Effects of Oxidative Stress on Circadian Gene Expression during Aging by Tara Bonar

A THESIS

submitted to Oregon State University University Honors College

in partial fulfillment of the requirements for the degree of

Honors Baccalaureate of Science in Biology (Honors Biology)

> Presented March 3rd 2016 Commencement June 2016

AN ABSTRACT OF THE THESIS OF

Tara Bonar for the degree of Honors Baccalaureate of Science in Biology presented on March 3rd 2016. Title: <u>Effects of Oxidative Stress on Circadian</u> <u>Gene Expression during Aging</u>

Abstract approved: <u>Jadwiga Giebultowicz</u>

All organisms possess circadian clocks, which generate daily oscillations in clock controlled gene expression that regulate many essential biological functions in synchrony with the 24-hour light/dark cycles. When the genes that control the internal clock are weakened, accelerated aging symptoms are observed. Past research in Drosophila demonstrated that aging flies with disrupted clocks have higher levels of reactive oxygen species (ROS) and oxidative damage to proteins than age-matched control flies with normal clock function. To understand how functional clock protects the aging brain, genome-wide gene expression profiles were measured around the clock in heads of young and old flies using RNA-seq. The results revealed that many clock controlled genes lose rhythmicity with age; however, other genes actually become very rhythmic in old flies; we refer to them as late life cyclers (LLCs). The goal of this project was to investigate the mechanisms that induce rhythmic gene expression in old flies. We used hyperoxia to generate oxidative damage in young flies. We measured gene expression levels and determined that these five LLCs exhibited induced rhythmicity in young flies exposed to hyperoxia, suggesting that they may be beneficial for the aging process. We observed LLC expression levels under hyperoxia in mutants with disrupted clock and as well in constant darkness. Our findings revealed a multifaceted regulation of these stress response genes involving both clock and light. Because oxidative stress has been linked to a variety of neurodegenerative diseases and also to cancer, our discovery of the novel role of the circadian system in defense against oxidative damage may help to understand mechanisms regulating healthspan in humans.

Key words: oxidative damage, circadian rhythms, reactive oxygen species, late-life cyclers

Corresponding e-mail address: bonart@oregonstate.edu

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APPROVED:

Jadwiga Giebultowicz, Mentor, representing Integrative Biology

David Hendrix, Committee Member, representing Department of Biochemistry and Biophysics and Department of Electrical Engineering and Computer Science

Sergei Filichkin, Committee Member, representing Department of Botany and Plant Pathology

Toni Doolen, Dean, University Honors College

I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

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1 Introduction

1.1 Roles and Importance of Circadian Rhythms

Nearly all organisms possess biological clocks, which generate rhythms in essential biological functions in synchrony with the solar 24-hour light/dark cycles (Young and Kay, 2001). Biological clocks create daily oscillations in gene expression which, in turn, impose rhythmicity on many of life's functions including behavioral, hormonal, neural, and metabolic processes. These fluctuations are generated by the endogenous circadian clock ("circa"- about and "diel"- day). The persistence of internal rhythms with a circa 24h period, even in the absence of 24-hour external cues in laboratory experiments is the defining attribute of circadian clocks (Allada et al., 2001). However, in nature, extrinsic cues such as the solar, 24-hour day and temperature fluctuations entrain these endogenous cycles to 24-hour periodicity. Circadian rhythms are crucial for synchronizing organisms to their environment and for providing temporal regulation of gene expression, and consequently of metabolic, endocrine and cellular functions. Disruption of such rhythms has been proven to be detrimental with regard to metabolic homeostasis and may be a causative factor in major neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's, which are characterized by the debilitating loss of sleep/wake patterns (Reddy, Hastings and Goedert, 2013; Musiek, 2015).

1.2 Molecular Clock Mechanism and its Conservation across Animal Kingdom

The internal clock mechanism consists of a group of core genes known collectively as the "clock genes". The first clock gene was discovered in the genetic model organism, *Drosophila melanogaster* (Konopka, 1971), subsequent research showed that core clock genes form a feedback loop. In Drosophila, this loop consists of two transcription factors encoded by the genes *Clock (Clk)* and *cycle (cyc)* (See Figure 1). The CLK/CYC proteins form heterodimers, which bind to the sequence in promoter region (known as the E-box) of *period (per)* and *timeless (tim)* genes and activate their transcription and thus production of PER and TIM proteins soon after darkness. PER protein is unstable on its own, and quickly degrades unless it is bound to TIM protein, making it an obligate heterodimer. Accumulation of PER/TIM heterodimers occur in the cytoplasm and is followed by their nuclear translocation late in the night (Hardin, 2011). PER protein binds to CLK/CYC heterodimer, inactivating its transcriptional activity. Consequently, PER and TIM protein production stops as CLK/CYC is no longer bound to the promoters of *per* and *tim* genes.

The input signal to this clock mechanism is provided by 24-hour light/dark cycles via a photoreceptor protein (CRY) which is encoded by the *cryptochrome (cry)* gene. Exposure to light causes the activation of this protein in the early morning hours. Once active, CRY binds to and causes the degradation of TIM, breaking the heterodimer and leaving unstable PER to degrade as well. Without the PER/TIM inhibition, CLK/CYC heterodimer are free to act again as transcriptional activators. Fluctuations in PER, TIM, and CLK proteins in the cell create the 24-hour molecular rhythms. CYC protein does not fluctuate within the circadian system, but is necessary for clock function. Flies with null mutation of the *cyc* gene do not produce any functional CYC protein and consequently are unable to have a functional clock (Rutila, 1998). The clock mechanism drives rhythmic expression of many clock-controlled genes (CCGs), which regulate a variety of cellular functions.



