do not completely support the hypothesis that WT *Drosophila* possess a circadian rhythm in contrast sensitivity, a strong circadian rhythm is presented by the TTFL mutant fly *Clk^{Jrk}st^{1}*. These results indicate that the correct function of molecular clock component dCLK is not essential for the retention of circadian rhythmicity in visual contrast sensitivity.

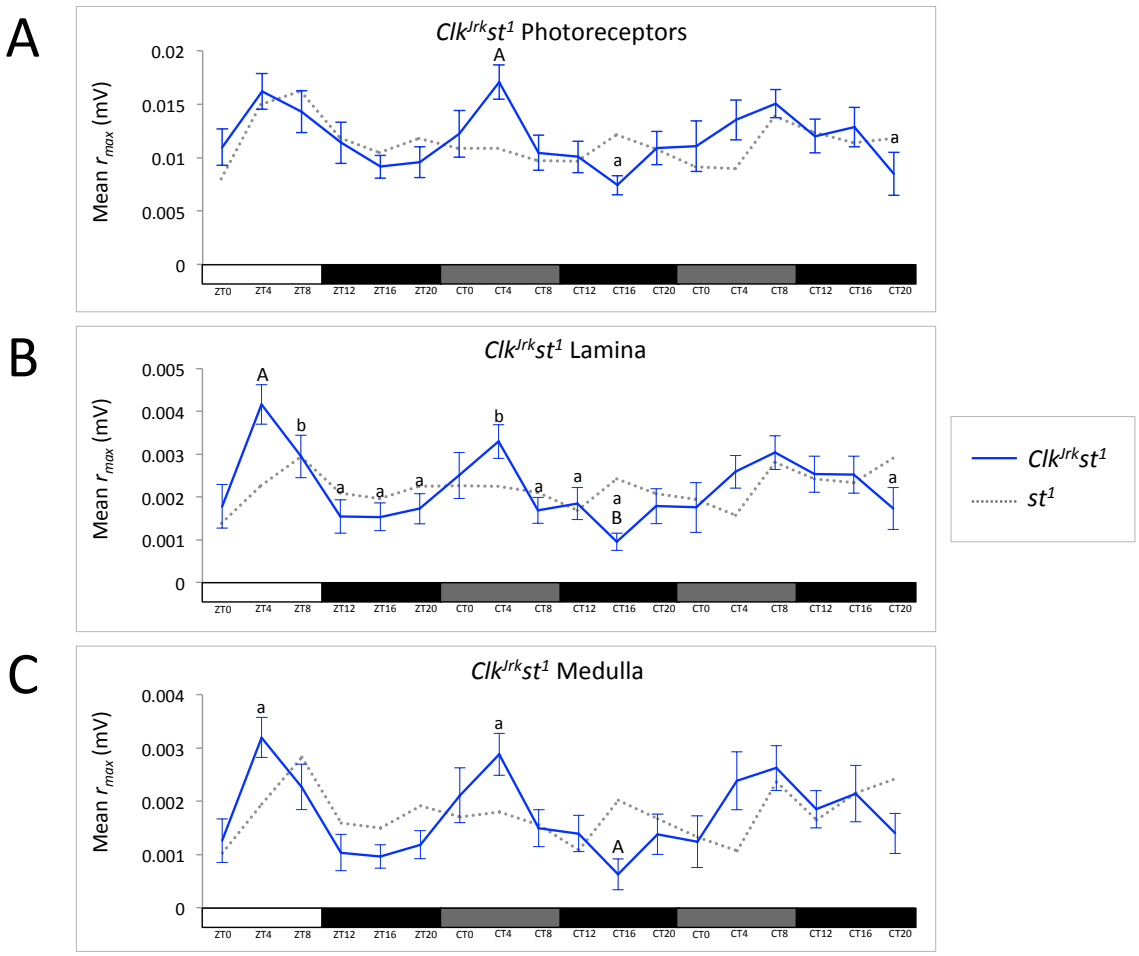

Figure 9: Locomotor arrhythmic clock mutant (*Clk*^{Jrk}*st*^{1}) *Drosophila melanogaster* possess a circadian rhythm in mean visual contrast sensitivity in the photoreceptors, lamina, and medulla. Male flies (n ≥15 for each time point) were photoentrained in 12:12 hour LD cycles for 6 days and had visual responses recorded via the SSVEP assay on LD6, DD1 or DD2. New flies were used for every reading and readings were taken at intervals of 4 hours over 3 days. The mean R_{max} in contrast sensitivity is plotted versus time in Zeitgeber or circadian time. Shown are the mean responses in the photoreceptors, lamina, and the medulla. Also shown are the results from the control fly, *st*^{1} (dashed grey line).

3. 3. Circadian rhythmicity in the visual contrast sensitivity of per^0

In this experiment a second TTFL mutant, the *period* gene null per^0, was tested for rhythmicity in visual contrast sensitivity. As another mutant of a core component of the molecular clock its responses can provide further evidence for the role or lack thereof of control over this visual rhythm. The control for this study, the isogenic-crossed wild type strain $CantonS / iso^{4147}$ carries no known mutations, unlike the eye colour defective control st^1 and so should offer a more accurate assessment of the WT *Drosophila* response. As in the previous experiment, visual contrast response was measured by way of the SSVEP assay on flies that had been photoentrained in 12:12 LD for ~5/6 days immediately following eclosion (LD6 readings), and on those that were also kept for a further 24 or 48 hours under constant conditions (DD1 or DD2 readings).

