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Abstract approved:

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The free-radical theory of aging proposes that intracellular accumulation of oxidative damage caused by reactive oxygen species (ROS) is responsible for the process of senescence. ROS are produced during normal aerobic metabolism and have been shown to cause numerous cellular and sub-cellular defects that lead to termination of the cell cycle and, in extreme cases, to apoptosis (cell death). Previous research has suggested that important antioxidant enzymes and other low molecular weight antioxidants may exhibit differential levels of expression throughout the day/night cycle. These expression trends appear to be under the control of biological clock genes, which interact to generate circadian rhythms within organisms. There are several clock genes that are essential for proper clock function in *Drosophila melanogaster*, the most important of which are *period (per)* and *timeless (tim)*; a null mutation in either of these genes causes a loss of clock function in the organism. Since biological rhythms regulate many important processes, they may be involved in the antioxidant defense system. The overarching hypothesis of this study was that the loss of biological rhythmicity exhibited by mutant flies may render them more susceptible to the negative effects associated with ROS exposure. The main objective of the current study was to investigate the intersection of circadian rhythmicity, oxidative stress, and the process of aging in *Drosophila*. We show that *period* mutants have increased susceptibility to oxidative stress, when compared to their wildtype (CS^P) counterparts, indicating that the *period* gene is somehow involved in the oxidative stress response system. However, experiments on the enzyme catalase show that one rhythmically expressed enzyme cannot explain the physiological response exhibited by the organism. Accumulation of oxidative damage, as measured by protein carbonylation, appears to be rhythmic, suggesting a possible explanation for the differential susceptibility to exogenous oxidative stress.

You Can't Beat the Clock: Oxidative Stress and *period* Mutants in *Drosophila melanogaster*

By

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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.

Andrew J. Davis

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1. Introduction and Background

1.1. Circadian organization, rhythmicity, and molecular clock components

Circadian rhythmicity is a fundamental biological process that is found in all groups of organisms. Intricate timekeeping systems have been identified in bacteria, fungi, plants, and animals, and have been implicated in many important cellular and organismal functions (Young & Kay, 2001). Circadian oscillations create temporal variations in processes ranging from gene expression and metabolic activity to sleep/wake cycles and hormonal release patterns. Daily rhythms in both physiology and behavior are endogenously generated via the interactions of various genes and proteins and are entrained by a variety of external cues such as light and temperature. Persistence of the rhythm in the absence of these external cues is characteristic of the biological clock, and further indicates the precise nature of the internal timekeeping system (Allada et al., 2001). By definition, circadian ("circa" – about, "diel" – day) rhythms operate on an approximately 24-hour cycle; thus, the light/dark cycle of the earth acts as a synchronizing mechanism that keeps organisms in tune with their environment (Allada et al., 2001).

Two transcription/translation feedback loops produce rhythmic behavior

The basic mechanism underlying circadian timing lies with a group of genes known collectively as "clock genes," which interact in two transcription/translation feedback loops (Figure 1). In *Drosophila melanogaster*, there are two "core" clock genes – *period* (*per*) and *timeless* (*tim*) – which have been shown to be rhythmically transcribed. These genes encode the proteins PER and TIM respectively; unsurprisingly, both of these proteins also exhibit rhythmic

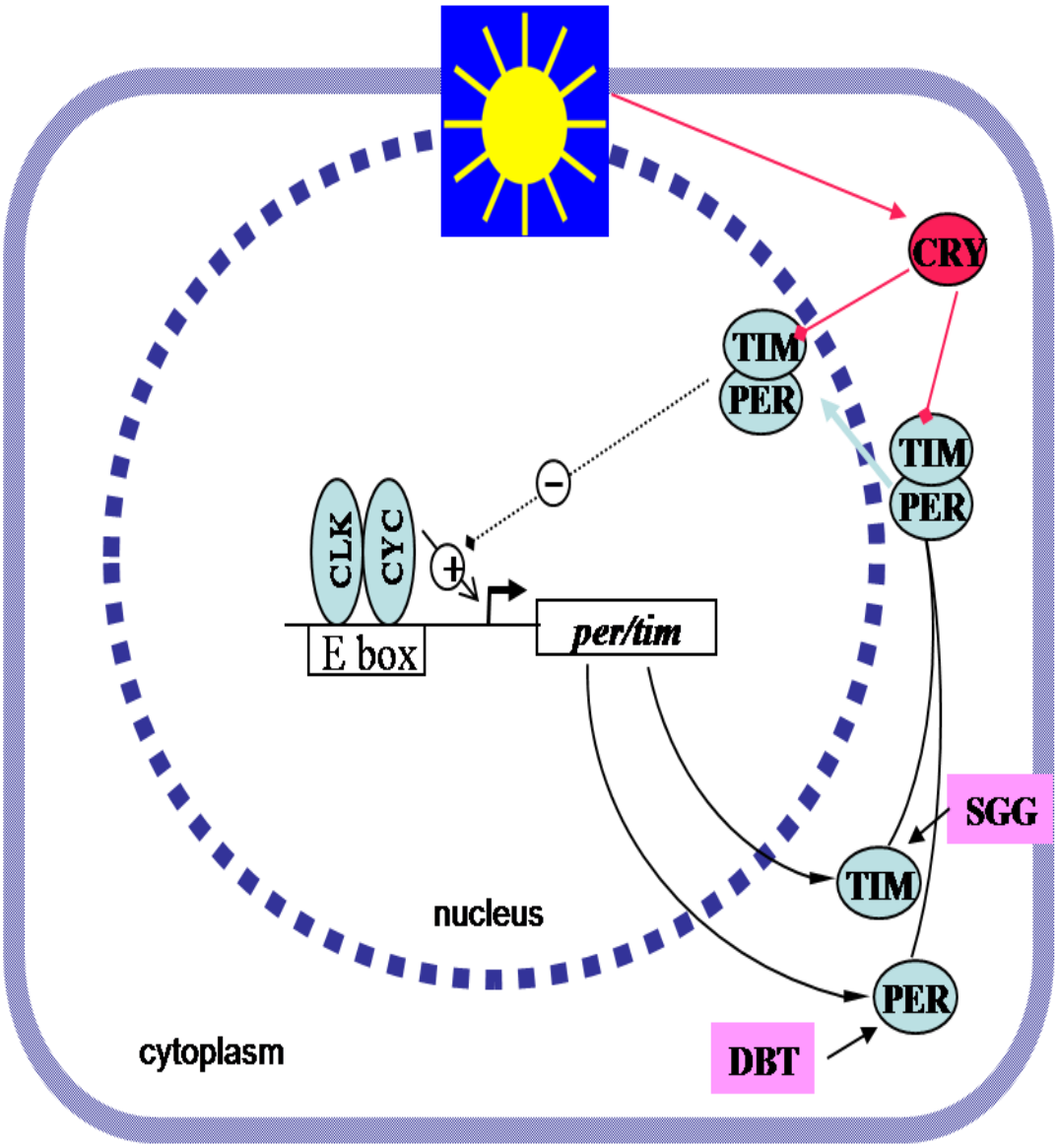

Figure 1: The basic molecular clock mechanism of PER and TIM

Transcription of the genes *per* and *tim* is activated by the positive elements CLK and CYC. PER and TIM proteins heterodimerize in the cytoplasm and localize to the nucleus in the evening, where they act in a negative fashion – interfering with the function of CLK and CYC – thus repressing their own transcription. The photoreceptive protein CRY rapidly causes the degradation of TIM in the presence of light, while the protein kinases DBT and SGG phosphorylate PER and TIM respectively.

expression (Young & Kay, 2001). As *per* and *tim* mRNA levels rise during the day, there is a corresponding accumulation of PER and TIM protein in the cytoplasm of the cell (Dunlap, 1999). Both of these proteins are negative elements of the clock that localize in the nucleus and suppress the function of two additional clock genes, *Clock* (*Clk*) and *cycle* (*cyc*), by interfering with their protein products, CLK and CYC (Scully & Kay, 2000). In order for PER and TIM to enter the nucleus, however, they must first form heterodimers in the cytoplasm. PER is known to be unstable on its own, and consequently needs this interaction with TIM to stabilize and promote nuclear localization (Dunlap, 1999). Nuclear localization of PER and TIM does not occur immediately, rather, these compounds appear to stay in the cytoplasm until the evening hours, when there is a mass migration into the nucleus (Scully & Kay, 2000). Recently, the model that PER and TIM translocate to the nucleus as obligate heterodimers has been cast into doubt. Single cell imaging assays monitoring fluorescent forms of PER and TIM have shown that PER and TIM may independently translocate to the nucleus (Mayer et al., 2006). Clearly, the mode of PER and TIM translocation remains a controversial issue within the scientific community.

Once the PER and TIM complexes have entered the nucleus, they interact with, and suppress the action of, CLK/CYC heterodimers. These CLK/CYC compounds act as positive elements that promote *per* and *tim* transcription by binding to E-boxes in the promoter regions of these genes (Young & Kay, 2001). While the exact mechanism of PER/TIM interference is unknown, it has been suggested that the PER/TIM complexes form trimers or tetramers with the CLK/CYC complexes, resulting in a diminished capacity of CLK/CYC to bind to the E-boxes (Scully & Kay, 2000). It should also be noted that PER alone may be sufficient to repress the activity CLK/CYC (Chang & Reppert, 2003). As with *per* and *tim*, *Clk* is also rhythmically transcribed, and the amount of CLK has been shown to vary throughout the day. These two rhythms appear to be

in antiphase (while *per* and *tim* are most actively transcribed, *Clk* is relatively dormant, and vice versa).

Additional clock genes refine the basic clock functionality

Several additional genes are also involved in the functioning of the clock. These genes — *double-time* (*dbt*), *vrille* (*vri*), *shaggy* (*sgg*), and *cryptochrome* (*cry*) — have a variety of functions. Both *dbt* and *sgg* are protein kinases that are constitutively expressed. *Double-time* associates with the PER/TIM complexes and also promotes the degradation of cytoplasmic standalone PER by phosphorylation (Young, 2000). *Shaggy*, on the other hand, phosphorylates TIM to promote the nuclear translocation of the PER/TIM complexes. Accumulations of the protein encoded by *vri* serve to repress the transcription of *Clk*, creating yet another regulatory element of the clock. Finally, the protein encoded by the *cry* gene serves as a photoreceptor specific to the clock; upon light exposure, CRY complexes with TIM, promoting rapid cytoplasmic degradation of TIM via proteasomes (Ashmore, 2003).

1.2. Circadian clocks influence physiology

While the molecular mechanism of the clock is relatively well understood, the pathways that link biological timekeeping with specific cellular and physiological processes are not. These pathways include clock-controlled effector genes which are expected to be rhythmically expressed. The search for rhythmically expressed genes has recently become possible with the advent of genome-wide expression analysis via microarrays. Several research groups have studied global gene expression as a function of time of day in various tissues of both mice and *Drosophila* (Duffield, 2003). These studies revealed that approximately 10% of the genes probed were rhythmically expressed; these clock controlled genes belong to many gene families, covering many cellular functions. Among the genes showing daily oscillations in expression were

genes involved in neural function, metabolism, protein folding and turnover, and the regulation of the redox state (Duffield, 2003). One of the studies compared patterns of circadian gene expression in *Drosophila* heads and bodies (Ceriani et al., 2002). This study revealed highly rhythmic expression of the enzyme catalase, especially in *Drosophila* bodies (Figure 2). Rhythmic expression of copper-zinc (cytoplasmic) superoxide dismutase was also noted. Both enzymes are known to be involved in the antioxidant defense system, which protects organisms from oxidative stress.

1.3. Circadian rhythmicity, oxidative stress and antioxidant defense systems

Circadian organization, by nature, may lead to the rhythmic formation of reactive oxygen species (ROS) and other stressors (Hardeland et al., 2000). The periodic formation of these harmful molecules could be a consequence of rhythmic control of metabolism, as organisms are known to endogenously produce these molecules as byproducts of normal metabolic processes, most notably cellular respiration. Organisms are also known to exhibit higher levels of cellular respiration during periods of activity, providing further support for the notion that ROS may be rhythmically generated. The environment is known to contribute to ROS formation as well; these exogenous sources of ROS may however also exhibit circadian periodicity due to organismal activity rhythms timed to avoid the deleterious effects of light and free oxygen (Sharma, 2003).