Figure 1. Molecular Model of the circadian clock in *Drosophila melanogaster* CLK and CYC form a heterodimer and bind to the E-box element of the circadian clock genes *per* and *tim* and activate their transcription during the day and early evening. As *per* and *tim* mRNAs peak, PER and TIM proteins accumulate, form a PER/TIM complex, and translocate into the nucleus to repress their own transcription during the late night. During the day, PER and TIM are degraded by light-dependent photoreceptor protein, CRY, thus allowing a new cycle of transcription to start. Figure created by Nandita Kumar.

1.3 Alterations to Circadian Rhythms in Aging

With age, there is a decline in robustness of circadian rhythms in both humans and rodents (Froy and Miskin, 2010; Klerman, 2005; Kolker et al., 2003). Fruit flies exhibit similar transitions as they age. This is evident when comparing the activity rhythms of young and old fruit flies. As fruit flies age, they transition from a state of long, consolidated bouts of sleep-like rest to shorter, more frequent bouts of rest interspersed with locomotor activity (Koh et al., 2006). This decay in behavioral rhythms is associated with weakened oscillations in the expression of some core clock genes (Rakshit et al., 2012; Luo, 2012). The observation that clock components decline with age could be causally connected to aging because animals with disrupted clocks show accelerated aging. Fruit flies with mutated clock elements display accelerated senescence and age-related pathologies. Male flies with a genetic mutation of one of the core clock genes, either *per* (Klarsfeld and Rouyer, 1998), or *cyc* (Hendricks et al., 2003), exhibit shorter lifespans than control flies. In addition, aging phenotypes such as decreased climbing ability, accelerated accumulation of reactive oxygen species (ROS), and neurodegeneration have been reported in flies with genetically disrupted clocks (Krishnan et al., 2009; Krishnan et al., 2012). Similarly, in mammals the decline or absence of properly functioning clocks may lead to premature signs of aging, including increased levels of ROS (Kondratov et al., 2006; Yu and Weaver, 2011). Although physiological consequences of impaired clocks have been reported,

very little is known about how daily rhythms in clock-controlled genes are affected by aging.

1.4 Reactive Oxygen Species and Antioxidant Defense Systems

Among processes that had been shown to be regulated by the circadian clock is production of reactive oxygen species (ROS) in fly heads (Krishnan, 2008) and mouse liver (Gong, 2015). ROS are formed as a byproduct during the metabolism of oxygen. Consequently, aerobic organisms are known to produce ROS from endogenous sources. Normal metabolic reactions, namely the electron transport chain (ETC) in the mitochondria, produce highly reactive molecules such as peroxide (O_2^2), superoxide (O_2), hydroxyl radical (•OH), and singlet oxygen (O_2). Other metabolic processes contribute to ROS production such as activity of certain enzymes in both the cellular and subcellular membranes and cytosol and lipid metabolism in the peroxisome. Ionizing radiation and UV light as well as certain chemicals and toxic substances have been implicated as exogenous sources of ROS (Finkel & Holbrook, 2000).

Approximately 90% of endogenous ROS is generated in the mitochondria (Balaban et al., 2005). More specifically within the mitochondria, the production of ROS can be isolated to two main structures within the ETC, complex I (NADH dehydrogenase) and complex III (ubiquinone-cyctochrome c reductase) with complex III being the main culprit (Finkel & Holbrook, 2000). This is due to the inevitable "leakiness" of the ETC. This energy-dense process is bound to result in side reactions that form due to incomplete electron transfer (Anderson, 2004).

Janus-nature of ROS

As ROS levels rise above the homeostatic conditions, they begin to damage cells causing cytotoxicity (Korsloot et al., 2004). Accumulating ROS causes a pro-oxidative cellular environment resulting in damage to proteins, nucleic acids and membranes (Korsloot et al., 2004) and induction of various pathologies (Cross et al., 1987). Lipids, proteins, and DNA form distinct products after undergoing reactions with ROS; proteins form carbonyls, lipids form peroxidation products, and nucleic acids form oxidized bases. Each of these serve as biomarkers of ROS-related oxidative damage (Coto-Montes & Hardeland, 1999).

While ROS are often associated with the deleterious effects of oxidative damage, in recent years, the viewpoint has shifted towards a more diverse understanding of the many roles of ROS (See Figure 2). Important biological pathways are activated by ROS signaling at physiological levels, which supports the idea that ROS are involved in the maintenance of homeostasis (Finkel, 2011). They are involved in many essential pathways ranging from antibacterial defense (Hardeland et al., 2003), innate and adaptive immune response (West et al., 2011; Kaminski et al., 2013), and stem cell differentiation (Owusu-Ansah & Banerjee, 2009).



Figure 2. Janus nature of ROS

Studies over the past several decades have expanded our understanding of the various roles of ROS. They are no longer seen simply as molecules that invoke cellular damage through lipid peroxidation, protein carbonyls and denaturation of DNA, but under appropriate levels have been found to be important signaling molecules in various physiological responses. The amount and compartmentalization of ROS determines which of these two roles it will take on. (Figure adopted from Hekimi et al, 2011)

Antioxidant defense systems

To combat dangerously high ROS levels, cells possess different defense mechanisms. One of those mechanisms is the use of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) to detoxify ROS (Valko et al., 2006). These enzymes make up the majority of the defense against ROS. The SOD enzyme employs a catalytic metal to dismutate the superoxide anion into hydrogen peroxide which can be broken down into oxygen and water by other enzymes. The Catalase enzyme is found in peroxisomes (Korsloot et al., 2004), and both catalase and glutathione peroxidase catalyze the conversion of hydrogen peroxide to water. Interestingly, glutathione peroxidase is not found in insects. Instead, they use another enzyme, glutathione S-transferase which functions as a peroxidase (Hardeland et al. 2000). New studies show that increased ROS may stimulate mitochondria to produce peptides that increase production of ROS-scavenging enzymes, the process known as mitohormesis (Held, 2015).