The isogenic-crossed WT fly exhibits a highly significant relationship between time-of-day and the R_{max} of its visual contrast sensitivity in all three orders of neurons assayed ($p>0.005$) (Figure. 10). In the photoreceptors there was a significant difference between peak values at ZT4, ZT8 and ZT16 and the lowest value measured at DD1 CT16 (Figure. 10A). There was a difference of ~39% between the peak and trough values. The lamina neurons showed a similar response, but without a significant peak at ZT8 (Figure. 10B). No significant comparisons were identified in the medullary neurons (Figure. 10C). The FFT results (not shown) indicate that the most likely number of rhythmic cycles undergone over the 3-day time course is 7. By looking at the graphs, although 7 significant peaks are not found by the Bonferroni comparison of means, the trend of the data does appear to reflect the results of the FFT, and could therefore suggest that the WT fly has a biphasic rhythm in contrast sensitivity, and a period shortened to slightly under 24 hours under free-running DD conditions, leading to 7 peaks over 3 days.

The TTFL mutant per^0 also exhibits a highly significant relationship between time-of-day and the R_{max} of its visual contrast sensitivity in all three orders of neurons assayed ($p>0.005$) (Figure. 11). In the photoreceptors peak levels occurred at ZT8, DD2 CT8 and DD2 CT16, with the lowest values at ZT16 and DD1 CT0 (Figure 11A). Peak values were ~59% higher than the lowest recorded sensitivity values. In the lamina neurons, the lowest values also