Production of ROS

Aerobic organisms are known to produce reactive oxygen species as byproducts of normal metabolism (endogenous sources), while the environment is also known to contribute to ROS production (exogenous sources) (Figure 3). Cellular metabolic reactions, most notably the electron transport system (ETS) found in the mitochondria, are mainly responsible for the

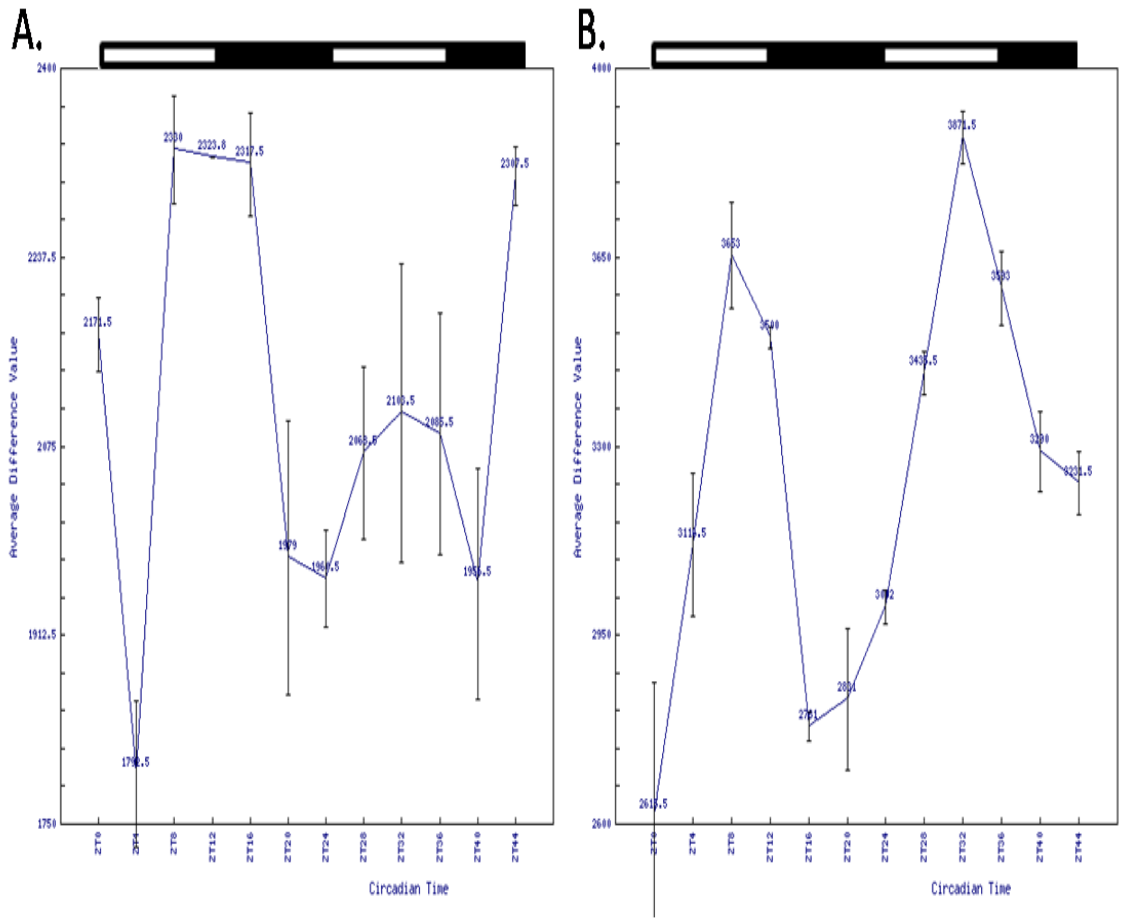

Figure 2: Rhythmic expression of catalase mRNA

A. Expression of catalase mRNA in *Drosophila* heads. Some cycling is suggested, but standard error measurements cast doubt on the precise nature of expression. **B.** Expression of catalase mRNA in *Drosophila* bodies. A distinct pattern of expression is seen, with mRNA levels rising during the day and falling during the night. Data from Ceriani et al (2002).

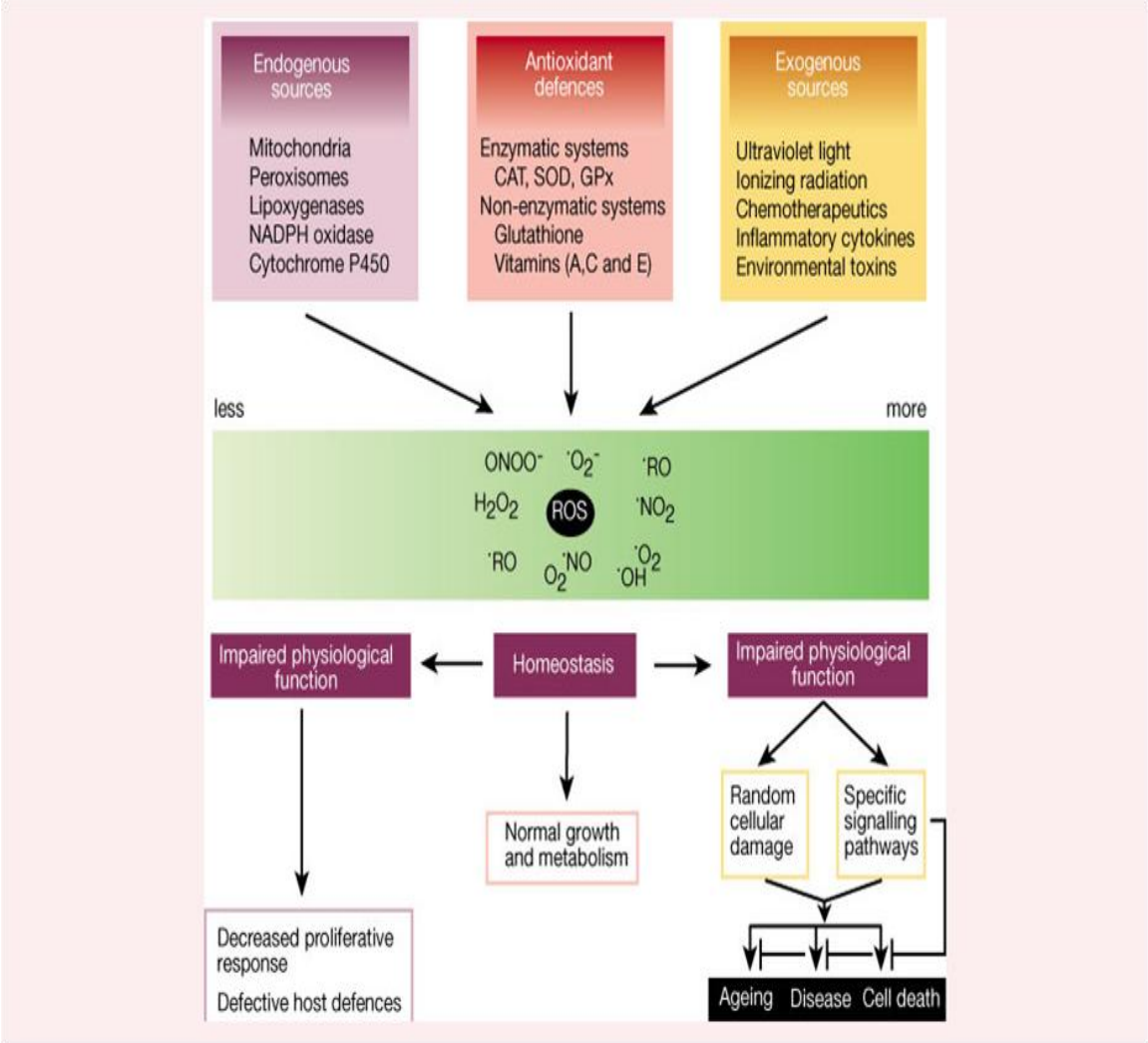

Figure 3: ROS sources, molecules, and effects

Reactive oxygen species are produced both endogenously and exogenously. At normal levels these molecules are important as second messengers and in the maintenance of homeostasis. At extremely low or extremely high levels, ROS have negative effects; aging is thought to occur as a result of ROS accumulation and the resulting cellular damage caused by these molecules. Figure from Finkel and Holbrook (2000).

endogenous production of these highly reactive molecules, although other biological processes such as lipid metabolism in the peroxisome and the activity of certain enzymes in both the cytosol and the various cellular and sub-cellular membranes have also been implicated in ROS production. Exogenous sources of ROS include UV light and ionizing radiation as well as chemicals and other toxic substances found in the environment (Finkel & Holbrook, 2000).

It is estimated that 90% of cellular ROS are produced in the mitochondria (Balaban et al., 2005). We now know that ROS production can be pinpointed even more specifically within the mitochondria — complex I (NADH dehydrogenase) and complex III (ubiquinone-cytochrome *c* reductase) are the main culprits, with the majority of ROS production occurring at complex III (Finkel & Holbrook, 2000). It has been estimated that 1-2% of the total daily oxygen consumed by the mitochondria is ultimately transformed into superoxide anion. While this may not seem like much, a 60 kg woman would produce between 160 and 320 mmol of superoxide anion per day while a man weighing 80 kg would similarly produce between 215 and 430 mmol due to cellular respiration alone (Cadenas & Davies, 2000).

The Janus-nature of ROS

There are numerous ROS molecules, the most biologically significant of which are superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), nitric oxide radical ($NO^{\bullet}$), singlet oxygen ($^1O_2$), and most importantly, hydroxyl radical ($OH^{\bullet}$). Some of these molecules, such as superoxide anion and hydroxyl radical, are extremely unstable and thus short-lived species; others, notably hydrogen peroxide, are relatively stable and long-lived species. ROS, like many things, are beneficial in moderate amounts. At physiological levels, reactive oxygen species are used as signaling molecules (especially nitric oxide radical) and are important in the maintenance of homeostasis as well as in normal growth and development. ROS have also been

shown to be important contributors to antibacterial defense (Hardeland et al., 2003). New evidence implicates ROS in numerous stress pathways, and it has been suggested that this indicates an important step as organisms evolved to live in an aerobic environment. The importance of ROS in stress pathways points to the conclusion that rising levels of oxidants may act in a broader fashion as signals of all types of cellular stress (Finkel & Holbrook, 2000).

As ROS levels rise above normal concentrations, they react randomly and indiscriminately with cellular components, causing cytotoxicity, damage to cellular structures, apoptosis, or necrosis (Korsloot et al., 2004). Of the ROS molecules mentioned, hydroxyl radical is considered to be the most dangerous in biological systems. This molecule is known to attack proteins, causing fragmentation and denaturation, nucleic acids, causing base alterations and strand breaks, and lipids, causing peroxidations (Korsloot et al., 2004). For reasons of proximity, superoxide anion produced from complex I of the ETS is known to react quite often with nearby iron-sulfur clusters, forming iron radical, which can produce hydroxyl radical through Fenton chemistry (Cadenas & Davies, 2000). Iron-sulfur clusters are not, however, the only targets of ROS. Proteins, lipids, and nucleic acids can also undergo reactions with reactive oxygen species, and each of these reactions forms a characteristic product: proteins form carbonyls, lipids form peroxidation products, and nucleic acids form oxidized bases. Protein carbonyls, lipid peroxidation products, and oxidized bases can all be used as biomarkers of oxidative stress. Lipid peroxidation products are known to react extensively with themselves and with other cellular components, making them problematic as biomarkers of damage due to ROS (Coto-Montes & Hardeland, 1999). Protein carbonyls, on the other hand are very reliable as biomarkers of oxidative damage, as the addition of the carbonyl group represents the end of the reaction chain.

Antioxidant defense systems

To ensure ROS do not wreak internal havoc, cells possess two essential defense mechanisms: a suite of antioxidant enzymes and a set of low molecular weight antioxidants (LMWAs) — small, non-enzymatic molecules — that are used to scavenge and neutralize highly reactive oxidant molecules. The majority of ROS defense falls to the enzymatic systems. Enzymes of the superoxide dismutase, catalase, peroxiredoxin, and glutathione peroxidase families are all important contributors to oxidant scavenging and neutralization. On the non-enzymatic side are a number of compounds such as glutathione, urate, pyruvate, and vitamins A, C, and E (Finkel & Holbrook, 2000). These antioxidant defenses work together to maintain normal levels of ROS, thus ensuring that an organism is not overwhelmed by a surfeit of oxidants and does not undergo the rigors of oxidative stress.

The enzyme superoxide dismutase (SOD, EC 1.15.1.1) is perhaps the first line of defense against ROS production. This enzyme dismutates superoxide anion into oxygen and hydrogen peroxide via a catalytic metal. SOD is known to exist in two forms — a manganese form located in the mitochondrial matrix, and a copper/zinc form found in the cytoplasm. Both forms of SOD catalyze the reaction converting superoxide anion to hydrogen peroxide, which is then broken down to water and oxygen by catalase (Figure 4). Alternatively, hydrogen peroxide can be broken down by the glutathione peroxidases (GSH-$P_{x,}$ EC 1.11.1.9) to form glutathione and water; interestingly, it seems that insects and other arthropods do not possess glutathione peroxidase and as such must rely upon the services of another related enzyme, glutathione S-transferase (GST, EC 2.5.1.18)), which also exhibits a peroxidase activity (Hardeland et al., 2000). The enzyme catalase (EC 1.11.1.6) is found in peroxisomes, and it has been shown that *Drosophila melanogaster* also possess this enzyme in the cytoplasm (Korsloot et al., 2004).

Cytoplasmic catalase, along with the peroxidase functionality of GST, may help to alleviate the lack of GSH-P_x (Korsloot et al., 2004).

1.4. Oxidative stress and aging

The process of aging has intrigued researchers for many years. Many scientists have focused on this important phenomenon, but it is still poorly understood. One of the first theories proposed to explain the process of aging is known as the rate of living theory. This theory, posited in 1928 by Raymond Pearl, was based upon the observation that organisms with a faster metabolic rate led correspondingly shorter lives. Specifically, Pearl proposed that the metabolic rate of an organism ultimately determines that organism's maximum lifespan. The main criticism of Pearl's theory was that it was based entirely upon observation and had no support from molecular data; however, in 1956 Denham Harman suggested a biochemical explanation. His theory — the free radical theory of aging — proposed that cellular accumulation of oxidative damage, caused by free radicals and other ROS, results in the cessation of the cell cycle and, if the damage is too great, in apoptosis (cell death). The accumulation of cellular damage is due to oxidative stress, which is defined according to the balance of oxidants and antioxidants; when the relative number of oxidants outweighs the number of antioxidants, the organism experiences oxidative stress. According to the theory, this molecular and cellular damage ultimately disturbs homeostasis to such an extent that senescence is the consequence. Further research on aging and oxidative stress has shown that organisms with higher metabolic rates tend to produce more ROS, and consequently, have a shorter maximum lifespan. Perhaps not surprisingly, mitochondria, and specifically mitochondrial DNA (mtDNA), have been shown to be especially susceptible to damage from ROS; this is thought to be due in large part to the