Another defense mechanism against oxidative damage is the use of molecular chaperone proteins, such as heat shock proteins, for example HSP22. Chaperones are involved in protein folding, translocation, and also regulation of transcription factors and protein kinases (Georgopuolos & Welch, 1993). A chaperone's primary function is to ensure the proper folding of polypeptides by way of binding to reactive hydrophobic surfaces and preventing aggregation of misfolded proteins. While chaperones were originally discovered as cellular defense against excessive heat, recent data show that they are upregulated upon exposure to oxidative stress (Landis, 2004; Grunewald, 2009). The small heat shock protein, HSP22, is localized in the mitochondria. The mitochondria have been described as the "powerhouse of the cell" by generating valuable energy in the form of ATP, but it is also known as a powerful source of ROS due to the "leakiness" of the electron transport chain. Studies are not conclusive on whether overexpression of *Hsp22* extends or shortens lifespan of *Drosophila* (Morrow, 2004; Tower & Yang, 2008). In response to oxidative stress, *Hsp22, CG7130,* and *CG15784* have been observed to be upregulated in *Drosophila*, suggesting its crucial role in defense against accumulation of ROS (Gruenewald, 2009).

The cells are capable of making metabolic adjustments in response to oxidative stress. Organisms redirect their flux from glycolysis to the pentosephosphate pathway which provides cells with reducing power (NADPH) it needs to combat dangerously high ROS levels (Grant, 2008). This is important because several of the antioxidant enzymes rely on NADPH to maintain their effectiveness. Lactate dehydrogenase (LDH) is the mammalian equivalent of *Drosophila*'s *ImpL3*. This enzyme catalyzes the conversion of lactate to pyruvate and back, also using NADH or NAD⁺ in the process. In response to hyperoxia, *ImpL3* has been observed in flies to be upregulated, suggesting its involvement as an oxidative response gene (Gruenewald, 2009).

1.5 Aging and Oxidative Damage

The Free Radical Theory of Aging states that aging is caused, at least in part, by the buildup of ROS from the mitochondria, causing oxidative damage. This damage has been recorded in biomolecules such as nucleic

acids, lipids, proteins and carbohydrates. Due to the high concentrations of polyunsaturated fatty acids in the brain, it is highly susceptible to the deleterious effects of ROS. Thus, ROS are implied as the culprit in the development of many neurodegenerative diseases including Alzheimers's, Parkinson's, mild cognitive impairment, amyotrophic lateral sclerosis, and Huntington's (Mariani et al., 2005). Age-related declines of the antioxidant defense systems enhance the brain's susceptibility to oxidative damage. In addition to lipid peroxidation and antioxidant shortages, studies reported an increase in protein oxidation across the lifespan in mammals (Smith et al., 1991) and flies (Krishnan, 2009). Overall, excess ROS tends to be detrimental to brain function as we age, but base levels of ROS are required for essential cell signaling. Finally, it should be noted that recent research and clinical trials demonstrated that anti-oxidant supplementations or overexpression of ROS-removing enzymes does not extend lifespan or delay neurodegenerative symptoms in Alzheimer's disease patients (Ristow, 2014). Therefore, more research is needed to understand relationships between ROS and aging. Studies showing higher age-related accumulation of ROS in mutants without functional clocks (Krishnan 2009, Musiek 2015) suggest that the circadian clock plays a role in protecting organisms from ROS.

1.6 Background for Thesis Objective

It is hypothesized that many clock controlled genes (CCGs) lose rhythmicity with age, and this was confirmed in some cases in flies (Klichko,

2015) and mammals (Gong, 2015). However, there is a lack of genome-wide studies investigating how CCG profiles change during aging. This important question was recently addressed in a collaborative project between the Dr. Giebultowicz and Dr. Hendrix labs. The goal was to perform genome-wide expression studies with RNA-Seq around the clock on heads of young and old flies, with samples collected every 4h for 24h in two biorepeats. Surprisingly, these data revealed that some genes that were arrhythmic and expressed at low levels in young flies became very rhythmic with high levels of expression in old flies; these genes were named Late Life Cyclers (LLCs) (Figure 3A). Interestingly, the most robust oscillations were detected in a subset of genes that peak at ~Zeitgeber time (ZT) 12-14 and have been previously suggested to have neuroprotective effects based on their upregulation under hyperoxic conditions which leads to neurodegeneration (Gruenewald, 2009). These genes include the small heat shock protein *Hsp22*, lactate dehydrogenase (*ImpL3*), cytokine *bnl*, chaperone *CG7130*, and *CG15784*. We then confirmed the RNA-Seq results with Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) in the heads of young, old, and very old flies. As seen in Figure 3B, the LLCs gene expression is rhythmically induced with increasing peak expression as flies age. These new, exciting data generated several questions regarding the functional significance and mechanism regulating rhythmic LLC expression, some of which were addressed in this project.