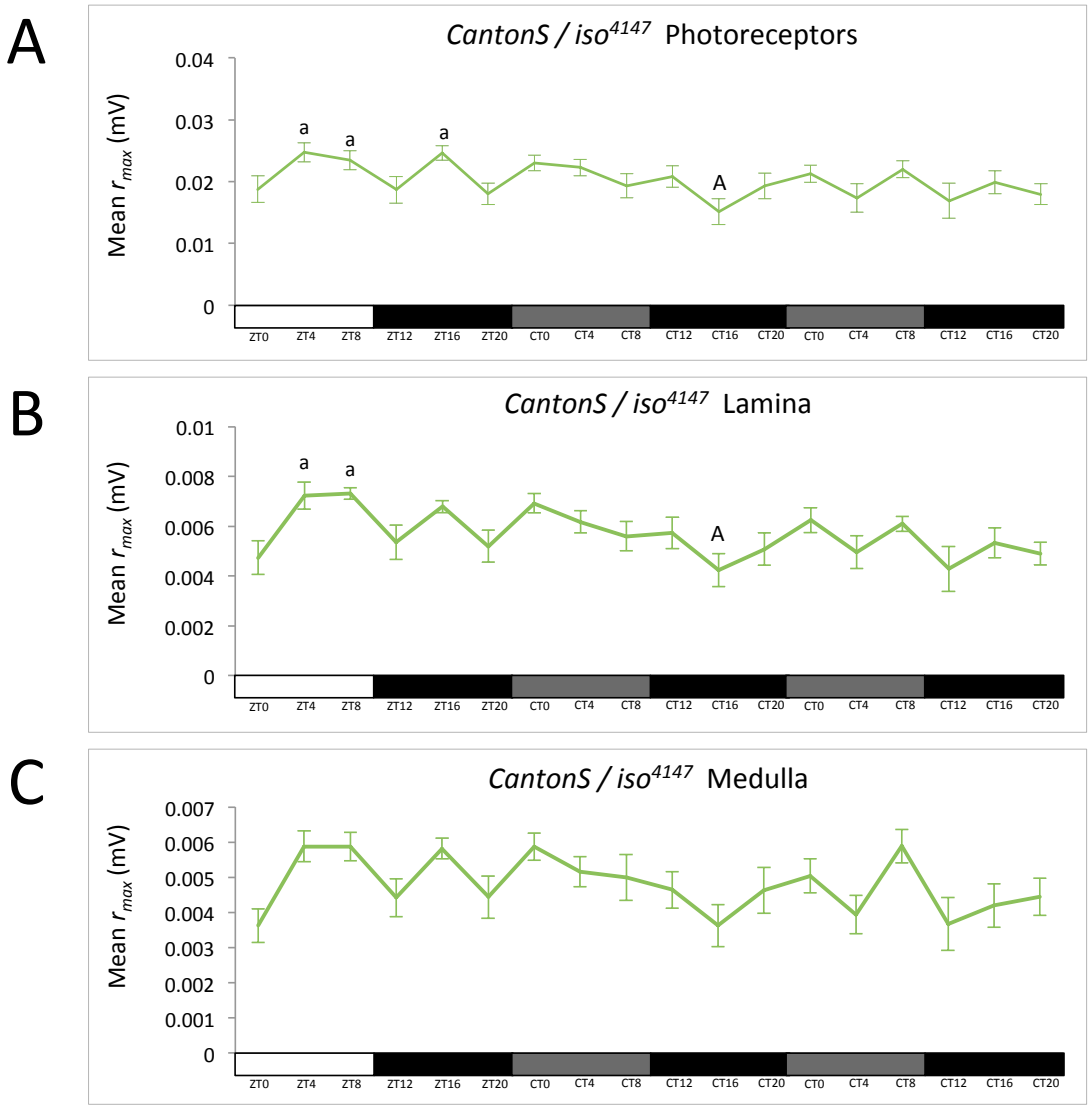

Figure 10: Isogenic crossed wild type (*CS / iso*^{4147}) *Drosophila melanogaster* demonstrate some evidence of rhythmicity in mean visual contrast sensitivity in the photoreceptors and lamina. Male flies (n ≥15 for each time point) were photoentrained in 12:12 hour LD cycles for 6 days and had visual responses recorded via the SSVEP assay on LD6, DD1 or DD2. New flies were used for every reading and readings were taken at intervals of 4 hours over 3 days. The mean R_{max} in contrast sensitivity is plotted versus time in Zeitgeber or circadian time. Shown are the mean responses in the photoreceptors, lamina, and the medulla.

occurred at ZT16 and DD1 CT0, with peaks at DD2 CT0 and DD2 CT8 (Figure 9B). Several more multiple comparisons were highlighted in the medullary neuronal response, with peaks identified at ZT4, ZT8, DD1 CT20 and DD2 CT8, and lowest levels of contrast sensitivity at ZT12, ZT16, DD1 CT0, DD1 CT4, and DD1 CT20. The results of the FFT (not shown) suggest that over the 3 days assayed, the per^0 flies undergo either just one compete cycle (or in other words, there is no repeating rhythm to be seen) or 8 complete cycles. It is possible that the per^0 fly has a shortened period length in its rhythm in contrast sensitivity as well as locomotor activity, and that it, like the WT experiences a biphasic rhythm in its visual response (i.e. resulting in 8 peaks over 3 days). Certainly the mutant and control seem to be in phase with one another under LD conditions, and appear to share an increase in sensitivity on DD2 CT8, however statistically there is no repeating rhythm to be found in the response of the per^0 fly in spite of a clear relationship between time-of-day and contrast sensitivity overall. Another possible conclusion therefore is that the per^0 fly has little to no control over contrast sensitivity when under constant DD conditions.

The results of this experiment suggest that the WT *Drosophila* possesses a circadian rhythm in visual contrast sensitivity that is biphasic, and peaks twice per cycle, from ZT4-ZT8, and again in the middle of the dark phase at ZT16. This rhythm appears to decrease slightly in period length when under constant conditions. While this rhythm may persist under LD conditions in the absence of *per* expression, the regularity of peak sensitivity values under constant darkness seem to be *per* dependent.

