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Circadian regulation of response to oxidative stress in *Drosophila melanogaster*

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Abstract

Circadian rhythms are fundamental biological phenomena generated by molecular genetic mechanisms known as circadian clocks. There is increasing evidence that circadian synchronization of physiological and cellular processes contribute to the wellness of organisms, curbing pathologies such as cancer and premature aging. Therefore, there is a need to understand how circadian clocks orchestrate interactions between the organism's internal processes and the environment. Here, we explore the nexus between the clock and oxidative stress susceptibility in *Drosophila melanogaster*. We exposed flies to acute oxidative stress induced by hydrogen peroxide (H₂O₂), and determined that mortality rates were dependent on time at which exposure occurred during the day / night cycle. The daily susceptibility rhythm was abolished in flies with a null mutation in the core clock gene *period* (*per*) abrogating clock function. Furthermore, lack of *per* increased susceptibility to H₂O₂ compared to wild-type flies, coinciding with enhanced generation of mitochondrial H₂O₂ and decreased catalase activity due to oxidative damage. Taken together, our data suggest that the circadian clock gene *period* is essential for maintaining a robust anti-oxidative defense.

Keywords

Circadian rhythms; catalase, hydrogen peroxide, oxidative stress; period; protein carbonylation

Introduction

Many life processes display daily rhythms, such as rest/activity cycles in animals, including humans. Daily rhythms are entrained by light/dark (LD) cycles but also persist in constant conditions with a periodicity of circa 24 hours as they are generated by endogenous mechanisms called circadian clocks. Several genes acting in a cell-autonomous manner are involved in circadian timekeeping [1]. In *Drosophila melanogaster*, clock genes are expressed in approximately 150 brain neurons as well as in glia, sensory neurons, and many peripheral organs [1-3]. The basic feedback mechanism forming clockwork in *Drosophila* involves Clock (*Clk*) and Cycle (*cyc*) genes encoding proteins that activate transcription of *period* (*per*) and *timeless* (*tim*) genes. The PER and TIM proteins accumulate in cell nuclei, where PER represses CLK/CYC activators, leading to suppression of *per* and *tim* transcription [1].

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While the circadian system controls several behavior-related rhythms in *Drosophila*, less is known about physiological rhythms in this model organism [1-3]. Genome-wide microarray studies have suggested rhythmic expression of genes involved in various metabolic pathways and stress resistance in flies [4,5] and mammals [6]. These molecular rhythms are emerging as important modulators of circadian susceptibility to xenobiotics in mammals [7,8] or to pathogens in flies [9].

Circadian organization of rest/activity cycles implies fluctuations in the level of reactive oxygen species (ROS) that are generated as byproducts of fluctuations in activity and metabolic rates [10]. Microarray studies have suggested daily rhythms in the expression of some antioxidant enzymes such as catalase, SOD, or GST. It has been suggested that these rhythms may protect the organism from excessive levels of ROS and the resulting damage to biological macromolecules [11,12] but there is only scant experimental evidence supporting this hypothesis [13].

To obtain insight into crosstalk between the circadian clock and ROS homeostasis, we queried whether the response to acute oxidative stress is modulated by the circadian system in *Drosophila melanogaster*. We discovered that mortality after 4 h exposure to hydrogen peroxide varied significantly according to the time of oxidant application. These findings set the stage to explore the functional links between the circadian clock and oxidative stress defenses. We demonstrate that a mutation in the clock gene *period* renders flies more susceptible to oxidative stress induced by H₂O₂ and this is correlated with the elevated H₂O₂ production by mitochondria as well as increased accumulation of carbonylated catalase compared to flies with a functional circadian clock

Materials and Methods

Fly rearing and strains

Flies were reared on yeast-cornmeal-molasses diet at 25°C in a 12-hour light/12-hour dark cycle (LD). Time points relative to LD are expressed as Zeitgeber Time (ZT); by convention, ZT0 is the time of lights-on while ZT12 is the time of lights-off. We used 5-day-old male flies of the wild type strain, Canton-S (CS), and mutant *per*⁰¹ [14] which do not produce PER protein. The *per*⁰¹ flies were backcrossed to the CS flies six times to equalize the genetic background; isogenized CS flies were designated CS^P. To test the rescue of *per*-null phenotypes, we used transgenic flies carrying a wild-type copy of *per* (designated as *per*^G) in a *per*⁰¹ background, which rescues locomotor activity rhythms [15]. Females with two copies of *per*^G (*y w per*⁰¹; *per*^G:32.1); were crossed with *per*⁰¹;+;+ males, and F₁ males containing one copy of the rescue construct were used for this study. These heterozygous males have rhythmic locomotor activity as well [15].