A) 48h mRNA profiles of young (dashed) and old (solid) flies. LLCs have low, arrhythmic expression in young flies, but were found to be rhythmically overexpressed in old flies in these RNA sequencing data. B) Relative mRNA expression of young, old, and very old flies confirmed age-dependent changes in LLCs. Young flies have relatively low expression, whereas old and very old display rhythmic expression of LLCs, peaking near ZT12. Stars indicate significance from the trough of each respective condition (test of rhythmicity) as calculated by *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

1.7 Objectives

Molecular pathways underlying age-dependent diurnal rhythms in LLCs are not understood. Past research in *Drosophila* demonstrated that aged flies with disrupted clocks have higher levels of ROS and oxidative damage to proteins than young flies (Krishnan et al., 2009). In young flies, the expression of LLCs is low and arrhythmic while in old flies, their expression is higher and rhythmic. In light of these data, we hypothesized that age-induced rhythms in LLC genes are generated in response to an increase in endogenous oxidative stress by the mechanism that involves the circadian clock. The goal of this project was to test experimentally the above hypothesis. We also investigated if the circadian clock regulates rhythms of oxidative stress responsive LLCs. *Drosophila melanogaster* is an ideal model organism due to the variability and accessibility of mutants, and has a sequenced genome. Also, core clock genes and ROS detoxification genes are highly conserved between flies and mammals.

2 Materials and Methodology

2.1 Fly rearing and strains

In the experiments performed in these studies, the *white* (w^{1118}) genotype of *Drosophila melanogaster* was used for the wildtype strain with a functioning circadian clock. Flies with a loss-of-function mutation for the gene *cycle*, cyc^{01} (Rutila et al., 1998), were also used. Flies with the cyc^{01} mutation were in the *white* genetic background. All flies were grown on a diet consisting of 1% agar, 6.25% cornmeal, 6.25% molasses, and 3.5% Red Star yeast at 25°C. All flies were entrained to 12h light: 12h dark (LD) cycles. Time of lights-on is denoted Zeitgeber Time (ZT) 0, and lights-off by ZT 12. Flies were sorted by sex according to genotype 2-3 days after emergence in groups of 50 females held in wide 28.5mm diameter plastic vials for hyperoxia experiments or 8 oz. inverted polypropylene bottles (Genesee Scientific, San Diego, CA) for longevity experiments. Five-day-old mated female flies were used for all hyperoxia experiments.

2.2 Longevity in Hyperoxia

To determine longevity under hyperoxia between *white* and cyc^{01} , we exposed 5-day old flies to 100% (medical grade) oxygen flow at a constant rate (200 mL min⁻¹). Four cohorts of 50 flies of each genotype were placed in vials in a Plexiglass chamber filled with oxygen at atmospheric pressure (1 atm). Control flies were transferred to vials with diet and were kept in the same conditions except under normoxia. Diet was changed on alternate days without anesthesia and mortality was recorded twice daily every 12 hours until no flies remained alive.

2.3 Hyperoxia gene expression experiment

Flies of both *white* and *cyc⁰¹* strains were exposed to the same hyperoxia conditions as described above. 50 flies of each genotype were placed in a Plexiglass container filled with oxygen. Normoxia control flies were also housed in wide vials with diet and placed next to hyperoxia chamber. Flies collected on "Day 3" were placed in hyperoxia chamber two days prior to the day of collection, and "Day 4" flies were hence placed three days prior as shown in Figure 4. All flies, including controls, were collected on the same day at 4h intervals starting with ZT 0 and ending with ZT 24, samples were frozen at -80°. Samples taken during lights-off or constant darkness (DD) were collected using a red light, which *Drosophila melanogaster* are insensitive to.



Figure 4 Scheme of Hyperoxia Experiments

Young *white* flies to hyperoxic conditions in 12h light: 12h dark (LD) for 0-72h. For all LD experiments, flies continued in LD for the collection on day 4. For the consant darkness (DD) experiment, after 72h of exposure, we transferred the flies into DD while maintaining hyperoxia exposure. Normoxia flies were also transferred into DD simultaneously. On the 4th day of hyperoxia, which also corresponds to the first cycle in DD, we collected samples every 4h for 24h for mRNA expression analysis.

2.4 Quantitative Real-Time PCR (qPCR)

Whole female flies collected around the clock from hyperoxia gene expression experiments were placed into a 15mL conical tube in liquid nitrogen. Fly heads were separated from bodies by alternate bouts of vortexing and cooling with liquid nitrogen, which caused frozen flies to break at the most fragile point, the neck. The heads and bodies were placed in a sieve with liquid nitrogen and separated based on size (the heads are small enough to fall through the holes of the sieve whereas the bodies are not). 50 frozen heads were homogenized in 500µL of Trizol Reagent (Life Technologies, Grand Island, NY, USA) with a motorized pestle. RNA extraction was performed by ethanol precipitation. The samples were treated with DNAse (Takara, Mountain View, CA, USA), and DNAse was deactivated by use of phenol/chloroform extraction. Samples were purified with sodium acetate. RNA concentration was measured using a Nanodrop spectrophotometer. cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to manufacturer's protocol.

Quantitative real-time PCR was performed using the Power SYBR Green Master Mix (Life Technologies) on an Applied Biosystems Step-One Plus real-time machine. All primers used in this study were obtained from IDT Technology (Coralville, IA, USA) and their efficiencies were > 96%. Data were normalized to reference gene Dcp2 and analyzed using the standard $2^{-\Delta\Delta CT}$ method. GraphPad Prism 6 (San Diego, CA, USA) was used to perform statistical data analysis.