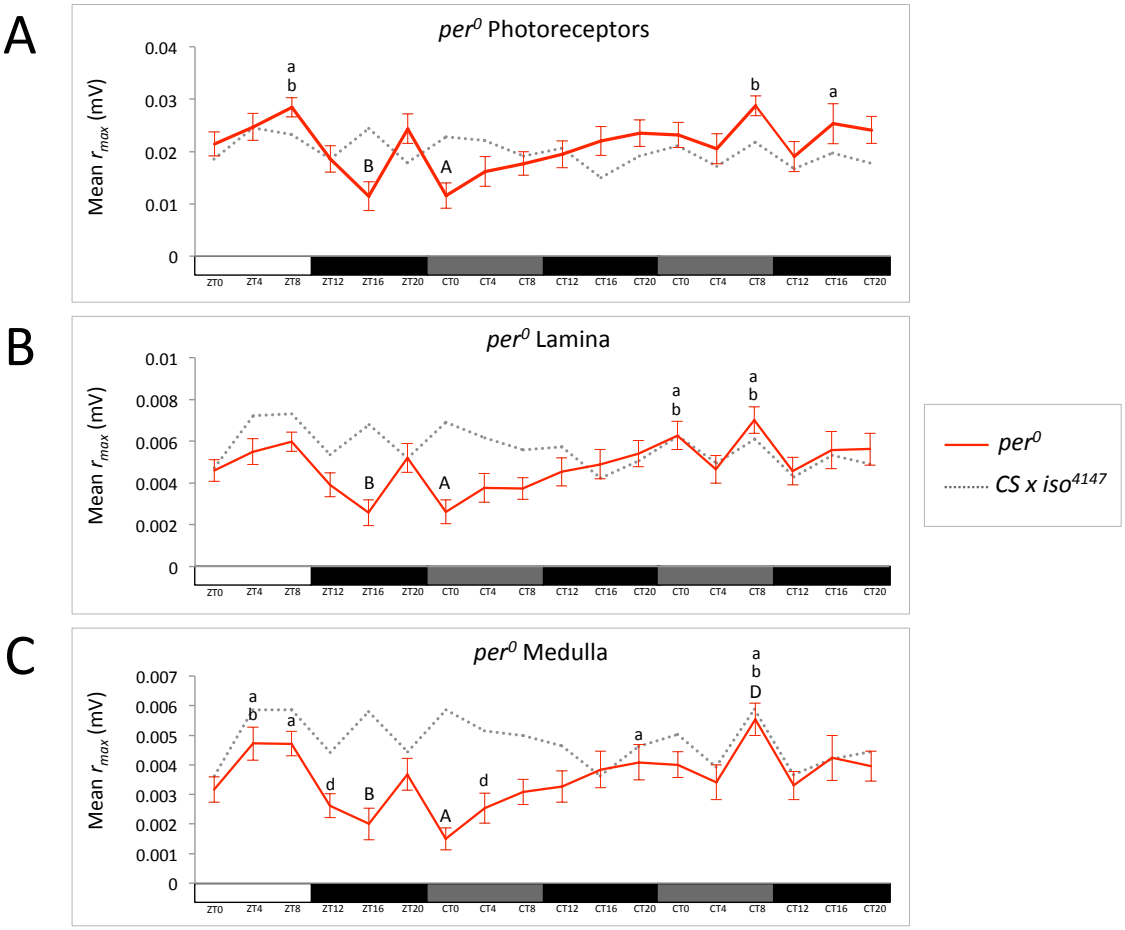

Figure 11: Locomotor arrhythmic clock mutant (*per*^{0}) *Drosophila melanogaster* demonstrate some evidence of rhythmicity in mean visual contrast sensitivity in the photoreceptors, lamina, and medulla. Male flies (n ≥15 for each time point) were photoentrained in 12:12 hour LD cycles for 6 days and had visual responses recorded via the SSVEP assay on LD6, DD1 or DD2. New flies were used for every reading and readings were taken at intervals of 4 hours over 3 days. The mean R_{max} in contrast sensitivity is plotted versus time in Zeitgeber or circadian time. Shown are the mean responses in the photoreceptors, lamina, and the medulla. Also shown are the results from the control fly, *CS / iso*^{4147} (dashed grey line).

3. 4. Circadian rhythmicity in the visual contrast sensitivity of w^{1118}

Discrepancies between the conclusions drawn from the control fly strains st^1 and CS / iso^{4147} make it difficult to ascertain the true WT phenotype of rhythmic contrast sensitivity in *Drosophila melanogaster*. The aim of this experiment is to measure the effects of time-of-day on the R_{max} of contrast sensitivity in a third control line, the white-eyed WT fly, w^{1118}, and so provide further indication of the true WT phenotype. As in the previous experiments, visual contrast response was measured by way of the SSVEP assay on flies that had been photoentrained in 12:12 LD for ~5/6 days immediately following eclosion (LD6 readings), and on those that were also kept for a further 24 hours under constant conditions (DD1 readings).

The w^{1118} flies show a highly significant relationship between time-of-day and contrast sensitivity R_{max} in the photoreceptors, lamina and medulla as determined by univariate ANOVA ($p>0.005$) (Figure. 11). In the photoreceptors, peak values were recorded from CT0-CT8 on DD1, during the anticipated light period of the first day under constant darkness (Figure. 11A). These peak levels were found to be significantly higher than almost all other time points measured and sensitivity was 55-94% higher at these times. These peak values from CT0-CT8 were also seen in the lamina and medullary neurons, were all time points but ZT4 elicited a significant difference to at least one of the DD1 subjective daytime values (Figure. 11B and C). The results of the FFT (not shown) indicate that the w^{1118} flies underwent only one complete cycle over 2 days (i.e. no detectable repeating rhythm) that peaked on DD1.

The results of the white-eyed control fly's visual responses suggest that while the fly has a highly significant relationship between time-of-day and contrast sensitivity, there is no repeating rhythm to be seen over 2 days, and as such, no likely circadian rhythmicity, despite highly significant peaks being identified under free-running DD conditions.