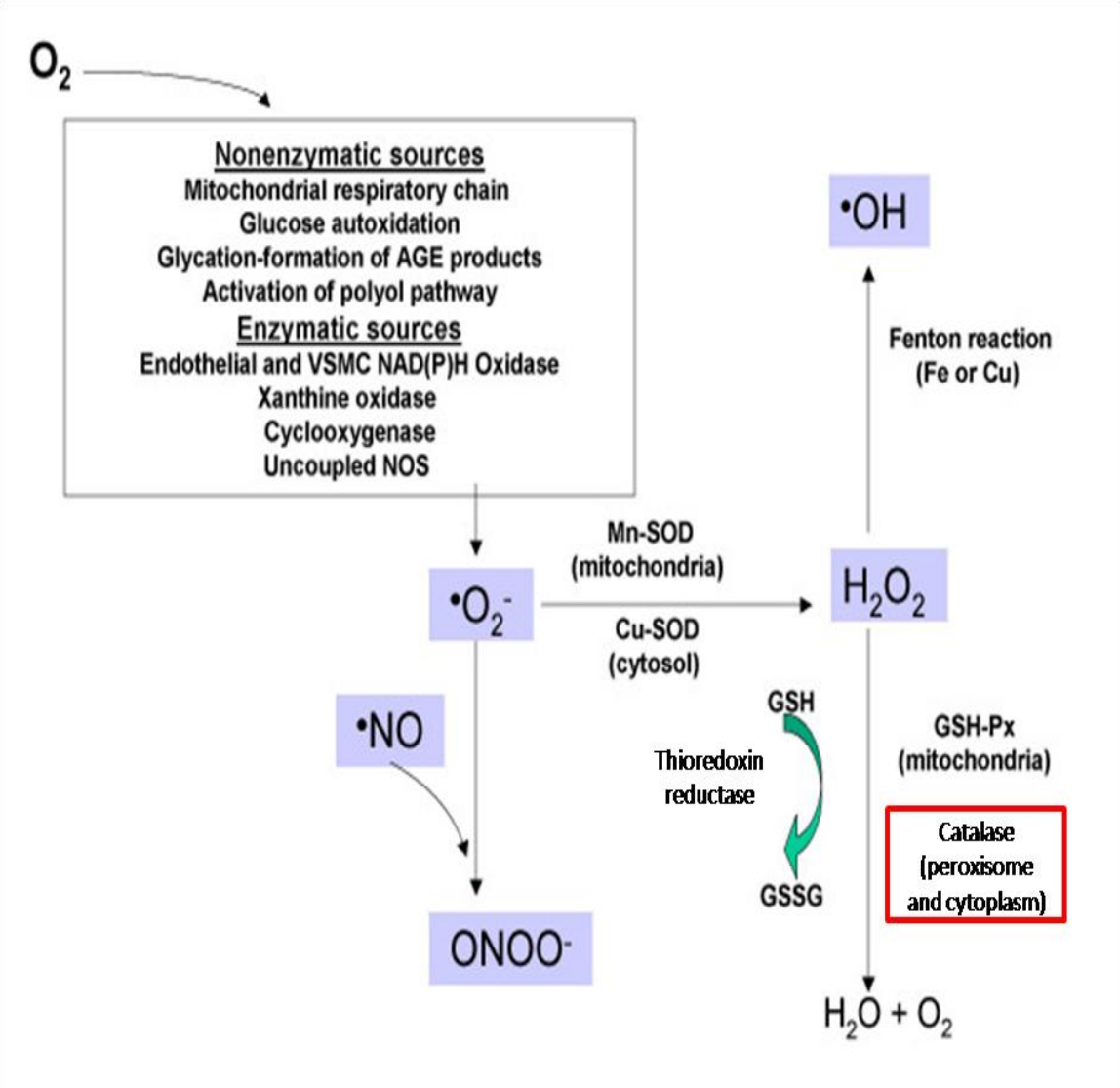

Figure 4: ROS interactions with defensive enzymes

Superoxide anion ($O_2^{\bullet-}$) is an important progenitor of other ROS, notably hydroxyl radical ($OH^{\bullet}$). $O_2^{\bullet-}$ can nitric oxide radical ($NO^{\bullet}$) to form peroxynitrite ($ONOO^-$), which then decomposes to $OH^{\bullet}$ in the presence of iron. Alternatively, superoxide dismutase can enzymatically dispose of $O_2^{\bullet-}$, forming hydrogen peroxide (H_2O_2). H_2O_2 is then either enzymatically neutralized (by catalase or other defense enzymes) or converted to $OH^{\bullet}$ by the Fenton reaction. Catalase, which was the focus of this study, is highlighted. Figure from Johansen et al (2005).

proximity of mtDNA to sources of ROS and the fact that mtDNA is not covered in protective histone proteins (Balaban et al., 2005). This situation creates a vicious cycle: as organisms age, their mitochondria become increasingly damaged, which in turn decreases the mitochondrial efficiency (thus increasing the amount of ROS released), leading to yet more damage (Finkel & Holbrook, 2000). The fact that some genes involved in the antioxidant defense system, such as SOD and catalase, exhibit rhythmic expressions raises the possibility that the circadian clock may be involved in stress resistance and the prevention of aging.

1.5. Objectives

The fact that many important organismal processes are influenced by the biological clock, including those processes that are responsible for ROS production, along with the ideas contained in the free radical theory of aging, suggests that there may be important links between circadian organization, oxidative stress, and aging. The major objective of this thesis was to address, at least in part, the juncture between these three topics of research using the model organism, *Drosophila melanogaster*. The ubiquitous fruit fly is an excellent model for this type of research for a number of reasons, including, short generation time, ease of rearing and manipulation, availability of mutants, and access to massive numbers of individuals. Primarily, I sought to examine if there was any differential response to oxidative challenge between individuals with a functioning circadian clock mechanism and those without. If the answer to this primary inquiry was yes, my next objective was to elucidate why this was the case. Finally, I was interested in determining if different methods of oxidative challenge (hydrogen peroxide stress, ingestion of hydrogen peroxide, hyperoxia, etc.) produced similar responses between the genetically different flies.

2. Materials and Methods

2.1. Fly rearing and strains

In these experiments, we used flies of the wildtype strain, Canton-S (CS), and mutant *per*^{01} flies (Konopka & Benzer, 1971) which do not produce PER protein. The per-null flies were backcrossed to the CS flies six times to equalize the genetic background of both strains; isogenized wildtype flies were designated CS^{P}. The null mutation in the *period* gene destroys the clock function in all cells of the animal. Flies were reared on standard yeast-cornmeal-molasses diet. Three separate light regimens were used during our experiments: a 12-hour light/dark cycle (LD), a 24-hour light cycle (LL), and a 24-hour dark cycle (DD). We designated time of day using the Zeitgeber Time (ZT) standard; by convention, ZT0 is the time of lights-on while ZT12 is the time of lights-off. All flies were reared in LD from the egg stage and then transferred to their respective light regimes two days after adult emergence. The flies were maintained at a constant temperature of 25°C in all light regimes. In all experiments only male flies were tested.

2.2. Chemicals and reagents

All chemicals used in the experiments and assays were of reagent grade and obtained from Sigma Chemical Co., USA unless otherwise stated.

2.3. Longevity test

In order to elucidate differences in lifespan between CS^{P} and *per*^{01}, male flies of both strains were placed on standard diet and kept under optimal conditions. The flies were transferred onto fresh diet every two days and monitored for mortality until no individuals remained alive.

2.4. Oxidative stress tests

2.4.1. Hydrogen peroxide exposure stress

In order to test for differential susceptibility to acute oxidative stress, 5 day old male flies of both genetic strains were exposed to treatments of 880µmoles hydrogen peroxide (Mallinckdrodt Chemicals, USA). Flies were transferred into glass scintillation vials containing a small filter paper soaked in 100µl of hydrogen peroxide. Flies were left in this environment for 4-hours before being transferred back to normal conditions (standard diet). This procedure was repeated every 4-hours; the experiment lasted a total of 24-hours, resulting in six different treatment groups. The first flies were treated at ZT0 (9am) and the last group at ZT20 (5am). Mortality was scored approximately 48 to 72-hours post-treatment.

2.4.2. Hyperoxic stress

In order to test for differential susceptibility to chronic oxidative stress, male flies of both genotypes (CS^{P} and per^{01}) were exposed to hyperoxic conditions. The flies were placed in an enclosed oxygen chamber of plexiglass under normal atmospheric pressure and exposed to 100% oxygen at a flow rate of approximately 300ml/min. In one set of experiments, flies were exposed to hyperoxia on a continuous basis and were monitored for mortality. In a second set of experiments, flies were exposed to the hyperoxic conditions for varying lengths of time viz 24-, 48-, 72- and 96-hours. Following exposure to hyperoxic conditions all flies were then transferred to a normoxic environment on standard diet and monitored for mortality and longevity. The flies were flipped onto fresh diet every two days.

2.4.3. Hydrogen peroxide dietary stress

In order to test for differential susceptibility to dietary oxidative stress, male flies of both genotypes were exposed to hydrogen peroxide supplemented to the diet. To ensure exposure and consumption, flies were starved for 12-hours and then transferred into vials containing a filter paper soaked in 5% sucrose solution and a variable amount of hydrogen peroxide ranging from 0.01 to 1%. Following exposure to the stressor for 12-hours, flies were transferred to vials containing the standard diet. The flies were transferred onto fresh diet every two days and monitored for mortality daily.

2.5. Assays

2.5.1. Catalase activity assay

This assay was performed to measure the activity of catalase in both CS^{P} and per^{01} flies. Flies were frozen throughout the day (every 4-hours starting at ZT0 and ending at ZT24). Frozen samples were separated into heads and bodies and assayed separately. In a separate experiment to study the induction of catalase activity, flies were collected at 0-, 4-, and 12-hours following exposure to hydrogen peroxide at ZT8 and ZT20 and subsequently assayed for catalase activity in heads and bodies separately. Catalase (EC 1.11.1.6) was assayed in individual heads and bodies of flies essentially following the method of Orr and Sohal (1992) with some modifications for microtitre plate-based assay. Briefly, individual heads or bodies were homogenized in 100mM K-PO_4 (pH 7.0) with 0.1% Triton X-100 by a motor driven homogenizer. Homogenates were centrifuged at 13,000g for 5-minutes and supernatants were used for assay. The

substrate was 10mM H_2O_2 in K-PO_4 buffer. The decrease in absorbance due to decomposition of hydrogen peroxide was monitored at 240nm in a SpectraMax 190 microtitre plate-reader using UV-transparent microtitre plates. The activity of catalase was expressed in $\mu mol.min^{-1}.mg^{-1}$ protein using the extinction coefficient of $39.4mM^{-1}cm^{-1}$ for H_2O_2 (Aebi, 1984). Protein content of supernatants was estimated using the BCA reagent (Stoschek, 1990).

2.5.2. Protein carbonyl assay

This assay was performed to quantify the amount of protein carbonyls between genotypes. Flies were frozen at ZT8 and ZT20; frozen samples were separated into heads and bodies and assayed separately. Carbonyls were quantified after their reaction with 2,4-dinitrophenylhydrazine (DNPH) (Levine et al., 1994) with some modifications by Krishnan et al (2007) for a microtitre plate-based assay. 25-30 heads or bodies were homogenized by sonication (Branson Sonifier 150) in 50mM K-PO_4 buffer (pH 7.0). Homogenates were centrifuged at 10,000g for 15-minutes at 4°C. Supernatants were treated with 10% streptomycin sulfate to precipitate nucleic acids. Samples were then treated with 7mM DNPH in 2M HCl (experimental sample) or 2M HCl (control). After incubation in the dark at 37°C for 1-hour, proteins were precipitated with 28% trichloroacetic acid (TCA) and centrifuged at 15,000g for 10-minutes, again at 4°C. The pellet was then re-suspended in 5% TCA on ice and centrifuged again. Precipitated proteins were re-suspended manually, with ethanol/ethylacetate (1:1) and dissolved in 6M guanidine hydrochloride. The carbonyls were quantified at 370nm in a SpectraMax 190 ELISA microtitre plate reader. Results were expressed as $nmol.mg^{-1}$ protein using an extinction coefficient of $22,000M^{-1}cm^{-1}$. A bovine serum albumin (BSA) standard curve

was used in the absorption measurements (280nm) of protein concentrations in guanidine solutions. Protein carbonyl values were corrected for interfering substances by subtracting the A_{370}/mg protein measured in control samples.

2.5.3. Western blots

Western Blots were performed to determine if the levels of catalase protein were differentially expressed throughout the day in CS^{P} and *per*^{01} flies. Flies were frozen throughout the day (every 4-hours starting at ZT0 and ending at ZT24). Frozen samples were separated into heads and bodies and assayed separately. 10-15 heads or bodies were homogenized by sonication in lysis buffer (protein extraction buffer and protease inhibitor cocktail). SDS-PAGE was performed using a 15% gel; proteins were separated for approximately 6 hours at 125 volts. Proteins were transferred to PVDF Immobilon membranes overnight at 4°C using a constant voltage of 30V. Membranes were blocked for 2-3 hours using Odyssey Blocking Buffer (Li-cor Biosciences, Infrared imaging systems, USA) and then transferred into primary antibody. Two primary antibodies were used: anti-catalase, produced in rabbits, isolated in 1999 and 2000, and generously provided by Dr. S. Radyuk (Radyuk et al., 2000); and anti-alpha-tubulin (clone DM1A), produced in mice and obtained from Sigma Chemical Co. (St Louis, MO, USA). A secondary antibody cocktail containing both goat anti-mouse (IR800) and goat anti-rabbit (IR680) was used for detection of proteins (Li-cor Biosciences, Infrared imaging systems, USA). Membranes were then visualized using an Odyssey Infrared Scanner.

2.5.4. Western blotting for protein carbonyls

Western Blots for protein carbonyls were performed using the OxyBlot protein oxidation detection kit (Chemicon International, USA). The carbonyl groups in the protein side chains were derivatized to 2,4-dinitrophenylhydrazone by reaction with 2,4-dinitrophenylhydrazine. The DNP-derivatized protein samples were separated by polyacrylamide gel electrophoresis followed by western blotting onto PVDF Immobilon membranes overnight. The membranes were incubated with primary antibody specific to the DNP moiety of the proteins followed by goat anti-rabbit secondary antibody treatment. The membranes were then visualized using an Odyssey Infrared Scanner.

2.6. Statistical analysis of data

Survival curves were generated using Kaplan-Meier survival analysis followed by Log-rank (Mantel-Cox) Test and in certain cases the Gehan-Breslow-Wilcoxon Test to analyze if the curves were significantly different for CS^{P} and per^{01} flies. One-way ANOVA followed by Tukey or Bonferroni post test was used in analysis of some data whereas for others a non-parametric Student's t-test was used assuming unequal variance. Dose-response data was calculated using non-linear regression. All statistical analysis and graphs were generated using GraphPad Prism 5 (San Diego, USA) and in certain cases using GraphPad Instat 3 (San Diego, USA).