Gene	Forward Sequence (5' – 3')	Reverse Sequence (5' – 3')	
per	GCGCGACCGGACAACTAC	GTGTGGCGTATGGCGAACT	
tim	GTGCTTCTGCTGAGGCGTTTCAAT	GGCGAATGGTTTGACATCCACCAA	
Dcp2	CCAAGGGCAAGATCAATGAG	GCATCGATTAGGTCCGTGAT	
ImpL3	CGTTTGGTCTGGAGTGAACA	GCAGCTCGTTCCACTTCTCT	
Hsp22	GTCAACAAGGATGGCTACAA	CACCAGGACAACGCTCTC	
CG7130	TCCAGTTCTTCAACCGGAAA	AGGTACCCGAGATTGCTTCA	
CG15784	ATGGCCGTCGACTAGAGAGA	AACTGGAGCTGGAACTGGAA	
bnl	GGAACCCAGGACGAGAATAG	GCGTCCATGCAGAGATACAG	

Table 1. I filler sequences for qK1-1 Cr	Table 1: Prime	r sequences	for o	aRT-PCR
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3 Results

3.1 Clock genes per and tim function under hyperoxic stress

The goal of this project was to investigate expression profiles of clock genes and late life cyclers (LLCs) in young flies exposed to oxidative stress in the form of constant hyperoxia (100% oxygen). It was necessary to first determine how core clock genes were functioning under this stress. Knowing that mRNAs of both *period (per)* and *timeless (tim)* genes accumulate during the day peaking at ZT 12, we collected flies for a preliminary analysis at ZT 0 and ZT 12. This collection began at ZT 0 on day 2, after young flies had already been under hyperoxic conditions for 24h. If core clock genes continue to function under hyperoxia, we would expect their mRNA levels to oscillate from low at ZT 0 to high at ZT 12 for *per* and *tim* and in reverse patterns for *Clk* mRNA. We found that both *per* and *tim* continue to oscillate under oxidative stress, similar to the normoxia controls (Figure 5). However, per was observed to be upregulated at ZT12 on day 4 in hyperoxia in the first biorepeat, while in the second biorepeat, no upregulation of *per* was detected. Expression of *tim* was found to be suppressed at all time points in one biorepeat, but was not found to be significantly different when both biorepeats were combined. Additional studies were performed to determine how mRNA levels were changing in a more detailed experiment (see next section). *Clk* and *cyc* mRNA levels were not found to be significantly different at any of the time points in hyperoxia compared to normoxia (Figure 5); thus their 24h profiles were not studied.





Clock gene expression levels were measured in heads of flies maintained under 100% oxygen (HO) and collected every 12 hours at ZT 0 and ZT 12. A trend toward increased expression was observed in *per* mRNA under HO (A), while decreased expression was observed in *tim* mRNA (B). Both *cyc* (C) and *Clk* (D) showed very similar expression levels in HO compared to N controls.

To further investigate whether expression of the negative clock elements *per* and *tim* changed significantly in response hyperoxia, we performed qRT-PCR on the heads of young flies exposed to 100% oxygen and collected at 4h intervals. The 24h mRNA expression profiles on days 3 and 4 in hyperoxia revealed that indeed *per* expression was significantly (p<0.0001) upregulated in response to oxidative damage at the ZT12 peak on day 4 (Figure 6A). Remarkably, this pattern was very similar to the *per* overexpression found in old flies (Figure 6C). Interestingly, *tim* expression was found to be significantly suppressed in hyperoxia compared to normoxia controls (Figure 6B) and similar trends observed in old (day 50) and very old (day 75) flies (Figure 6D).

3.2 LLCs are upregulated in response to hyperoxia in young flies

As discussed in the introduction, a select group of genes designated late life cyclers (LLCs) display age-dependent rhythms (Figure 7). We hypothesized that these rhythms are generated in response to an increase in endogenous oxidative stress. To test this hypothesis, we mimicked oxidative damage that may accumulate during aging (Krishnan et al., 2008) by exposure of young flies to hyperoxia. After two days under oxidative stress, we collected flies around the clock for a gene expression analysis.

We measured the expression of five LLCs during exposure to constant hyperoxia. We determined that the expression of these genes did not continue to steadily increase with prolonged exposure to hyperoxia, but instead





Expression levels measured every 4h for 48h show that *per* and *tim* continues to oscillate in hyperoxia (HO) with a phase similar as in normoxia (N). (A) On day 4 in HO, *per* was found to be significantly higher at ZT 8 and ZT 12 in HO, very similar to its expression trend in old flies (C). Expression of *tim* (B) was significantly reduced on day 3 and 4 in HO and this pattern was comparable to the reduction found in old flies (D). Figures (C) and (D) were generated from other lab member's data. Stars indicate significant difference compared to normoxia (N) control according to two-way ANOVA *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

showed daily fluctuations. We determined that both CG15784 and bnl showed diurnal 24h profiles, with a phase similar to the rhythms found in old flies (Figure 7). bnl was significantly upregulated compared to its normoxia trough on both day 3 (p< 0.05) and day 4 (p<0.0001) at ZT 12 and ZT 16. CG15784 also had significant peaks on days 3 and 4 at ZT 12 and ZT 16. The fold change between the trough and peak was greater on day 4 than day 3 for both genes.

Other LLCs, namely *ImpL3, Hsp22,* and *CG7130*, displayed phaseshifted peaks in hyperoxia compared to rhythms in old flies. Their mRNA levels peaked at approximately ZT4-ZT8, especially after 72h hours in constant hyperoxia (day 4). *ImpL3* displayed significant (p<0.001) upregulation on days 3 and 4 at ZT 4 and ZT 8 and on day 4 at ZT 12. Both *CG7130* and *Hsp22* showed peak expression on day 4 at ZT 4. These genes were rhythmically expressed in response to hyperoxia; however their peak expression exhibited an advanced phase shift compared to old flies under normoxia.