Hydrogen peroxide exposure

To test susceptibility to acute oxidative stress, 5-day-old males were exposed to 88 μM H₂O₂ (Sigma Chemical Co., USA). Flies were transferred into glass scintillation vials containing filter paper moistened with 100 μl of H₂O₂ and left there for 4-hours before being returned to the rearing vial; this treatment results in partial mortality within 1–2 days after exposure [16]. H₂O₂ was administered at 4 h intervals: the first group of flies were treated at ZT0 (9 am) and the last group at ZT20 (5 am) resulting in six groups each treated at different time of day. Mortality was recorded 2 days post-treatment.

Total protein carbonyl assay

The amount of protein carbonyls was quantified at ZT8 and ZT20 in heads and bodies of CS^P and *per*⁰¹ flies under unstressed conditions after reaction with 2,4-dinitrophenylhydrazine

(DNPH) as described before [17]. Results were expressed as nmol.mg^{-1} protein using an extinction coefficient of $22,000 \text{ M}^{-1}\text{cm}^{-1}$ at absorbance maxima of 370 nm in a SpectraMax 190 microtitre plate-reader. BSA standard curve was used for protein concentrations in guanidine solutions (Abs 280 nm). Protein carbonyl values were corrected for interfering substances by subtracting the A_{370}/mg protein measured in control samples.

Endogenous hydrogen peroxide production by mitochondria

Mitochondrial H_2O_2 production from 25 heads of CSP and per^{01} males was measured using the Amplex Red reagent (Invitrogen, USA). Individual 100 μl reactions included 5 μg of mitochondrial protein, respiration buffer (40 mM glycylglycine, 10 mM KH_2PO_4 , 5mM MgCl_2 , 120 mM KCl, 1.25 mg/ml fatty acid free BSA, pH 7.4) containing Complex I substrates (5 mM proline and 5 mM pyruvate) or Complex III substrates (5 mM *sn*-glycerol 3-phosphate), 100 μM Amplex Red reagent, and 0.1 U/ml horseradish peroxidase. H_2O_2 production (average of Complex I and III) was measured as an increase in absorbance at 560 nm every 2 min for 1 hr at 25°C using a Biotek Synergy II microplate reader and the linear phase was used to calculate the rate of H_2O_2 production per mg of mitochondrial protein.

qRT-PCR of catalase mRNA

Total RNA was isolated from 25 heads of CSP and per^{01} flies using Tri-reagent (Sigma Chemical Co., USA). RNA was purified using RNeasy kit (Qiagen, USA). cDNA was synthesized using iScript cDNA synthesis kit (BioRad, USA). PCR was conducted using SYBR green qPCR mastermix (BioRad) with the following primers for Catalase: Forward: 5'—AGA TGC TGC ATG GTC GTC TGT TGT TCT-3' and Reverse: 5' TCC ATC CCG CTG GAA GTT CTC AAT-3'. Gene *rp49* was used as an endogenous control, and the clock gene *tim* was used to validate our methods (expected profiles were confirmed, data not shown). Reactions were performed on ABI Prism 7300 and data analyzed using the $2^{-\Delta\Delta\text{Ct}}$ method for fold changes in mRNA expression levels.

Catalase activity assay

Catalase (EC 1.11.1.6) was assayed in individual heads of flies [18] with modifications for a microtitre plate-based assay. Briefly, individual fly heads were homogenized in 100 mM KPO_4 (pH 7.0) with 0.1% Triton X-100, centrifuged at 13,000g for 5-minutes and supernatants were mixed with 10 mM H_2O_2 in K- PO_4 buffer. The decrease in absorbance due to decomposition of H_2O_2 was monitored at 240 nm in a SpectraMax 190 microtitre plate-reader. The activity of catalase was expressed in $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein using the extinction coefficient of $39.4 \text{ mM}^{-1}\text{cm}^{-1}$ for H_2O_2 . Protein content of supernatants was estimated using the BCA reagent.