3. Results

3.1. Differential mortality induced by oxidative challenge

In order to test flies under stressful conditions, they were subjected to 4-hour treatments with exposure to 880µmoles (100µl of 30% H_2O_2) hydrogen peroxide. This concentration was arrived at by using a dose-response test (data not shown) with concentrations ranging from 150µmoles to 880µmoles. The highest concentration resulted in substantial mortality in the shortest time. Therefore, this treatment concentration was selected for the exposure studies. Significant differences ($p<0.001$) in mortality were observed between wildtype (CS^P) and *period* mutant (per^{01}) males at all time points with the exception of ZT0 (lights on) (Figure 5). Mutant flies were markedly more susceptible to oxidative stress at ZT8, which is late in the day, than at other time points. In both genotypes there seems to be a peak of mortality at ZT8 and a trough at ZT20 (which is late in the night). Based upon this data, ZT8 and ZT20 were selected as particular points of interest for future experiments.

3.2. Differential mortality via oxidative challenge under different light regimes

Having gathered data on susceptibility to hydrogen peroxide induced oxidative stress in LD conditions (12-hours of light followed by 12-hours of dark), we decided to investigate mortality under different light regimes. In addition to re-examining mortality in response to hydrogen peroxide exposure at ZT8 and ZT20 in LD conditions, we also investigated mortality responses at equivalent times in both constant light (LL) and constant dark (DD) (Figure 6). Constant light conditions are known to disrupt the clock function by preventing the accumulation of TIM protein; this occurs through the photoreceptive protein CRY. Constant darkness, on the other hand, allows us to test whether or not a rhythm is truly circadian and not

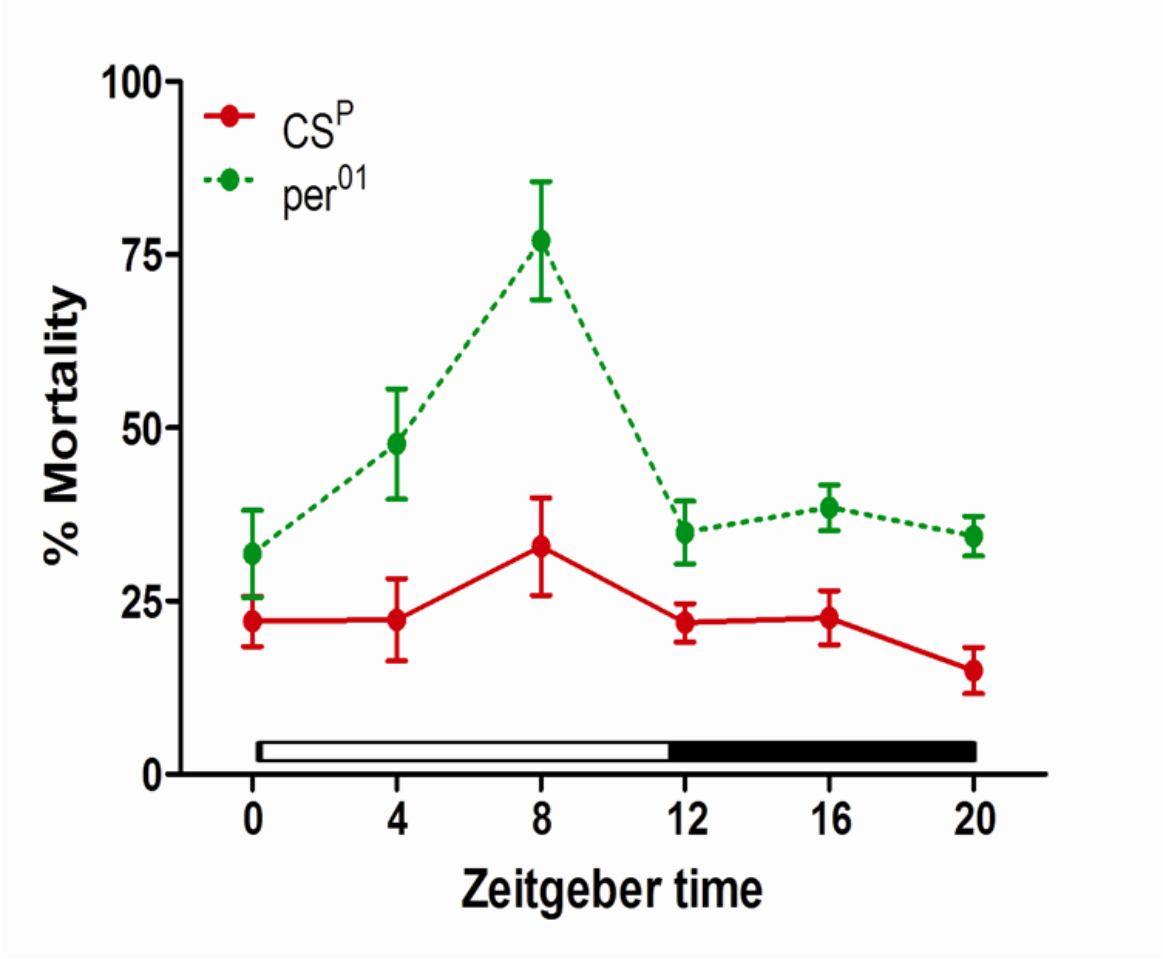

Figure 5: Mortality of male flies after 4-hour exposure to H_2O_2 at different times of day

Five-day old male flies were exposed to H_2O_2 for 4-hours before being returned to normal conditions. The data points were plotted based upon the beginning of the treatment (the points at ZT0 represent flies that were treated beginning at ZT0 and ending at ZT4). Each point represents the average of three separate vials of flies with approximately 16 individuals in each vial. Error bars represent the standard error of the mean.

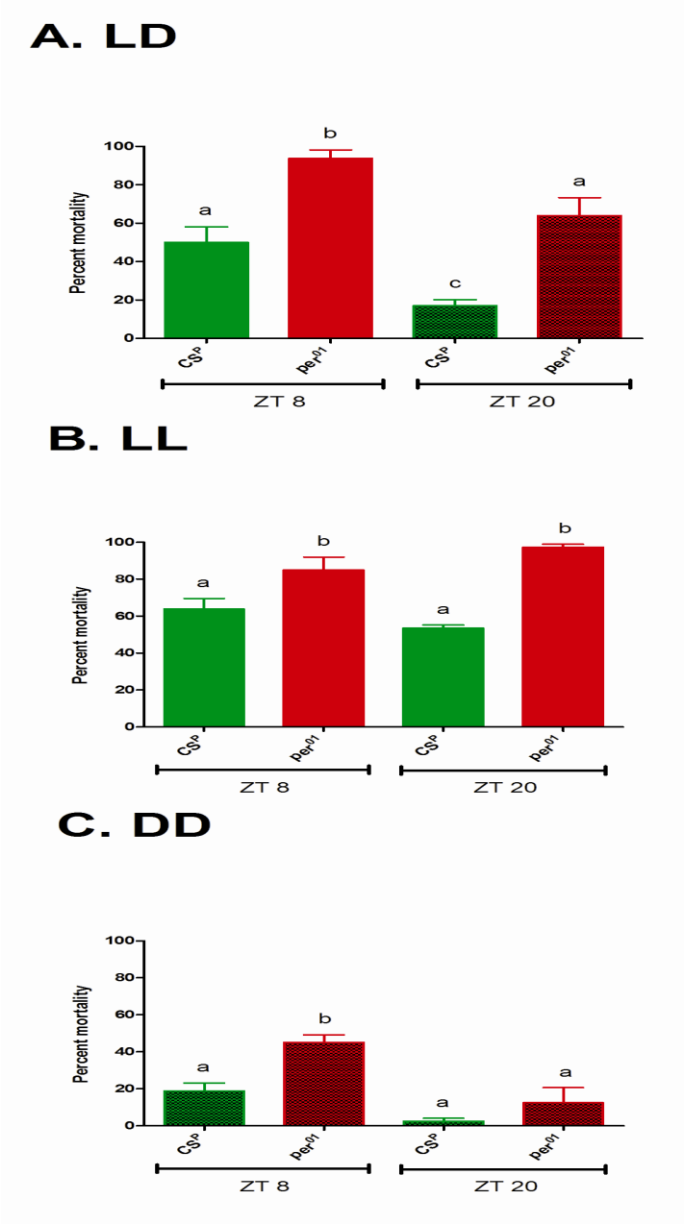

Figure 6: Longevity of male flies after exposure to H_2O_2 in various light conditions

Five-day old male flies were exposed to H_2O_2 as in previous experiments. Data plotted as ZT8 indicates flies were treated from ZT8 to ZT12, while data plotted as ZT20 indicates flies were treated from ZT20 to ZT24. Each column represents the average of five vials of approximately 16 flies each. Error bars represent the standard error of the mean. Columns representing data from the dark are patterned while those representing data from the light are not. Columns with different alphabetical superscripts represent significant differences. Under LD, a differs from b at $p<0.01$, a differs from c at $p<0.05$, and b differs from c at $p<0.001$; under LL, a differs from b at $p<0.05$; under DD, a differs from b at $p<0.05$. Significance levels were calculated by One-Way ANOVA with Tukey's Multiple Comparison Test (LD) or One-Way ANOVA with Bonferroni's Multiple Comparison.

simply a response to light (Young, 1998). The previous results were confirmed in the LD treatment: significantly ($p<0.05$) higher mortality was observed in both genotypes at ZT8 compared with ZT20. Significant differences were also observed between genotypes ($p<0.05$), with the *period* mutant flies displaying much higher mortality than their wildtype counterparts. Under LL conditions significant differences ($p<0.05$) were observed between genotypes (per^{01} showing higher mortality) but not between the time points. Similarly, under DD we observed higher mortality in per^{01} than in CS^{P}. These differences were significant ($p<0.05$) during the day (ZT8), however, they were not significant at night (ZT20). The non-significant nature of these differences may be due to the fact that we observed zero mortality in a number of experimental replicates. Intriguingly, flies exposed to light always exhibited higher rates of mortality, irrespective of genotype as compared to flies in the dark. In general, susceptibility to oxidative stress was elevated in the light while resistance to oxidative stress was elevated in the darkness.

3.3. Levels of catalase protein as a function of time of day

To explore the mechanism underlying the differences in mortality between flies treated with H_2O_2 at ZT8 and ZT20, we measured the levels of catalase protein in wildtype males using Western blots. When the blots were analyzed using the Odyssey Infrared Scanner analysis software no apparent differences were observed in the levels of catalase protein as a function of time of day (Figure 7). There was, however, significantly more catalase protein present in the fly bodies than in the fly heads per microgram of total protein. Unfortunately, similar analysis of the relative catalase protein levels in *period* mutant males was not successful, so no comparison between genotypes can be made at this time.

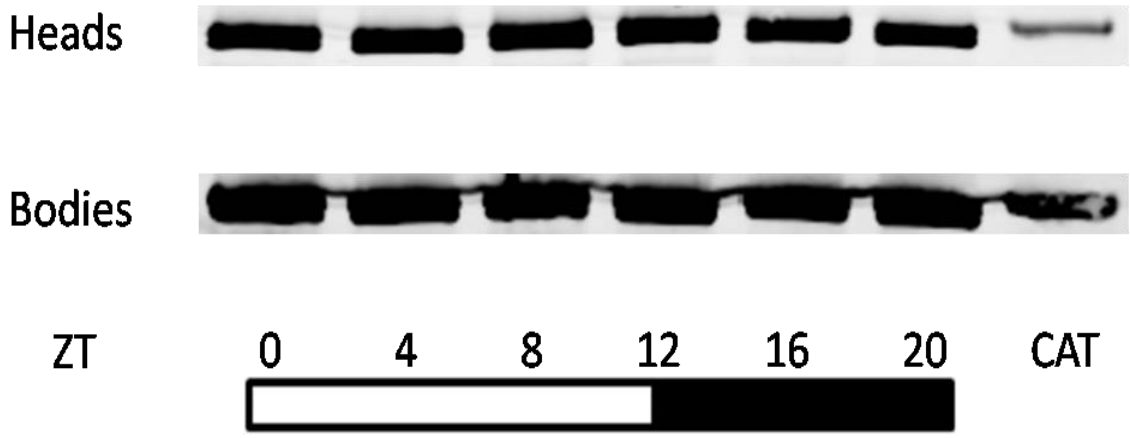

Figure 7: Relative levels of catalase protein in wildtype males

Flies were collected and frozen at each of the time points indicated; the assay itself was performed later. The homogenate of heads was prepared using 15 heads, while the homogenate of bodies was prepared using 10 bodies. Concentrations of total protein were assayed, and equal amounts of protein were loaded (~1µg) in all wells.

3.4. Catalase enzyme activity under optimal conditions

Since there appeared to be no differences in relative levels of catalase protein at different times of day, we reasoned that there may be daily fluctuations in factors affecting the activity of this enzyme. Therefore, we set out to examine whether there may be differences in the levels of catalase activity in unchallenged animals. The activity of this enzyme was assayed in both CS^{P} and per^{01} males in the heads and bodies separately (as with the Western Blot analysis) over various ZT times (ZT0-ZT28) (Figure 8). This experiment led to several observations. First, we show catalase activity in fly heads to be approximately 50% lower than in fly bodies, irrespective of genotype. This observation correlates well with previous results showing more catalase protein in fly bodies than in fly heads (Figure 7). Second, there were significant day/night fluctuations in the catalase activity in both heads and bodies. We observed an increase in CAT activity during the day followed by a decrease in CAT activity during the night; these changes were more pronounced in the fly bodies. Third, CAT activity in both heads and bodies was higher in CS^{P} than in per^{01} flies over most time points, but these differences were not statistically significant.

3.5. Catalase enzyme activity is not induced following hydrogen peroxide stress

Our data did not reveal any clear cut relationship between susceptibility to exogenous oxidative stress and the levels of catalase activity in untreated flies. In an effort to understand daily fluctuations in susceptibility to oxidative stress, we considered the possibility that CAT may be induced by H_2O_2 exposure and this induction may depend on the time of day. To test this hypothesis, we assayed the enzyme's activity in all three light regimes at 4- and 12-hours post-exposure to hydrogen peroxide in addition to assaying activity prior to oxidative challenge. In all cases there was more activity in the bodies than in the heads, again confirming earlier results.