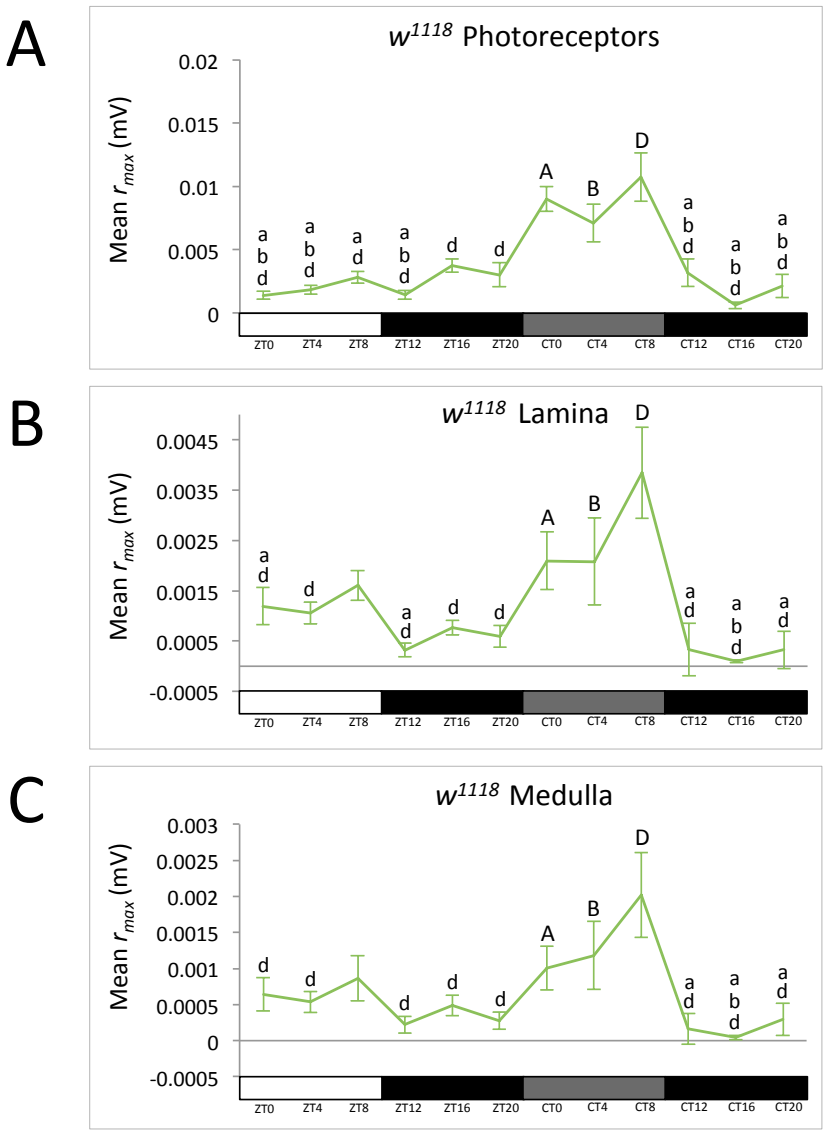

Figure 12: White-eyed control (w^{1118}) *Drosophila melanogaster* exhibit a rhythm in mean visual contrast sensitivity in the photoreceptors, lamina, and medulla under DD conditions. Male flies (n ≥15 for each time point) were photoentrained in 12:12 hour LD cycles for 6 days and had visual responses recorded via the SSVEP assay on LD6, DD1 or DD2. New flies were used for every reading and readings were taken at intervals of 4 hours over 3 days. The mean R_{max} in contrast sensitivity is plotted versus time in Zeitgeber or circadian time. Shown are the mean responses in the photoreceptors, lamina, and the medulla.

4. Discussion

4.1. Overview

In this study the Steady State Visually Evoked Potential assay enabled the characterization of *Drosophila melanogaster*'s rhythmicity in visual contrast response in the photoreceptor, lamina, and medullary neurons in a highly sensitive manner. Given that previous work on *Drosophila* has reported a circadian rhythm in visual pigment levels and ERG-measured luminance sensitivity of both wild type and *period* gene mutant strains under free-running conditions (Chen et al., 1992), one might have expected to uncover a similar circadian rhythm in visual contrast sensitivity that can function independently of the core molecular clock. As such, entering this study our hypothesis was that the wild type phenotype of *Drosophila melanogaster* would be a circadian rhythm in contrast sensitivity, that would persist in the absence of a functional molecular clock, a state represented by the TTFL-impaired mutants $Clk^{Jrk}st^{1}$ and per^{0}. While admittedly the lack of an obvious trend in the rhythmicity of contrast sensitivity amongst the wild type and eye colour mutant strains makes the matter of characterizing the wild type phenotype somewhat problematic, the high degree of regularity in the phase and periodicity of the *dClk* mutant $Clk^{Jrk}st^{1}$ both under LD and DD conditions offers strong support for the hypothesis, demonstrating an alternative timekeeping mechanism that can persist for at least 48 hours under constant darkness independently of normal TTFL function.

4. 2. Characterization of the WT contrast response rhythm

In this study the results of three data sets contribute to the characterization of *Drosophila melanogaster's* wild type rhythm in visual contrast sensitivity. These three control genotypes are the scarlet-eyed st^{1}, the white-eyed w^{1118}, and the brick red-eyed isogenic crossed CS/iso^{4147}. As previously stated, due to the visual nature of the SSVEP assay primarily used in this study, eye colour control genotypes of all mutants used were employed. Neither of the two eye colour mutations have had any previous association with dysfunctional circadian rhythmicity. It is quite possible that different visual eye pigments migrate in a temporal fashion resulting in changes in the phase or amplitude of a rhythm in contrast sensitivity. A rhythm in levels of visual pigment absorbance in white-eyed *Drosophila* was previously found to coincide with a circadian

rhythm in luminance sensitivity (Chen et al., 1992). If different eye colour pigments migrate at different speeds or cycle out of phase with one another then this could account for differences seen between the contrast sensitivity rhythms of fly strains with varying eye colour. This however makes the matter of characterizing a wild type rhythm more challenging as one must look searchingly to find any common features between the three data sets.

To begin with, one could argue that the *CantonS/iso*^{4147} line, being a true wild type with no eye colour defects in addition to having been crossed with an isogenic line should be the most likely of the three controls measured to represent the true wild type phenotype. While the ordinary *CS* line is highly inbred, this outcrossed line may offer heterotic vigour in the hybrid offspring, and thus a more reliable view of WT rhythmicity. The fly has a fairly regular rhythmicity and a period of only slightly less than 24 hours. The rhythm is biphasic, with an FFT identifying 7 likely peaks over 3 days and a Bonferroni multiple comparisons test highlighting two of the peaks and one of the troughs in this 3-day rhythm. Peaks occur during the subjective day at ZT4-8 as well as around midnight, at ZT16. Many of the documented rhythms in the *Drosophila* visual system are described as being unimodal (Chen et al., 1992; Górska-Andrzejak et al., 2013; Weber et al., 2009) although there is some precedent for bimodality also (Damulewicz et al., 2013). I do believe it is worth noting however that while numerous studies of rhythmic changes in morphology or protein expression include broad statements regarding the phase and modality of the rhythms uncovered, one cannot ultimately take too much stock in such claims as all too often the time points of such studies are irregularly spaced, with no results between ZT4 and ZT13, or even just a comparison between midday and midnight. As such, these may not accurately reflect a potentially more complex rhythm. The *CantonS/iso*^{4147} line demonstrated textbook rhythmicity in locomotor behaviour levels and so is a good candidate for potentially defining the wild type rhythm. It does however conflict with the results of the other two wild type lines, particularly being the only line to demonstrate visual rhythm bimodality, and a recurring nighttime peak. Furthermore the *CantonS* rhythm is as or less robust in the rhythmicity of its contrast response than the other controls measured, with very few significant comparisons. Inadequacy in the number of time points is an ever present concern in chronobiological studies, as more time points may reveal more significant peaks that are sharp enough to be missed by infrequent