Figure 7. Hyperoxia induces rhythmic expression of LLCs in young flies mRNA levels of *bnl* (A) and *CG15784* (B) showed significant rhythm on day 3 and 4 in hyperoxia (HO) with a peak at ZT12-16. (C) *ImpL3* was induced in both day 3 and day 4 in HO, but with a broad peak at ZT4-12. HO also induced rhythmic expression of *Hsp22* (D) and *CG7130* (E) with significant peaks at ZT4 on day 4 only. All LLCs were induced on day 3 and rhythmic on day 4 in HO. Stars indicate significance from the trough of each respective condition (test of rhythmicity) as calculated by two-way ANOVA *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

3.3 Effects of hyperoxia on LLC expression in flies with disrupted clock

Given the clear rhythms found in LLCs in response to oxidative damage, we next tested whether or not those rhythms could be attributed to the clock that functions in hyperoxia. To study the clock's involvement, we measured gene expression under hyperoxia in both wild type flies and mutant flies missing the core clock gene, *cyc*. This gene encodes a positive element of the clock feedback loop. The cyc^{01} flies are behaviorally arrhythmic both in LD cycles and constant darkness (DD) (Rutila et al., 1998). The cyc^{01} flies were placed under the same conditions as the *white* controls with an intact clock. We predicted that if the clock was involved in the expression of LLCs, then we would see an induced rhythmicity for wild type flies and the absence of such rhythms in *cyc* mutants.

We measured diurnal profiles of two genes, *bnl* and *ImpL3*, in wildtype flies and *cyc* mutants under oxidative stress. In the case of *bnl*, the significant rhythm in response to hyperoxia was lost in *cyc⁰¹* mutants (Figure 8A). In contrast, *ImpL3* showed significant (p<0.05) rhythms in *cyc⁰¹* flies, while *white* flies did not show significant oscillations in *ImpL3* mRNA levels (Figure 8B). Because of the unusual lack of rhythms of LLCs in control flies, expression of other LLCs in *cyc⁰¹* mutant flies were not measured.

Due to the variable response to hyperoxia at the gene expression level in cyc^{01} flies, we were interested in survival under hyperoxia in 5 day old *white* and cyc^{01} female flies. These flies were placed in a hyperoxia chamber and their survival was recorded starting at ZT12 on the first day in hyperoxia and every 12 hours thereafter until no flies remained alive. As previously reported (Gruenewald et al., 2009), hyperoxia overall significantly decreases the lifespan of flies. There was no significant difference in survival between *white* and *cyc* mutant flies. As depicted in the survival graph in Figure 8C, by the end of day 4 (96h in HO) there was about an 80% survival rate for *white* and *cyc*⁰¹ mutant flies. By the end of day 5 (120h in hyperoxia), no flies remained alive. The fact that survival was similar between *white* and *cyc*⁰¹ mutants may be related to the fact that one of the LLCs was lower in *cyc*⁰¹ while the other was higher.

3.4 Effects of constant darkness has differential effects on LLC expression in hyperoxia

Data from Figure 7 suggests that both the role of clock and light could contribute to the expression of the LLCs. Based on the fact that the mRNA levels of three of the LLCs raised sharply within 4 hours of lights on, we hypothesized that light is involved in the activation of these genes. To test this, we exposed young *white* flies to hyperoxic conditions in LD for 0-72h, as described in the previous experiments. After 72h of exposure, we transferred half of the flies into constant darkness (DD) while maintaining hyperoxia exposure (see Figure 4 from Methods). Normoxia flies were also transferred into DD simultaneously. On the 4th day of hyperoxia, which also corresponds to the first cycle in DD, we collected samples every 4h for 24h for mRNA expression analysis.



Figure 8. Disruption of clock function has differential effects on LLC expression in hyperoxia

24h mRNA expression levels were compared on day 4 in hyperoxia (HO) of both *white* and cyc^{01} flies for *bnl* (A) and *ImpL3* (B). *bnl* was not significantly rhythmic in cyc^{01} mutants compared to induced rhythmicity of *white* flies. *ImpL3* continued to oscillate in HO in cyc^{01} mutants, peaking at ZT 4. C) Survival under HO was not significantly different between *white* and cyc^{01} flies. Stars indicate significance from the trough of each respective genotype (test of rhythmicity) according to two-way ANOVA *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

One LLC gene, *bnl*, was significantly rhythmic in DD (Figure 9A), but its peak expression was phase-advanced, peaking at ZT 8 in DD compared ZT12-16 in LD. The other four genes examined, *Hsp22*, *CG7130*, *ImpL3* and *CG15784*, did not display oscillations in their mRNA levels in DD (Figure 9B-E) while LD controls showed expected rhythms. We observed that *ImpL3* and *CG7130* diurnal expression was lost in constant darkness, displaying low and arrhythmic expression similar to normoxia (Figure 9 C and E). *CG15784* and *Hsp22* were non rhythmic, but still significantly (p<0.05) induced in DD hyperoxia compared to DD normoxia controls, but at levels much lower than those in LD.

Given that most LLC peaks were abolished under hyperoxia in DD, it was of interest to determine whether survival under hyperoxia was different for LD and DD flies. To test this, we exposed day 5 *white* female flies to hyperoxic conditions and recorded their survival every 12 hours until no flies remained alive. Interestingly, there was no significant difference in survival between LD and DD flies under hyperoxia (Figure 9F).