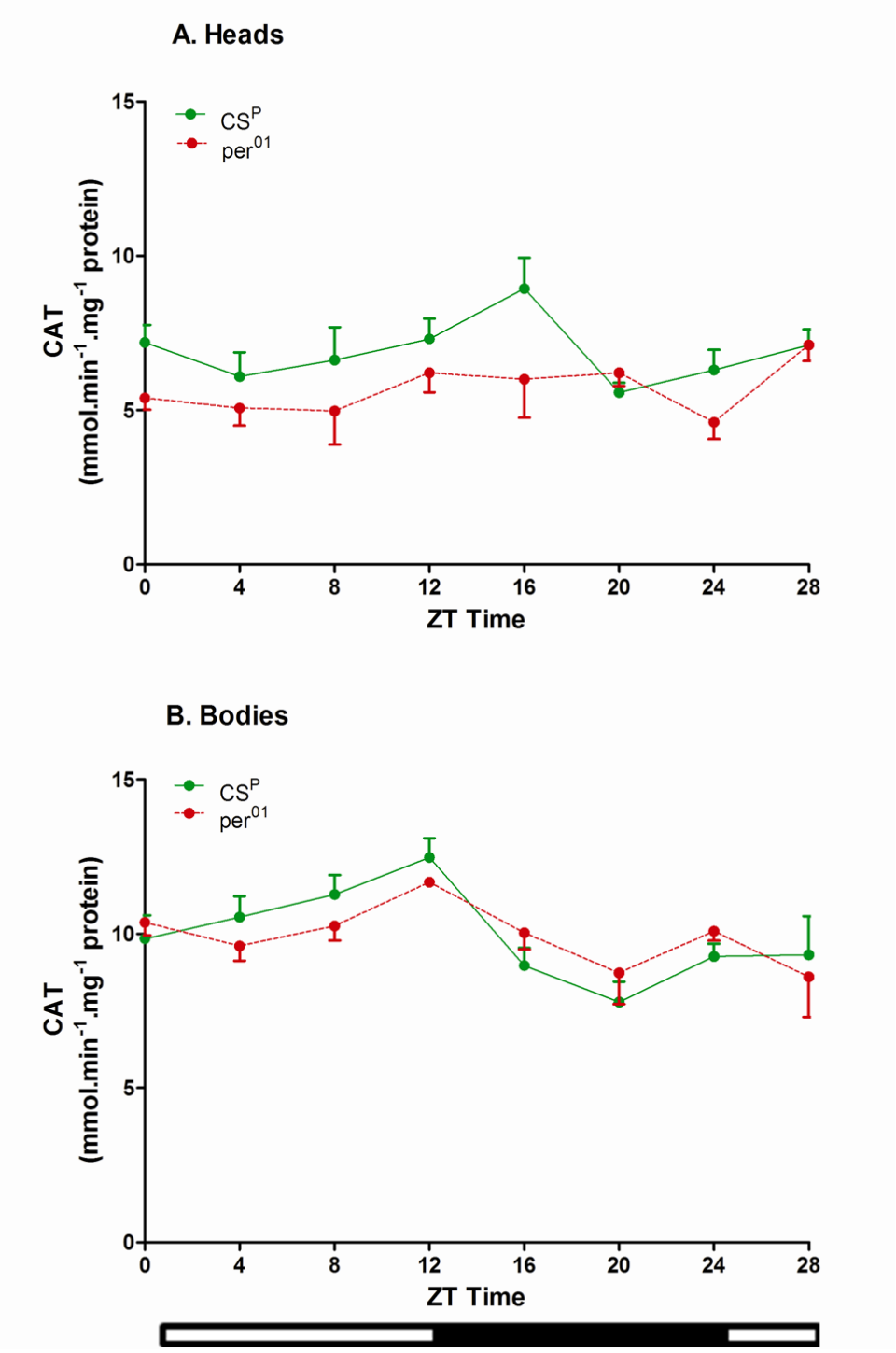

Figure 8: Activity of the antioxidant enzyme catalase

Flies were collected and frozen at each of the time points indicated. Each data point represents the average catalase activity level in 24 individual heads or bodies, normalized for protein content. Error bars represent the standard error of the mean.

There also appears to be more activity in the CS^P males than in the *per*^{01} males, but these differences were generally not statistically significant (Figure 9). In general, catalase activity did not change significantly at the end of the 4-hour treatments with H_2O_2, or 8-hours post-treatment. Surprisingly, catalase activity seems to decrease in some cases after H_2O_2 treatment; this trend is best seen under LD conditions in the head tissue at ZT8. Similar to the results from the light regime mortality experiments, there appears to be more enzyme activity during hours of darkness than during hours of light. While this result certainly does not explain the earlier result, it is interesting and perhaps merits further inquiry.

3.6. Protein carbonyl levels as a function of both time and genotype

In our initial experiments, we observed a differential response to H_2O_2 between CS^P and *per*^{01} flies, as indicated by mortality (Figure 5). Since we did not detect corresponding differences in the activity of the enzyme catalase between these two genotypes, we considered the possibility that there was more endogenous oxidative damage in the mutant strain, causing the differential physiological response to exogenous oxidants. An important biomarker of oxidative stress, protein carbonylation, was assayed to determine relative levels of oxidative damage in untreated flies. In samples from CS^P and *per*^{01} male heads we observed drastically lower levels of carbonylation at ZT20 than at ZT8 (Figure 10). The *period* mutants also displayed significantly more carbonylation at ZT8 than the corresponding wildtype males. In the bodies of CS^P males there was a significant ($p<0.05$) difference between carbonyl levels at ZT8 and ZT20; interestingly no corresponding difference was observed in *per*^{01} males.

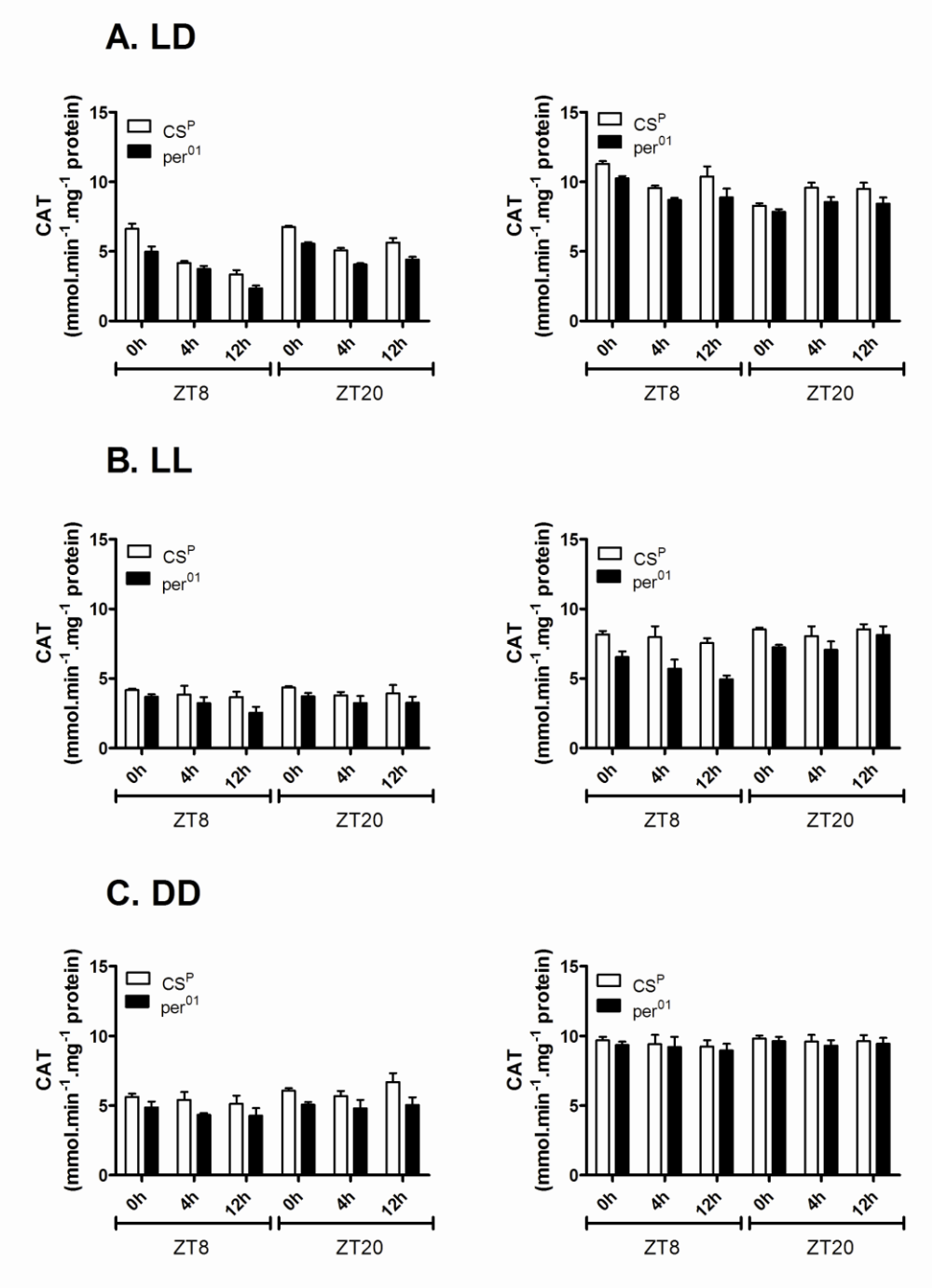

Figure 9: Activity of catalase 0-, 4-, and 12-hours after hydrogen peroxide exposure

Flies were collected and frozen at each of the time points indicated. Each column represents the average catalase activity level in 8 individual heads or bodies, normalized for protein content. Error bars represent the standard error of the mean.

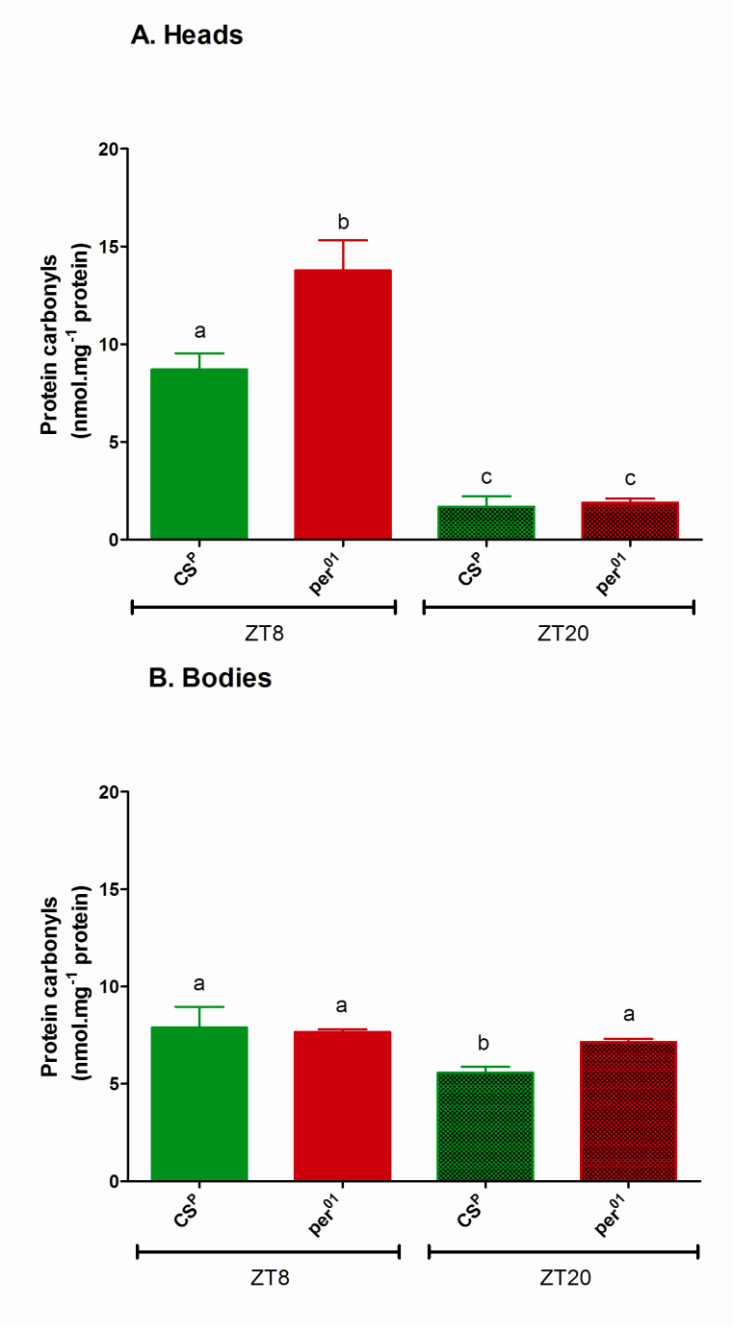

Figure 10: Protein carbonylation in both heads and bodies of CS^P and *per*^{01} males

Flies were collected and frozen at each of the time points indicated. Each column represents the average carbonyl accumulation level in 16 individual heads or bodies, normalized for protein content. Error bars represent the standard error of the mean. Patterned columns represent data taken during darkness. Columns with different alphabetical superscripts represent significant differences. One-Way ANOVA followed by Tukey's Multiple Comparison Test revealed p-values of less than 0.05.

3.7. Detection of carbonylated proteins by Western blot

In the assay of total protein carbonylation, we observed the most significant differences in the fly heads. To verify this result we assayed the protein carbonyl levels in fly heads using SDS-PAGE and the OxyBlot kit. In these results we observed more carbonyl accumulation in CS^{P} heads at ZT8 than ZT20 (Figure 11). Interestingly, there appears to be higher accumulation of carbonylated protein in per^{01} flies at both time points as compared with CS^{P} flies. Experiments are currently underway to determine if catalase specifically is differentially carbonylated between the genotypes and also between the time points.