measurements. It is therefore a possibility that the lack of distinction between *CS*'s 7 "peaks" indicates that there is no real rhythm to be seen, and that more frequent time points would reveal a sharp peak for instance during the subjective day which would better agree with the multitude of morning peaking unimodal visual rhythms previously recognized in *Drosophila*, as well as with the other control lines.

So let's address the rhythm measured in the scarlet-eyed control, st^1. This fly demonstrated decent rhythmicity in the locomotor assay, although less robustly than *CantonS*. It exhibited a rhythm in contrast sensitivity that was very regular, with a period shortened to less than 24 hours, causing the results of the FFT to support either 3 or 4 peaks over the 3 day time course. Contrast sensitivity peaked initially during the subjective day at ZT8, and although thereafter was not sufficiently robust to be highlighted by the multiple comparison of means test, appeared to repeat, albeit somewhat diminished, every 16 hours or so. And so this rhythm may be characterized as being unimodal, with a daytime peak and diminishing robustness under constant DD conditions. Whether or not a rhythm with period shortened to 16 hours under constant darkness can be termed circadian is debatable, but while the regularity of this rhythm is appealing as a benchmark for the WT phenotype, having previously deemed any rhythm in the locomotor assay with a period shorter than 21 hours to not be circadian, I must likewise dismiss the st^1 visual rhythm. In spite of overly shortened period, many features of the st^1 rhythm better correlate with what would have been expected of the WT visual contrast rhythm. For instance, as previously mentioned, unimodality has been seen to be common of most visual rhythms in *Drosophila* (Chen et al., 1992; Górska-Andrzejak et al., 2013; Weber et al., 2009), including in rhythms of luminance and contrast sensitivity in other model organisms. Luminance sensitivity rhythms in *Drosophila* and zebrafish have both been described as being unimodal and peaking around light offset (Chen et al., 1992; Li and Dowling, 1998). Meanwhile circadian rhythms in visual contrast sensitivity have been documented in *Xenopus* and murine models. These contrast rhythms also agree with one another, both being unimodal and peaking during subjective daylight (Hwang et al., 2013; Solessio et al., 2004). If from this we conclude that visual sensitivity rhythms are homologous across these models, then we must expect that the *Drosophila* contrast rhythm is also most likely to be unimodal with a daytime peak. And so although the period and robustness of the st^1 rhythm may be somewhat

dubious, given our previous frame of reference for rhythms in visual contrast it may reflect the true WT phenotype. Of course we must also acknowledge the possibility that like the *CantonS* fly, more frequent SSVEP readings may reveal more distinct peaks in sensitivity for instance around midnight, which would therefore support the *CantonS* rhythm as being more archetypal.

Thirdly we have the responses of the white-eyed control fly, w^{1118}. Strikingly this strain does not appear to have any peaks in sensitivity when under LD conditions, only exhibiting peak values on DD1 subjective daytime and significantly lower contrast sensitivity at all other times. While the significance of this increased acuity on DD1 appears highly robust compared to other fly lines due to the sheer number of significant comparisons found by Bonferroni testing, the FFT does not identify any likely cycling of the response over two days, and to the eye the responses on LD6 and DD1 bear very little resemblance to one another. While the peak in sensitivity on DD1 is much greater than any measured on LD6, the highest values of each day of recordings may coincide at 8 hours after light onset, at ZT/CT8, as although the ZT8 reading is significantly lower than that seen on DD1, it is not as low as any other time point on the same day. The white-eyed fly could then have a unimodal rhythm peaking towards the end of the subjective day, just as in the st^{1} line and in other models. This however a generous conclusion given that statistically there is no repeating rhythm, and the increased robustness of a possible rhythm when free-running compared to that of LD conditions is incompatible with the gradual decline in amplitude usually associated with circadian rhythms in DD. A second day's worth of DD readings could reveal a more obvious repeating trend, as could more frequent readings. It has been suggested previously that the white-eyed mutant fly is not only optomotor blind due to its lack of visual eye pigmentation, but is in fact dazzled by moderate daylight, showing drastically lowered courtship vigour in daylight in comparison both to control lines in daylight, and to its own vigour under dim red light (Krstic et al., 2013). And so in this study the mutant may be dazzled during daytime LD conditions, concealing the typical WT rhythm, which otherwise continues unhindered, with low contrast sensitivity at night, and high sensitivity during the subjective daytime under DD conditions when it is not dazzled by greater light intensity.