Figure 9. Constant darkness has differential effects on LLC expression in HO LLC expression measured in *white* flies on day 4 of HO in LD and DD. *bnl* (A) showed significant rhythms in DD. However, expression levels of *CG15784* (B), *ImpL3* (C), *Hsp22* (D) and *CG7130* (E) were increased and rhythmic in LD, but not DD. Expression of *Hsp22* and *CG7130* was significantly elevated in response to HO in DD, compared to DD normoxia (DD N). However, none except *bnl* were observed to be rhythmically induced in DD as seen in LD. Survival of *white* flies in HO in LD and DD were very similar, but significantly different (F). Stars indicate significance from the trough of each respective condition (test of rhythmicity) as calculated by two-way ANOVA *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

4 Discussion

4.1 Hyperoxia induces rhythmic expression of LLCs

Results of our study support our hypothesis that age-related oxidative stress may contribute to the rhythmic expression of late life cyclers (LLCs) in aging flies. We determined that LLC's pattern of expression from old flies can be mimicked in young flies exposed to hyperoxia. The five LLCs that we investigated have been shown to be upregulated in hyperoxia in a previous microarray study (Gruenewald, 2009); however, their expression was measured at one time point only and the mechanism responsible for the increased expression was not studied. Here, we show that these genes are not constantly upregulated in response to oxidative stress; rather they show robust diurnal rhythms in their mRNA levels. We also determined that the mechanism behind these oxidative stress response genes is multifaceted with both clock and light contributing to regulation of LLC expression in hyperoxia.

4.2 Expression of clock genes under hyperoxia

We determined that the clock is functional in young flies under hyperoxia. Clock genes were rhythmically expressed whith a phase similar as in normoxia control flies. However, the peak levels of *per* mRNA were significantly higher while peak levels of *tim* mRNA were significantly lower on the fourth day in hyperoxia compared to normoxia controls. Interestingly, this response of *per* and *tim* to hyperoxia is very similar to patterns obtained for both genes in old normoxia flies (Figure 6). These data suggest that endogenous oxidative stress may modulate the expression of these two clock genes in old flies.

The results obtained from the *white* and *cyc⁰¹* mRNA expression level experiments led to a shift of focus. We proposed a new hypothesis that light may be playing a part in the activation of the transcription of some LLCs. With this new objective, we exposed *white* flies to hyperoxia in both LD and DD and found that indeed light may have a role regarding LLC expression.

4.3 Response to oxidative stress of the bnl gene is controlled by the circadian clock

After determining that the clock is functional under hyperoxia, we asked whether it controls LLC rhythms observed under these conditions in young flies. Our data suggest that *bnl* is a clock-controlled gene. In support of this, a distinct diurnal rhythm was observed in *bnl* in response to HO in young flies with a phase similar as in the heads of old flies (day 75) (Figure 3).

We obtained a second line of evidence by studying flies with a null mutation of the clock gene *cycle*. The *bnl* rhythm was abolished in *cyc⁰¹* mutants with mRNA expression levels significantly lower at ZT12-16 compared to the peak found in *white* flies with a functioning clock. Lastly, we determined that *bnl* expression remained rhythmic in constant darkness, peaking at ZT8 rather than ZT12-16 as in LD. These data suggest that because *bnl* continues to oscillate in a circadian fashion without the presence of light, it is not regulated by light itself.

The results of these independent experiments suggest that *bnl* is under circadian clock control. Given the mechanism of the circadian clock (Figure 1, Introduction), and the decline of mRNA levels of *bnl* in *cyc*⁰¹ mutants, it is likely that the core clock genes indirectly control the expression of this stressresponse gene. To the best of our knowledge, circadian control of *bnl* has not been reported, most likely because previous studies of circadian gene expression were done in young flies in normoxia (Rodriguez, 2013). The best understood role of the *bnl* gene is in response to oxygen deprivation in hypoxia; its full name *branchless* was given because of the massive tracheal branching that occurs as a result of its overexpression in *Drosophila* (Sutherland, 1996). It belongs to fibroblast growth factor (FGF) family and was shown to be expressed in the mammalian nervous system. Understanding the significance of rhythmic *bnl* expression in aging flies will require further studies. An important next step would be to measure BNL protein levels under the same conditions to see if they exhibit similar rhythmic patterns and measure aging parameters in flies with genetically manipulated *bnl* expression.

4.4 Light may be involved in the regulation of several LLCs under hyperoxia

Despite the strong rhythms in mRNA levels of *Hsp22*, *CG15784*, *ImpL3*, and *CG7130* induced by prolonged exposure to hyperoxia (Figure 7) in young flies, they were not rhythmically expressed in constant darkness. These data suggest that light plays an important role in their induction. The fact that mRNA of these genes increased sharply within four hours of lights on also supports the regulatory role of light in regulation of LLCs under hyperoxia. In old flies, mRNA of these LLC genes show tendency to rise gradually through the 12 hours of light and then decline after lights off (Figure 3), suggesting that light also modulates expression of these genes in old flies. Our data provide a foundation for investigating the complex mechanism which mediates the effects of light, circadian clock, and oxidative stress on the expression of LLCs in old flies.

4.5 The roles and significance of rhythmic LLC expression

Our project revealed that LLCs are not only rhythmic in old flies, but are also rhythmically induced in young flies under hyperoxia. All 5 genes were previously reported to be activated by hyperoxia in young flies as well as in old flies under normoxia when measured at one unspecified time point (Landis, 2004; Gruenewald, 2009). Here we show that the expression of the genes is robustly rhythmic under both conditions.