3.8. Longevity of wildtype and *per*-null males under optimal conditions

Our data thus far suggests that *per*-null mutants are more vulnerable to oxidative stress than wildtype flies. Since oxidative stress has been linked to accelerated aging, we hypothesized that *period* mutants might have a shorter lifespan as compared to wildtype. Flies of both genotypes were first evaluated under optimal conditions (12-hour light/dark regimen, no stressors, low population density, fresh diet every 2 days) in order to assess their longevity. Mortality was scored once daily, at a specific time of the day, until the end of the experiment when all individuals had died. The survival curves were significantly different with a p-value of 0.005 using the Gehan-Breslow-Wilcoxon Test. However, when the same Kaplan-Meier survival curves were subjected to the Log-rank (Mantel-Cox) Test, there were no significant differences in survival (Figure 12). Interestingly, the median survival of both genotypes was the same: 59 days. This data indicates that a null mutation in the *period* gene has a relatively minor effect under optimal conditions. Knowing the wide range of processes under the influence of the circadian clock, this result prompts questions about the benefits a

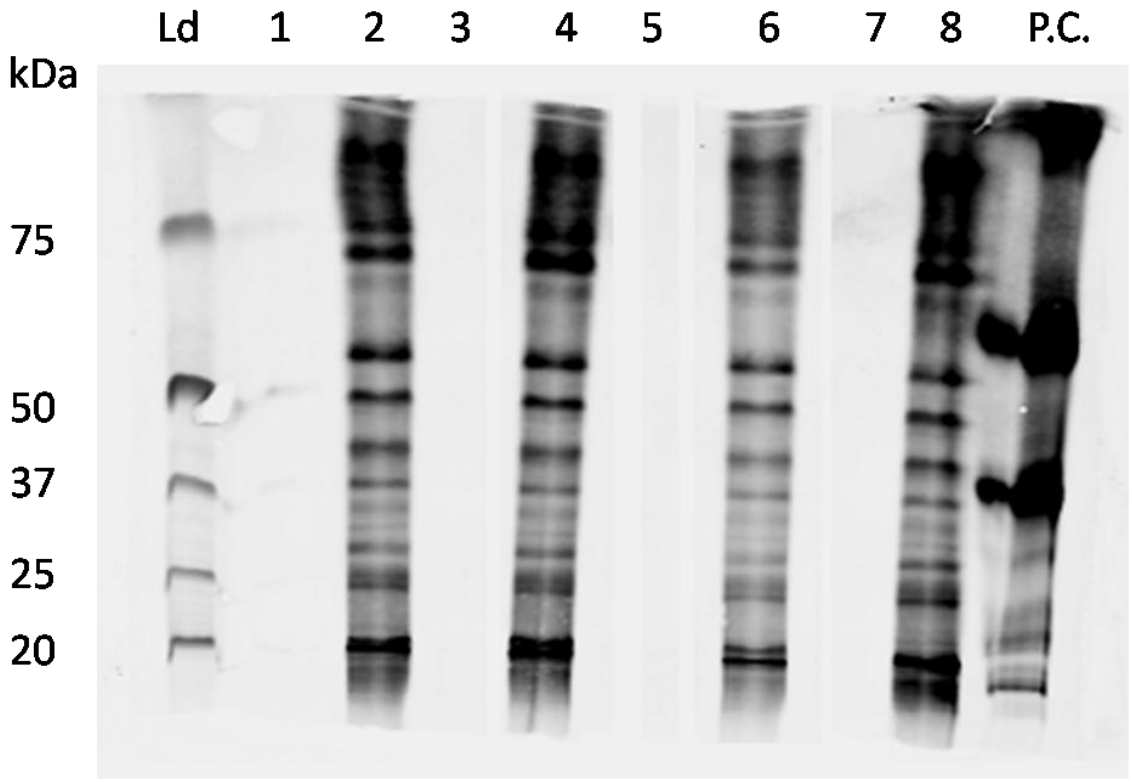

Figure 11: Western blot showing carbonylation in fly heads

Western blot performed using the OxyBlot kit for detection of protein carbonyls. Lanes 2, 4, 6, and 8 are samples; lanes 1, 3, 5, and 7 are the corresponding controls. Specifically, lane 2 is CS^{P} at ZT8, lane 4 is per^{01} at the same time; lane 6 is CS^{P} at ZT20, lane 8 is per^{01} at the same time. The very first and very last lanes are standards–the very first lane is the Odyssey standard, while the very last lane is the protein carbonyl standard provided with the OxyBlot kit. The Odyssey standards are label in kilodaltons. Concentrations of total protein were assayed and equalized so a consistent amount of protein was loaded in each lane (~5µg).

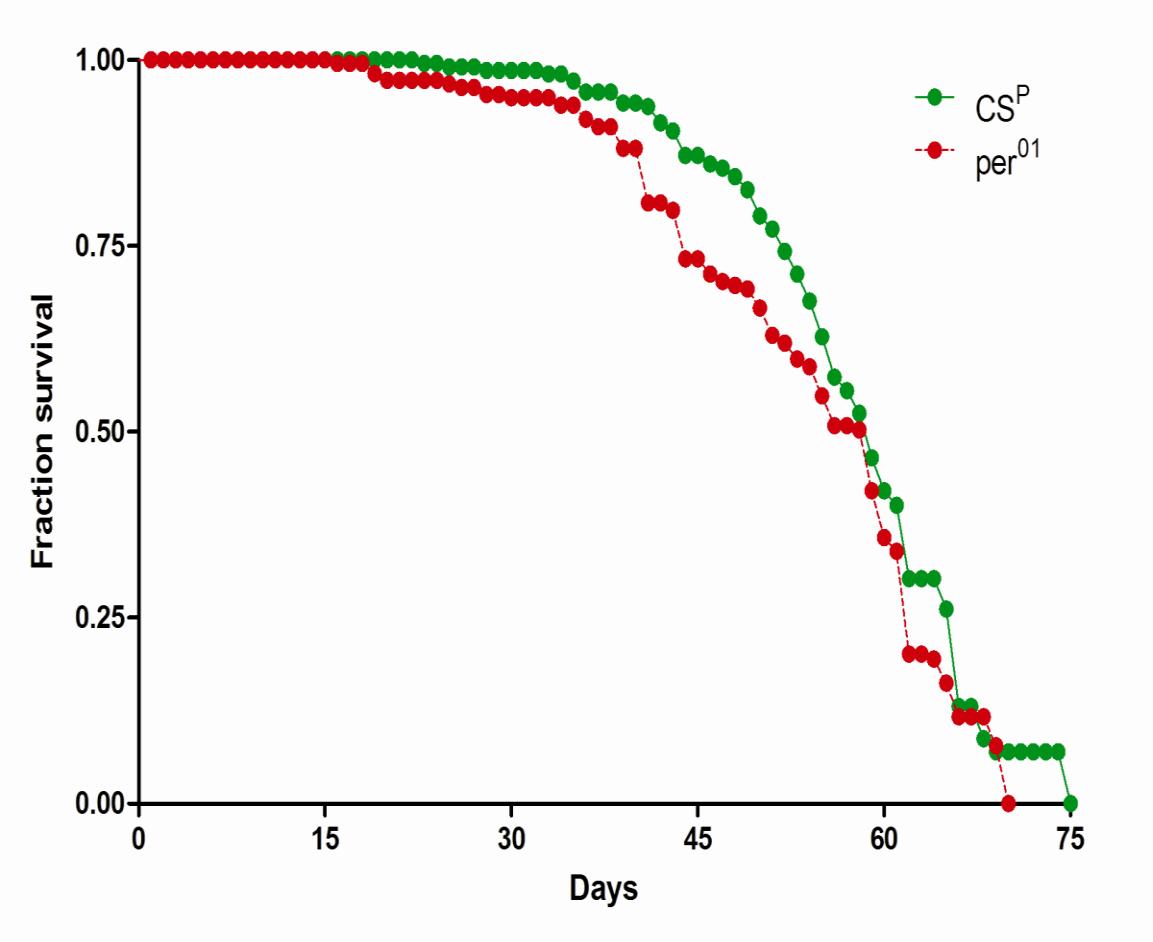

Figure 12: Longevity of male flies under optimal conditions

Male flies of both CS^P and *per*^{01} genotypes were placed in an environment with optimal conditions (normoxia, temperature of 25°C, low population density, standard diet) and monitored for mortality. Flies were transferred onto fresh diet every two days to avoid bacterial contamination. The median survival was 59 days for both genotypes. The maximum lifespan for CS^P was 75 days, while the maximum *per*^{01} lifespan was 70 days. Approximately 160 individuals of each genotype were used in this study.

functioning clock component such as the *period* gene bestows on an organism. In order to understand the possible role of *per* under sub-optimal conditions, we subjected CS^P and per^{01} flies to dietary and hyperoxic oxidative stress, hypothesizing that the effects of the clock may become apparent as the organisms experienced challenging circumstances.

3.9. Response to dietary hydrogen peroxide stress

We devised three experiments to assay survival and longevity of flies exposed to oxidative stress. In the first of these tests, we administered hydrogen peroxide to male flies of both genotypes via feeding. First, flies were starved for 12-hours to ensure feeding on the H_2O_2 solution, which was mixed with 5% sucrose and supplied on a filter paper. Seven doses of hydrogen peroxide were used in this test: 0, 0.01, 0.05, 0.1, 0.25, 0.5, and 1%. Survival was then monitored for forty days following this oxidative challenge. Based upon this dose response, we determined the EC_{50} of H_2O_2 administered in a sucrose solution to be 0.06% using a non-linear curve fit analysis (Figure 13). Using this dosage and our 40-day cut-off point, we detected significant ($p<0.05$) differences in mortality between CS^P and per^{01} males using the Mann-Whitney t-Test with Gaussian approximation.

3.10. Response to hyperoxia

In addition to hydrogen peroxide exposure, we also tested longevity under hyperoxic conditions. Two experiments were conducted using these conditions. First, chronic exposure to hyperoxia was assayed by exposing flies continuously to a 100% oxygen environment. Despite the fact that all individuals died within one week, there was still a significant difference ($p<0.05$) between survival curves of CS^P and per^{01} males (Figure 14). This difference was revealed using the Log-rank (Mantel-Cox) Test.

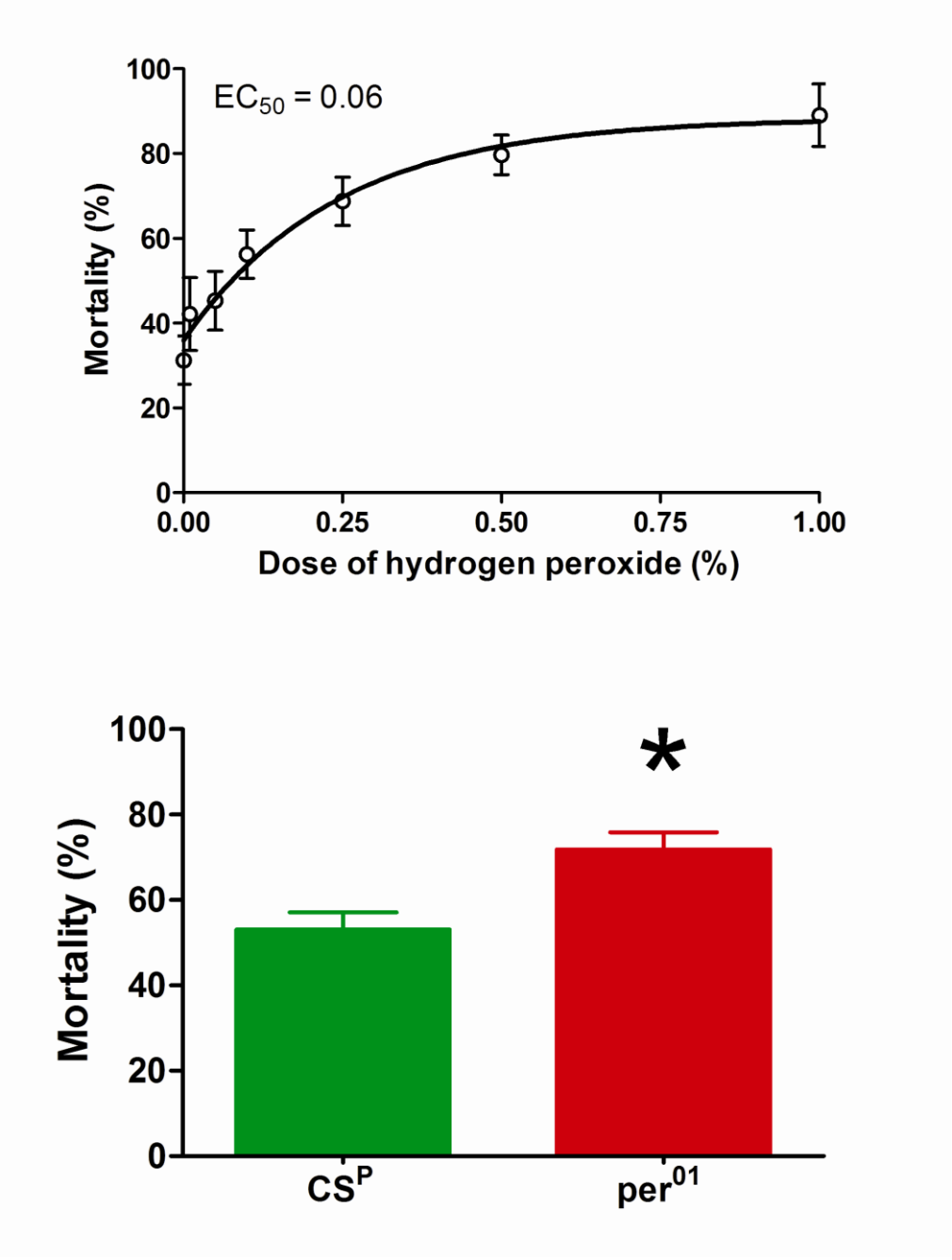

Figure 13: Mortality after feeding on 5% sucrose and variable concentrations of H_2O_2

Data in the upper panel shows a dose-response test performed using CS^P males. Seven different concentrations were administered orally with a 5% sucrose solution. Statistical analysis provided an EC_{50} of 0.06%, which was subsequently used to test both genotypes. Data in the lower panel shows that mutant males exhibited significantly ($p<0.05$) higher mortality than wildtype males at this concentration, after 40 days. Each column represents the average of five vials of flies, each vial containing approximately 16 individuals. Error bars represent the standard error of the mean.