And so we are left with two apparent options when characterizing the WT rhythm in contrast response; 1) a bimodal rhythm with daytime and nighttime peaks, supported only by data from the *CantonS / iso*^{4147} line and 2) a unimodal rhythm with a daytime peak supported by data from the *st*^{1} line, and potentially from the *w*^{1118} line. Given the homology seen amongst the recorded contrast sensitivity rhythms of other model organisms (Hwang et al., 2013; Solessio et al., 2004), as well as homology in the luminance response rhythm between *Drosophila* and zebrafish (Chen et al., 1992; Li and Dowling, 1998), it is possible that the WT rhythm is that which correlates with *Xenopus* and mouse models, and is here presented by the response of the *st*^{1} fly. Ultimately however, despite discrepancies between the control lines, one must argue that the *CantonS / iso*^{4147} flies, being heterozygous offspring of a classic *Drosophila* WT strain and an isogenic line are the most reliable example of wild type behavior, as evidenced by their exemplary locomotor rhythms. This should be confirmed by further examination of wild type *Drosophila melanogaster* with more frequent time points.

4. 3. Circadian rhythms in the contrast response of TTFL-compromised mutants

In order to determine the involvement of the molecular clock in the control of *Drosophila*'s rhythm in visual contrast sensitivity two Transcriptional-Translational Feedback Loop impaired mutant lines were used. The first of these, *Clk*^{Jrk}*st*^{1} carries a mutation deleting most of *dClk*'s C-terminal domain, disrupting normal function of the core molecular clock component and thus disrupting the TTFL. The second, per^{0}, is a genetic knock out of the *period* gene, another core component of the TTFL and so the mutant is also lacking correct TTFL function.

What may appear ironic at first, the circadian rhythmicity in the *Clk*^{Jrk}*st*^{1} mutant arguably demonstrates the most robust circadian rhythm measured in this study. This mutant, despite disrupted TTFL function, has a highly regular circadian rhythm. The rhythm appears to peak 4 hours after anticipated light onset on every day assayed, even under DD conditions, although the peak of DD2 is sufficiently diminished in amplitude not to be identified by a multiple comparison of means test, and significant troughs during the subjective night are found on all three days. The rhythm has a periodicity of approximately 24

hours as the FFT results indicate that 3 complete cycles occur over the three-day time course. And so the first of the TTFL impaired mutants has a clear and truly circadian rhythm in visual contrast sensitivity that peaks during the subjective day, and then significantly decreases during the subjective night, and so is unimodal. The rhythm gradually diminishes in amplitude each subsequent day under constant conditions. This rhythm is very similar in phase to that of the control, the st^1 fly. It is slightly longer than that seen in the control, which correlates with the results of the locomotor assay, in which the minority of $Clk^{Jrk}st^1$ flies found to be behaviourally rhythmic had a period that was slightly lengthened compared to the control. The amplitude of the rhythm is also slightly greater in the $Clk^{Jrk}st^1$ relative to that of the control.

The first conclusion we may take away from this is that although there is a loss of an evening or nighttime peak, the rhythm in contrast sensitivity seen in the WT persists in $Clk^{Jrk}st^1$, and thus must be partially capable of functioning independently of the molecular clock. The evening peak in the L1 and L2 monopolar cell swelling/shrinking rhythm has been proposed to be driven by evening release of Ion Transport Peptide (ITP) from the 5th sLNv (Hermann-Luibl et al., 2014), where ITP expression was also documented as being diminished in Clk^{AR}, a hypomorph mutant of *dClk*, and so it is likely that the $Clk^{Jrk}st^1$ mutant also experiences low ITP expression and thus a loss of the evening-driven peak in visual activity. Given that *dClk* is integral to TTFL function the retention of the daytime peak supports the idea of an alternative timekeeping mechanism, such as the more recently proposed metabolic oscillator (Bass and Takahashi, 2011; Causton et al., 2015; Edgar et al., 2012; O'Neill and Reddy, 2011). In this model autonomous cycling of redox metabolites produce circadian output independently of molecular clock gene transcription. In fact, rhythms in the oxidation state of peroxiredoxin proteins have been found to be conserved across all kingdoms of life, and to cycle in the very same $Clk^{Jrk}st^1$ mutant used in this study (a rhythm which also peaks between ZT0 and ZT4, and is unimodal) (Edgar et al., 2012). And so although some features of WT rhythm may have yet to be fully elucidated, one cannot deny that a robust and circadian rhythm is maintained in a TTFL-impaired mutant, and that this strongly suggests that the rhythm in visual contrast sensitivity's daytime peak is or can be governed exclusively by an alternative circadian oscillator. Given similarities in the phase and modality of this rhythm, it is likely driven by a metabolic oscillator that derives from the cycling state of

reactive oxygen species. In mice, the circadian rhythm in contrast sensitivity is described as being modulated by the CLOCK analog NPAS2 and D_4 retinal dopamine receptors in a dopaminergic signaling pathway in the retinal ganglion cells (Hwang et al., 2013). While *Drosophila melanogaster* expresses no second analog of dCLK, such as the pair CLOCK and NPAS2 in mice it may have some as-of-yet unidentified timekeeping component that regulates the contrast response in a similarly dopaminergic pathway. Additionally $Clk^{Jrk}st^{1}$'s increased rhythmic amplitude relative to the control st^{1} may then be accounted for by $Clk^{Jrk}st^{1}$'s high levels of tyrosine hydroxylase and subsequently dopaminergic signaling (Kumar et al., 2012).