Three genes in this group, *Hsp22, CG15784* and *CG7130*, are in a larger grouping of proteins called chaperone proteins. These proteins play a protective role during times of environmental stress, such as heat shock or hyperoxia. These molecular chaperones interact with a diverse set of misfolded proteins to aid in their proper folding and prevent protein damage

and apoptosis. Importantly, high levels of Hsps are detected in tumors. This is consistent with the idea that Hsps can be involved in preventing cell death (Jolly & Morimoto, 2000). Pathological stress can promote the formation of chaperonopathies (chaperones which function normally, but favor processes that lead to disease). This new concept has led to Hsps becoming the target for anticancer therapies (Rappa et al., 2012). On the other hand, chaperones can also be induced to fight against diseases by activating the immune system and providing a defense mechanism against stress (Rappa et al., 2012). We hypothesize that periodical expression of chaperones during aging can provide neuroprotection while preventing detrimental effects of constant overexpression. The same may be true for other genes investigated in this study. For example, *bnl* is an FGF activated by hypoxia to stimulate cell growth and divisions. Rhythmic expression of *bnl* could prevent uncontrolled cell proliferation.

Finally, we report a robust rhythm in *ImpL3*, a gene that encodes the sole lactate dehydrogenase in flies, an enzyme that catalyzes the conversion of lactate to pyruvate and vice versa. Mammals have two genes serving this function, *LDHA* and *LDHB*. Interestingly, expression of *LDHA* mRNA and activity of LDH enzyme display diurnal rhythms in the central clock neurons in mammals (Isobe, 2011). Recent data suggest that the expression of *LDH* is high in cancer cells, but low in aging brains, and especially in animal models of Alzheimer disease (Harris, 2014). These data are consistent with our

hypothesis that periodical expression of LLCs could promote healthy aging better than constant levels. Recent experiments in our lab suggest that genetic manipulations of *ImpL3* that caused either constant overexpression or constant reduction of this gene both led to significantly shorter lifespan compared to wild type flies (Eileen Chow, personal communication). (Akerfelt, 2010)

The genes associated with metabolism are often found to be expressed rhythmically. *ImpL3* is no exception in aging flies. An organism's ability to make metabolic shifts in allocation of energy in response to stress is a crucial mechanism. When organisms are exposed to dangerously high ROS levels, they redirect their flux from glycolysis to the pentose-phosphate pathway which provides the cell with much needed reducing power to combat oxidative damage (Grant, 2008). Lactate dehydrogenase (LDH) is the mammalian homolog of *ImpL3* in *Drosophila melanogaster*. LDH catalyzes the conversion of lactate to pyruvate, and vice versa. Not only is it an essential enzyme for metabolic purposes, but its upregulation in response to hyperoxia and in old mice (Poon et al., 2006) and flies (this research) suggests its role as a stressresponse gene. Changes in metabolism that occur due to aging may be linked to diseases such as cancer and neurodegenerative diseases, which rouses the study of metabolic regulatory enzymes such as *ImpL3* as novel targets for gene therapy (Harris et al., 2014).

4.6 Implications of this research for health and diseases

Circadian clocks are important regulators of many of life's functions including hormonal, neural, and temporal regulation. Disruption of such rhythms contributes to a variety of diseases (Reddy and O'Neill, 2009) while lack of these core clock genes altogether results in premature aging (Krishnan et al., 2009; Krishnan et al., 2012). Our data provide novel information regarding the involvement of the circadian clock and light in oxidative stress defense systems. ROS-related diseases can be due to either ROS depletion (e.g., chronic granulomatous disease, certain autoimmune disorders) or an excess of ROS (e.g., cardiovascular disease, cancer, Alzheimer's disease, and Parkinson's disease) (Brieger, 2012). The disruption of biological clocks, such as in swing shift workers, has been associated with increased rate of tumor formation (Antoch et al, 2008). Interestingly, increased levels of ROS have been detected in various types of cancer suggesting that ROS contributes to tumor expansion (Liou & Storz, 2010). This crucial connection between preservation of biological clocks and protection against disease is also seen in per genes and their role as tumor suppressors (Reddy & O'Neill, 2010). Data presented here suggest clock involvement in regulation of repair of oxidative damage caused by ROS. Our data provide the basis for investigations of whether or not manipulation of the clock and/or light results in reduced incidence of disease, improvement of quality of life, and promotion of healthy aging.

4.7 Future directions

Our research raises several questions that need to be addressed in future experiments. Now that it has been demonstrated the circadian clock genes regulate oxidative stress response gene *bnl* at the mRNA level, the expression profile of protein levels of BNL should be determined using a Western blot. The daily expression profiles of the other cytokines should be measured to determine if the circadian clock regulates all enzymes of this family.

The mechanism for a light-mediated response should also be further investigated. CG15784, CG7130, Hsp22, and ImpL3 all displayed lightdependent expression; it would be important to determine whether this is mediated through photoreceptor protein CRY or other light-sensitive proteins. To better understand the role of light, 24h mRNA expression profiles could be examined in differing light intensities. If light is involved in the regulation of these genes, we would observe corresponding expression levels with varying light intensities. Our data showed similar longevity in hyperoxia of young flies kept in DD and those in LD. Even in the absence of diurnal expression of the LLCs, flies kept in DD continued to survive just as well as those kept in the presence of light. This lack of difference in survival suggests that light may have detrimental effects to some degree. Recently, it has been determined that blue light irradiation induces the production of ROS, resulting in adverse effects on human gingival tissue (Yoshida et al., 2013). These data should stimulate future experiments regarding the

mechanisms behind both light and the circadian clock in the regulation of LLCs.

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