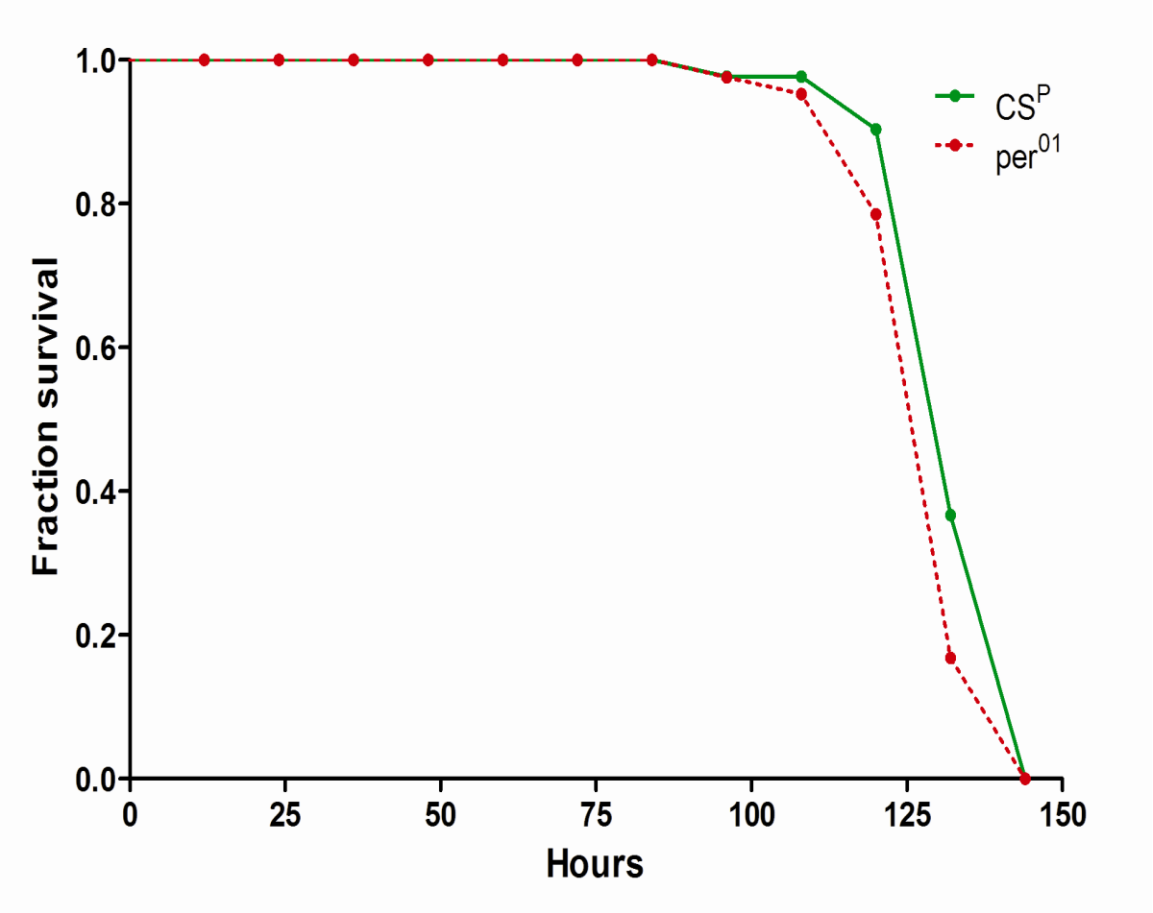

Figure 14: Survivorship during chronic exposure to hyperoxia

Male flies of both genotypes were placed into a hyperoxia chamber with an oxygen flow rate of 300ml/min. The flies were 5 days old. After treatment began flies were only removed from the chamber to transfer them to fresh diet (this occurred every two days). Approximately 160 flies of each genotype were used in this study.

Flies exposed to continuous hyperoxia died within one week, regardless of genotype, suggesting that continuous hyperoxic stress overwhelms the organisms and may mask the differential response to oxidative challenge. Therefore, we tested longevity under milder oxidative stress by exposing flies to the 100% oxygen environment for varying lengths of time. Flies were treated for 24-, 48-, 72-, and 96-hours before being returned to normoxia. Longevity was subsequently monitored and recorded (Figure 15). In all cases significant differences were observed (Log-rank (Mantel-Cox) Test, $p<0.01$ except for 48-hour treatment where $p=0.0106$) between CS^{P} and per^{01} flies, with *period* mutants exhibiting shorter lifespans. Also of note, as the duration of exposure to hyperoxia increased, there was a corresponding decrease in longevity for both genotypes. For example, the average lifespan after 48-hours of hyperoxia was 58 days for CS^{P} flies and 54 days for per^{01} flies; after 96-hours of hyperoxia, the average lifespans had shortened to 54 days for CS^{P} flies and 42 days for per^{01} flies. Taken together with earlier data (Figure 13), the results of these additional experiments confirms that *period* mutants show increased susceptibility to oxidative stress as compared with wildtype flies.

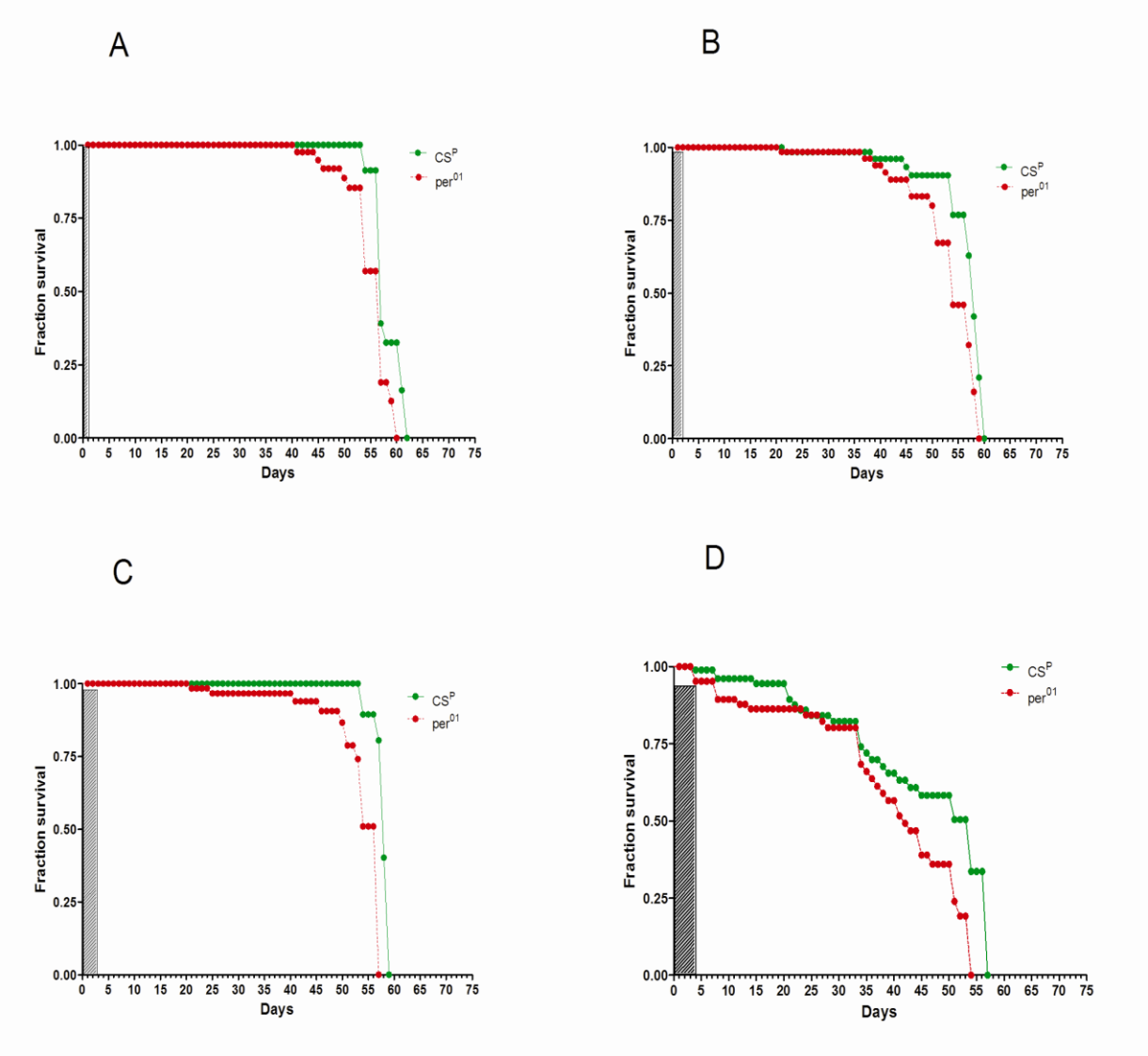

Figure 15: Longevity following differential exposure to hyperoxia

Male flies of both genotypes were placed into a hyperoxia chamber with an oxygen flow rate of 300ml/min. The flies were 5 days old. There were four experimental groups in this study: (A) 24-, (B) 48-, (C) 72-, and (D) 96-hours of hyperoxia exposure. In all cases *per*^{01} flies exhibited significant shortening of lifespan as compared to CS^{P}. Approximately 80 flies of each genotype were used in each experimental group.

4. Discussion

4.1. Adaptive advantages of circadian rhythmicity

The fact that circadian rhythms confer adaptive advantages to organisms has been proposed almost from the very beginning of research on this topic (Pittendrigh & Minis, 1972) (Went, 1960). Recent evidence corroborates such a hypothesis, showing that organisms with altered or disrupted circadian clocks are less adapted to their environment (Johnson, 2001) (Mori & Johnson, 2001) (Sheeba et al., 2000), more prone to genotoxic stress (Kondratov & Antoch, 2007), or have compromised reproductive fitness (Beaver et al., 2002).

The relationship between circadian rhythmicity and redox metabolism, and thereby the regulation of free radical generation, detoxification and also possibly its avoidance, strongly suggests an adaptive advantage for both cells and organisms to anticipate daily stresses. Direct evidence for such an advantage is missing in the literature. Benefits of the clock could be apparent only if mutations in certain clock components led to reductions in fitness in the natural environment. This may be extremely difficult to discern under optimal laboratory conditions. One of the ways to investigate if the fitness of an organism with a clock mutation is compromised is to subject it to stressful situations and to compare its response to wildtype counterparts with fully functioning clocks. We have taken this approach to investigate the role of the circadian clock in oxidative stress defense in the model organism, *D. melanogaster*. Using a combination of various stressors under different experimental situations, we sought to understand the utility of the circadian clock mechanism in the overall fitness of the individual. For the purpose of this discussion, we will use the term “fitness” to describe susceptibility to both acute and chronic oxidative stress.

In the investigations conducted here, a combination of experimental situations was employed to investigate the role of the clock in susceptibility to oxidative stress. These situations included exposure to acute oxidative stress at different Zeitgeber times, exposure to the same stressor under different light regimes at times of minimum and maximum susceptibility, and finally exposure to chronic stress to elucidate its effects on longevity.

4.2. Does mutation in the *period* gene affect susceptibility to oxidative stress?

In a number of independent experiments, it was observed that a null mutation in the *period* gene results in increased mortality when compared to wildtype individuals (Figures 5, 6, 12, 13, 14, and 15). This relationship holds across a number of stressors such as exposure to hydrogen peroxide, oral uptake of hydrogen peroxide, and exposure to hyperoxic conditions, as well as in three different light regimes (LD: 12L/12D, LL: constant light, and DD: constant darkness). The consistency of these results suggests that *period* is somehow involved in the antioxidant defense system. Our results are consistent with the previously reported data showing that a large number of genes are rhythmically expressed (Duffield, 2003), including those involved in the regulation of the redox state. Daily rhythms of protective antioxidant enzymes in various phylogenetically diverse organisms have also been reported by a number of authors (Albarran et al., 2001) (Collen et al., 1995) (Marheineke & Hardeland, 1997) (Samis et al., 1981). Of particular relevance to our study is the finding that the enzyme catalase exhibits rhythmic expression (Ceriani et al, 2002), as this enzyme is strongly involved in the response to oxidative stress (Figure 2). While molecular data are extremely important, their relevance to physiology is currently missing. Our data provides the first physiological evidence that susceptibility to oxidative stress is rhythmic. While preliminary results displayed only a weak mortality rhythm in response to hydrogen peroxide exposure, subsequent experiments

confirmed that there was indeed a rhythmic response in wildtype animals. A somewhat puzzling finding of our study is the fact that *period*-null mutants also showed a rhythm of susceptibility to oxidative stress, although their mortality was always higher than the wildtype animals. At this time we have no explanations for this unexpected result.

The novel finding of our study is that light by itself appears to have a significant impact on oxidative stress (Figure 6). In constant light conditions, we observed a substantial increase in mortality in both genotypes and at both time points surveyed. Conversely, in conditions of constant darkness we observe dramatically less mortality in both genotypes. This result mirrors findings from LD conditions: higher mortality in light as compared with darkness, especially in the *per*-null mutant. Perhaps this differential mortality is due not to the influence of the clock alone, but to other factors such as endogenous levels of ROS. In fact, in zebra fish cell lines, light has been shown to induce the formation of hydrogen peroxide, which may in turn lead to increased oxidative damage in turn (Hirayama & Sassone-Corsi, 2007). It remains to be seen whether a similar mechanism exists in *Drosophila*; if so, it could lead to increased oxidative damage, which could explain higher susceptibility to exogenous hydrogen peroxide.