Western blots

To determine the levels of catalase protein Western blots were performed in CSP and per^{01} flies. Heads (10–15) were homogenized in protein extraction buffer with protease inhibitor cocktail, separated on 15% SDS-PAGE gel and transferred to PVDF Immobilon membranes. Membranes were blocked for 2 hours using Odyssey Blocking Buffer (Li-Cor Biosciences, USA), and transferred into primary antibody. Two primary antibodies were used: anti-catalase, produced in rabbits [19]; and anti-alpha-tubulin (clone DM1A), produced in mice (Sigma Chemical Co., USA). A secondary antibody cocktail (Li-Cor Biosciences, USA) containing goat anti-mouse (IR800) and goat anti-rabbit (IR680) was used for detection of catalase using an Odyssey Infrared Scanner.

Western blot for carbonylated catalase was performed using the OxyBlot protein oxidation detection kit (Millipore, USA) according to manufacturer instructions. For positive control,

catalase from bovine liver (Sigma Chemical Co. USA) was oxidized at 37°C in the dark (5 hours) using 5 mg/ml protein sample in 25 mM HEPES buffer pH 7.2 containing 25 mM ascorbic acid and 100 μ M FeCl₃. The sample was then dialyzed against 50 mM HEPES and 1 mM EDTA. The carbonyl groups in the protein side chains were derivatized to 2,4-dinitrophenylhydrazone by reaction with 2,4-DNPH in sample homogenates from both genotypes as well as bovine catalase. The DNP-derivatized protein samples (25–30 μ g) were separated by SDS-PAGE on 7.5% resolving gel and transferred onto PVDF Immobilon membranes. Membranes were blocked for 2 hours using Odyssey Blocking Buffer and incubated overnight with primary rabbit antibody against DNP (1: 150), followed by goat anti-rabbit (IR680) secondary antibody (1:20,000). The carbonylated catalase was visualized using an Odyssey Infrared Scanner.

Results

Susceptibility to oxidative challenge is regulated by the circadian clock gene period

Exposure of male flies to 4 h H₂O₂ treatment at different times of day resulted in disparate rates of survival. The peak mortality was observed following H₂O₂ exposure at ZT8 during the day and significantly lower ($p < 0.05$) mortality was recorded in flies exposed during the night at ZT20 (Fig. 1A). To determine whether these differences were dependent on a functional circadian clock, we compared susceptibility to H₂O₂ between flies with normal and disrupted circadian clocks. In the first experiment, we exposed flies to constant light (LL) conditions, which disrupt the molecular clock mechanism by preventing accumulation of clock proteins TIMELESS and PERIOD [20], and cause behavioral arrhythmia. Flies kept in LD had significantly ($p < 0.001$) lower mortality following night exposure to H₂O₂ as compared to day exposure. In contrast, mortality of H₂O₂-exposed flies was similar at equivalent time points in LL (Fig. 1B). In the second experiment, we tested null mutants of the clock gene *period* (*per*⁰¹). There was no difference in mortality between *per*⁰¹ mutants treated with H₂O₂ at ZT8 versus ZT20 (Fig. 1C). Interestingly, these mutants showed significantly higher mortality (1.7 to 5 fold) after H₂O₂ exposure than control CS_P flies at both time points (Fig. 1C).

Flies with rescued *per* function displayed a rhythm in H₂O₂ sensitivity and their survival rates were similar to those in CS_P flies and significantly lower than those in *per*⁰¹, demonstrating that the effects observed in the mutant are functionally connected to the lack of PER protein (Fig. 1C).