There is proven homology between the dopaminergic neuronal network of fruit flies and vertebrates (Nässel and Elekes, 1992; Sanes and Zipursky, 2010). Dopaminergic signaling specifically modulates the contrast response of both vertebrates and flies in which a loss of dopaminergic signaling results in a loss of photoreceptor function (Chyb et al., 1999; Hindle et al., 2013). It has also been shown that due to dopamine's self-oxidising nature and proneness to generating reactive oxygen species (ROS), dopaminergic neurons in particular are highly sensitive to oxidative stress (Graham, 1978; Hald and Lotharius, 2005; Hanna et al., 2015), in which case the dopaminergic neurons of the *Drosophila* retina may be prime candidates for circadian control by a metabolic oscillator.

Meanwhile the per^{0} fly, our second TTFL mutant does not offer such informative responses. This mutant, while possessing significant changes in R_{max} both under LD and DD conditions, has no repeating rhythm, with the FFT suggesting a likely 1 or 8 cycles of 3 days. In contrast to the *CS* fly, there is no obviously shortened and repeating biphasic rhythm to support the idea of 8 distinct peaks having occurred, and more likely there is no rhythm at all. The *period* gene impaired mutant used in a study of *Drosophila* luminance sensitivity was found to have an unimpeded unimodal circadian rhythm, as was the *period* null mutant used in the study of circadian rhythmicity of PRX species (Chen et al., 1992; Edgar et al., 2012). This strongly supports the idea that expression *period* gene, as another core component of the molecular clock like *dClk*, is most likely not required for the maintenance of the circadian rhythm in contrast sensitivity under constant conditions. While in this study the *period* mutant was found to have a significant relationship between time-of-day and

contrast sensitivity R_{max} both under LD and DD conditions, the more distinct and telling peaks of this rhythm may have been overlooked due to insufficiently frequent time points.

4. 4. Local effects on circadian rhythmicity of the Drosophila *contrast response*

One of the benefits of the SSVEP assay is of course the ability to use a Fourier Transform to separate out and identify the contribution of the photoreceptor, lamina, and medullary neurons individually, thanks to their respective frequency tags. In this study therefore the contrast response of each genotype is separated into 3 parts, showing the contribution of these neuron orders. Previous studies in the *Drosophila v*isual system have uncovered numerous rhythmic changes in the morphology and expression patterns in specific cell types. For instance the expression of Bruchpilot, a protein found in the presynaptic active zone whose function involves organizing the release of neurotransmitter containing vesicles, undergoes a circadian rhythm in abundance peaking at ZT13 (Górska-Andrzejak et al., 2013; Kittel et al., 2006). It is probable that a corresponding rhythm in neurotransmitter release in the lamina would affect the phase of the local rhythm in visual sensitivity. However the phase in all three orders of neurons, appeared to be exactly the same in each of the genotypes used, for instance while differences were found in the rhythms of the $Clk^{Jrk}st^{1}$ and *CantonS* lines, in each of these lines, phase was no different in the photoreceptors, lamina, or medulla. From this we can conclude that despite local synaptic modifications that would likely affect the visual transduction pathways and so sensitivity, the fact that the photoreceptors, lamina and medulla are electrically linked in a feedback loop (Heisenberg, 1971) is preventing any local changes to the rhythm in contrast sensitivity. Any local circadian changes must contribute to the overall waveform of all three neuron orders.

4. 5. Concluding remarks

It is well accepted that the function of circadian rhythms is to allow for better adaptation to an environment that fluctuates itself in a circadian manner, and so allow an organism enhanced fitness and odds of survival (Sheeba et al., 1999; Yerushalmi and Green, 2009). In this instance it is proposed that *Drosophila* experience a circadian rhythm in visual contrast sensitivity that is bimodal, peaking twice during the day at ZT4 and ZT16. Given that fruit flies' rhythm in locomotor activity is also bimodal, peaking at ZT0 and ZT12 it is quite likely that the two rhythms have a similar phase and periodicity so that the flies may experience an enhanced ability to differentiate between two visual inputs when its activity levels are higher, as such an ability would confer an advantage in terms of detecting food, predators, or potential mates. This rhythm is out of phase with that of luminance sensitivity which peaks during the dark period/subjective night only (Chen et al., 1992). Presumably this trade-off between contrast and luminance sensitivity occurs so that during the dark period when activity levels are lower the fly is more sensitive to a light startle reaction. During the day when light levels are higher, the luminance sensitivity is an unnecessary metabolic cost, however regardless of light levels, increased contrast sensitivity remains worthwhile as long as activity levels are high.

In spite of the challenges in identifying *Drosophila*'s wild type rhythm in contrast sensitivity, the robustness of the $Clk^{Jrk}st^{1}$'s rhythm cannot be overlooked. The discovery of another circadian rhythm that is partially molecular clock independent adds additional weight to the recent theory of a metabolic oscillator and calls for a reexamination of the driving force behind circadian rhythmicity. Furthermore the results of this study strengthen the link between the dopaminergic pathway and circadian rhythmicity in the visual system. This improved understanding of the contributing factors to visual function provides knowledge that is vital for the treatment of both circadian and visual disorders in humans. In order to better understand these factors future routes of study should include looking for evidence of metabolic input to the contrast response locally, such as measuring for circadian rhythmicity in ROS and peroxiredoxin species in the retina.

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