4.3. Catalase alone does not explain mortality differences between CS^{P} and per^{01} flies

To understand the mechanism of susceptibility differences between genotypes to oxidative stress induced by exposure to hydrogen peroxide, the steady state levels of expression and activity of the antioxidant enzyme catalase (CAT) was investigated. CAT is chiefly involved in the breakdown of hydrogen peroxide to relatively innocuous water and oxygen. Previous microarray data suggests that CAT mRNA exhibits a robust rhythm in fly bodies (Ceriani et al., 2002), and this made CAT a likely candidate for involvement in differential responses to

hydrogen peroxide. Despite the reported rhythm in CAT mRNA, we did not find the rhythm in the relative levels of CAT protein throughout the circadian day as investigated by Western blots (Figure 7). However, a distinct difference in the amount of CAT protein was seen between the heads and bodies of male CS^P flies; this result may be explained by the amount of muscle, and the corresponding amount of mitochondria (and thus ROS) found in the fly body. Although the information and data on cycling mRNA concentrations of CAT is important from the view-point of genes under circadian control, our data prove that this is not sufficient to estimate the physiological significance of such a rhythm. It has been proven conclusively that a prominent rhythm of enzyme protein synthesis may not be detectable, and should it be detectable, it may exhibit a low-amplitude — especially when the protein is endowed with a long half-life (Hardeland et al., 1973)(Morris & Peraino, 1976) (Volknandt & Hardeland, 1984). In contrast, a flat or practically indistinguishable rhythm in mRNA concentrations does not exclude a translationally controlled high-amplitude rhythm in the protein level (Fagan et al., 1999) (Mittag, 2001) (Mittag et al., 1998) (Okamoto et al., 2001). Thus, protein concentrations determined by Western blots or other immunological techniques, may not be conclusive, since active centers or conformationally sensitive domains of enzymes may be destroyed, oxidatively modified, and eventually even reactivated by reductant-dependent mechanisms (Andersson et al., 1998) (Castro et al., 1998) (Gardner, 2002) (Liochev & Fridovich, 2002). Consequently only activity measurements could appropriately reflect the protective capacity of the enzyme.

When the steady state levels of CAT activity was investigated, we saw a trend similar to that observed in relative protein levels: two-fold higher activity in the body as compared to the head. This trend holds in both CS^P flies and per^{01} flies (Figure 8). In wildtype heads and bodies, we observed statistically significant oscillations in CAT activity; such oscillations were dampened in the mutant strain. In general, CAT activity was marginally higher and at some time points

significantly higher in CS^P flies than in per^{01} flies. Both the phase of the CAT activity rhythm and the lack of differences between genotypes lead us to the conclusion that steady state CAT activity alone cannot explain differential susceptibility to H_2O_2 as observed in physiological experiments. Thus, an investigation into the inducibility of this enzyme by H_2O_2 was merited.

Following exposure to hydrogen peroxide stress under various light regimes, the activity of CAT was monitored at 0-, 4-, and 12-hours post-exposure (Figure 9). The results were interesting: at ZT8, a definite and significant down-regulation of CAT activity was recorded post-exposure while at ZT20 a marginal up-regulation was observed under different light regimes. Also of note, under LL, a significantly lower activity of CAT in both head and body samples was recorded compared to LD and DD conditions. This leads us to question whether oxidative damage to tissues or to CAT is rhythmic and if so, does this damage increase under constant light and decrease in constant darkness? Certainly CAT is not the only enzyme up-regulated and functioning during periods of oxidative stress. A host of other enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APOX), glutathione-s-transferase peroxidase (GSTpx) and other low molecular weight antioxidants (LMWA) such as thiols would also be affected and participating in the antioxidant response. It has been reported that LMWAs are consistently found to be rhythmic—mainly reduced glutathione (GSH) or non-protein thiols (Bauman et al., 1988) (Belanger et al., 1991) (Bruckner et al., 2002). Amplitudes in GSH rhythms differ between organs, species, strains, and studies, from the borderline of demonstrability to maximum/minimum ratios of 1.8. In fact, the ratio of GSH to its oxidized form (GSSG) is a very reliable indicator of oxidative stress and has frequently been used in aging studies to demonstrate the link between oxidative stress and aging. GSH rhythms have been described for a beetle (Barros & Bechara, 2001) and two crayfish species (Duran-Lizarraga et al., 2001) (Fanjul-Moles et al., 2003). Studies have shown conflicting results on the beneficial impacts of the

overexpression of a variety of antioxidant defense enzymes. In one study, the simultaneous overexpression of copper-zinc (cytoplasmic) superoxide dismutase and catalase actually resulted in increased lifespan in *Drosophila* (Orr & Sohal, 1994), but other studies with a variety of overexpressed enzymes (catalase, thioredoxin reductase, and both manganese and copper-zinc superoxide dismutase) have shown no beneficial effects and even slight negative effects on lifespan (Orr et al, 2003).

The extensive work on rhythms of antioxidative enzymes and LMWAs appear to constitute an overwhelming body of evidence for a fundamental role of circadian organization in coping with externally induced and endogenously generated free radicals. Although this conclusion is justified, due caution is to be exercised with respect to details, especially concerning oversimplifications based on the activity of only one enzyme system. Thus, though CAT may not be the determining factor for the observed susceptibility of *period* mutants in *Drosophila*, it certainly may constitute an important component of the vast number of rhythmically expressed antioxidant enzymes and LMWAs involved in the process.

4.4. Circadian rhythmicity in oxidative damage

One would expect rhythms in oxidative damage, given that there are circadian/daily variations in ROS formation, activity of protective antioxidant enzymes, and LMWA concentrations (see previous section). We demonstrate clear differences in the oxidative damage between per^{01} mutants and their wild type counterpart CS^{P} flies. As mentioned earlier, in the course of their lifetime, organisms are constantly exposed to one or more factors that generate reactive oxygen species, which can damage proteins, nucleic acids, and lipids. Whereas the oxidative damage to nucleic acids is subject to repair by highly efficient excision/ insertion mechanisms, the repair of damaged proteins appears limited to the reduction of oxidized

derivatives of the sulfur-containing amino acid residues; repair of other kinds of protein oxidation has not been demonstrated. Instead, damaged proteins are targeted for degradation to amino acid constituents by various endogenous proteases including cathepsin c, calpain, trypsin and especially the 20s proteasome, whose activity is under metabolic control by diverse regulatory factors. These factors include the concentrations of enzyme substrates, ubiquitination, and various inhibitors (cross-linked proteins, glycation/ glycoxidation protein conjugates). Accordingly, the intracellular accumulation of the oxidized forms of proteins is a complex function of pro-oxidant and antioxidant activities, and the concentrations and activities of the proteases that degrade these oxidized forms.

The fact that carbonyl groups of proteins are major products of ROS-mediated oxidation reactions has led to the development of protein carbonylation test as a reliable biomarker for oxidative damage. In this study the levels of carbonyl groups in proteins was quantified in heads and bodies of flies of both CS^{P} and per^{01} (Figure 10). The results showed that per^{01} flies accumulated dramatically more carbonyl contents in their heads compared to CS^{P} flies at ZT8. Interestingly, at ZT20 there was no difference in carbonyl content in the heads of both genotypes. Again when the bodies were compared, at ZT8 there was no significant difference in either genotype, but at ZT20 a markedly lower accumulation of carbonyls was documented in CS^{P} flies compared per^{01} flies. This was also corroborated by Western blots (Figure 11). A similar result has previously been reported in *Drosophila* (Coto-Montes & Hardeland, 1999) (Coto-Montes & Hardeland, 1997), they observed the average level of protein carbonyl in the arrthythmic mutant (per^{0} and per^{s}) was elevated compared to the corresponding wildtype control. Unexpectedly, the mean protein carbonyl content was observed to be higher in the short-period mutant per^{s}. Thus, disturbances in internal timing due to clock mutations lead to an elevation of oxidative damage. These findings strongly suggest that appropriate circadian timing

contributes to an attenuation of oxidative stress. This gives rise to a further question: does protein carbonylation indicate a decline in biological fitness?

4.5. Oral uptake of hydrogen peroxide aggravates differences in susceptibility to oxidative stress between CS^P and per^{01} flies

Given our data that *per*-null mutants are more susceptible to acute oxidative stress and the fact that oxidative stress underlies the aging process, we sought to determine whether chronic oxidative stress would adversely affect the longevity of this mutant. First, we demonstrated that per^{01} mutants were more susceptible to dietary supplementation of H_2O_2 even at a concentration of 0.06% (Figure 13). This led to a final set of experiments to test the longevity of both genotypes under normoxic and hyperoxic conditions (100% oxygen, which is another form of oxidative stress).

4.6. Hyperoxia magnifies susceptibility differences between CS^P and per^{01} flies

Though longevity *per se* is not the sole indicator of biological fitness, it gives reliable estimates of the physiological state of an organism. Changes in longevity are thought to be caused by differences in the rates of oxidant production. In this investigation it was observed that under normoxic conditions per^{01} flies showed marginally reduced longevity compared to their wildtype counterparts (Figure 12). This has also been suggested by other groups reporting moderate reductions in the lifespan of *per* mutants (Klarsfeld & Rouyer, 1998). Similarly, another *Drosophila* clock mutant, cyc^{01}, is found to be much more short-lived, but this is seen only in males (Hendricks et al., 2003). When interpreting such data one should be aware of the complexities of both external and internal influences on longevity (Sheeba et al., 2000). Differences in life span are neither simply explained by oxidative damage by metabolic rates related to locomotor activity.

However, the obvious divergence between oxidative damage and its effects on lifespan should not preclude the hypothesis that rhythms in antioxidant defense mechanisms are a means to minimize both diurnal and cumulative oxidative stress effects. Thus, when both genotypes were subjected to hyperoxic conditions on a continuous basis, they survived only a few days and mean mortality minimally shorter for the *per*-null mutants (Figure 14). However, when flies were subjected to short exposure durations of 24-, 48-, 72-, and 96-hours of hyperoxic stress the effects of oxidative damage on longevity between the strains became much more evident (Figure 15). This indicates that when oxidant levels reach or exceed the limits of detoxification capacity as in constant hyperoxia one cannot expect marked differences in responses from either the clock compromised mutants or their wildtype counterparts. However, when flies were subjected to stress levels that were less severe, differences become obvious. The earlier finding that *Drosophila* lives longer in DD (Allemand et al., 1973) has been confirmed recently (Sheeba et al., 2000) and was also corroborated by our present observations in response to hydrogen peroxide stress under constant dark conditions. The exciting question of whether this is due to the presence or absence of light-induced oxidants remains to be addressed.

5. Conclusion

Circadian organization is, by all accounts, a fundamental process throughout the biological world. The ubiquity of this process suggests an important role in all aspects of an organism's life cycle, including stress defense. Oxidative stress is now thought to be one of the key stressors involved in aging, which points to the essential relationship between circadian rhythmicity and oxidative stress. In the current study, we have attempted to probe this nexus to cast new light on the interplay between circadian rhythms, oxidative stress, and the aging process. The data presented in the current study suggests a number of interesting conclusions.

First, we show that *per*^{01} mutant males are significantly more susceptible to oxidative stress induced by hydrogen peroxide exposure than their wildtype (CS^{P}) counterparts. Further experiments testing susceptibility to oxidative stress induced by hyperoxia and dietary hydrogen peroxide confirmed this result. This data implies that the *period* gene, and perhaps circadian rhythmicity in general, has a crucial role in the protective response of the organism. Intriguingly, the null mutation in the *period* gene seems to have only a minor effect under optimal conditions, leading to the conclusion that a functioning circadian clock is more important in situations of high stress than in optimal conditions. Our data also demonstrates significantly higher mortality during hours of light than during hours of darkness, irrespective of genotype; this data points to the exciting possibility that light plays a critical role in the response to oxidative stress.

Second, our investigations into the antioxidant enzyme catalase have shown no direct link between this enzyme and mortality. While we saw significant differences in mortality, we generally saw no differences in catalase protein levels and only moderate fluctuations in catalase activity levels as a function of time. We did, however, see much more catalase protein,

and a correspondingly higher catalase activity level, in fly bodies as compared with fly heads. Taken together, this data suggests that catalase alone is not directly responsible for physiological responses to oxidative stress, but it could function as an important component of a repertoire of antioxidant systems.

Finally, interesting differences in the levels of protein carbonyl accumulation were revealed in our study. In fly heads, there is significantly more carbonylation at ZT8 as compared with ZT20; in fly bodies the levels rhythm is less pronounced. This data suggests a rhythmic accumulation of oxidatively damaged proteins, and provides at least a partial molecular explanation for the differential mortality observed between the CS^{P} and per^{01} flies.

6. Future Directions

The body of work presented in this thesis is by no means comprehensive, and the data raises as many new questions as it answers. In order to more fully comprehend the complex interactions of circadian rhythmicity, oxidative stress, and aging, more work is needed. Since metabolism is known to be rhythmically controlled, cells most likely produce endogenous oxidants on a circadian basis. In this case, adding exogenous oxidants would only increase the total oxidant load on the organisms, possibly overwhelming them. To support this hypothesis, cellular and mitochondrial levels of hydrogen peroxide should be assayed throughout a 24-hour period. The overall metabolism of both CS^{P} and per^{01} flies should also be probed for differences, as these differences could contribute to oxidative stress in the organism. It is also important to note that experimenting on one enzyme system is not enough to draw broad conclusions about oxidative stress and circadian rhythmicity. Additional enzyme systems need to be investigated; prime candidates for investigation would be both (Mn and Cu/Zn) forms of superoxide dismutase and glutathione S-transferase peroxidase. The activity of the enzyme aconitase should be assayed; this enzyme of the citric acid cycle is susceptible to oxidative stress because of its cubane cluster and the relative susceptibility of one of the α-Fe units in its structure. This assay would in effect show if there is more endogenous production of ROS in *per* mutants, which is an important component of the overall ROS-circadian rhythmicity picture. Proteasome activity has been shown to decrease with age, leading to the possibility that as organisms age, they slowly lose the efficiency in degrading oxidatively damaged proteins. This avenue should be investigated more fully. Finally, the overall antioxidant capacity of both mutant and wildtype flies should be assayed to determine if per^{01} flies, or indeed other clock mutants, exhibit compromised antioxidant systems.

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