Daily rhythms in endogenous levels of protein carbonyls and hydrogen peroxide production

The difference in susceptibility to H₂O₂ between flies exposed at ZT8 or ZT20 prompted us to examine the endogenous levels of protein carbonyls, a reliable biomarker for oxidative stress. Total protein carbonylation in heads was significantly higher (2.6 fold, $p < 0.001$) at ZT8 than at ZT20 in CS_P flies whereas in *per*⁰¹ flies no significant differences were observed between the two time points; however, the levels were markedly (1.5 to 3 fold, $p < 0.001$) elevated compared to control flies (Fig 2A). We also analyzed protein carbonyls in fly bodies. The total protein carbonyl levels in bodies were approximately two-fold lower than in the heads; no differences were recorded at ZT8 between CS_P and *per*⁰¹ flies but carbonylation was significantly higher ($p < 0.05$) in *per*⁰¹ fly bodies at ZT20 (data not shown).

To test whether differences in protein carbonyls in fly heads might be related to the levels of endogenous H₂O₂ production by mitochondria, we compared daily pattern of total mitochondrial H₂O₂ production (Complex I and Complex III) between CS_P and *per*⁰¹ flies. In CS_P fly heads, the levels peaked during ZT4-ZT8 and declined during ZT12-ZT20. In *per*⁰¹ fly heads no significant rhythm was observed; H₂O₂ production was relatively constant, but significantly higher at all time points than in CS_P fly heads (Fig 2B).

Daily patterns of catalase transcription and activity in control and *per*-null flies

Daily fluctuations in H₂O₂ levels in wild type flies, and persistent elevation of this oxidant in *per*⁰¹ mutant could be related to changes in the main H₂O₂-scavenging enzyme-catalase. We tested this by measuring catalase transcription, activity and inducibility. The levels of *cat* mRNA did not differ significantly between the two genotypes and showed only minor daily fluctuations (Fig 3A). Consistent with mRNA data, the levels of CAT protein did not differ significantly between time points (data not shown).

In CSP flies the activity of CAT showed a tendency to peak at ZT16; but, was not significantly different (Fig 3B). In *per*⁰¹, the CAT activity was arrhythmic and significantly lower (*p*<0.05) compared to CSP at most time points.

In both CSP and *per*⁰¹ fly heads, there was no induction of CAT activity following exposure to H₂O₂, but rather a decrease with time (Fig. 3C). This inhibition of CAT activity was more pronounced in *per*⁰¹ flies than in CSP flies. Importantly, CAT activity in *per*⁰¹ fly heads was significantly lower than in CSP flies in both unchallenged (0 h) and H₂O₂-exposed flies (Fig. 3C).

Increased carbonylation of catalase in *per*⁰¹ flies

To test whether the reduced activity of CAT in *per*⁰¹ fly heads could be due to damage by carbonylation, we checked CAT protein carbonylation. Western blots revealed that *per*⁰¹ fly heads have distinctly more carbonylated CAT protein at both ZT8 and ZT20 compared to CSP flies under normal conditions (Fig 4).

Discussion

We demonstrate that *D. melanogaster* shows daily mortality rhythm in response to oxidative stress with a maximum susceptibility during the late light phase and a minimum during late dark phase. This rhythm was abolished in constant light demonstrating that a functional clock is necessary for such rhythms. The rhythm was also abolished in the *period* mutant which showed significantly increased susceptibility to H₂O₂ compared with control flies. This suggests that the physiological response to exogenous oxidative stress is regulated by the circadian clock in flies. While this is the first report of rhythmic susceptibility to oxidative stress, previous studies have reported a circadian response to other stressors such as xenobiotics or pathogens [7-9]. Thus, our data provide new evidence suggesting that circadian clocks may fine-tune defenses to exogenous stressors.

Our search for the basis of mortality rhythms in H₂O₂-exposed flies revealed significant differences in the levels of protein carbonylation in unchallenged flies. These levels correlated directly with differential mortality rates observed in CSP flies exposed during the day or night to exogenous H₂O₂. It has been established that protein carbonylation levels increase during aging [21]. Importantly, we demonstrate here that both protein carbonylation and the risk of death are under circadian control in non-aged flies. During late dark phase, when PER levels are normally high [1], flies have the least oxidative protein damage and the lowest risk of death. These data are interesting in view of reports that in humans the risk of death from various pathologies may vary with time of day [22]. We also show that lack of *period* gene results in excessive carbonylation, as reported earlier [13]. The mechanism of protective *per* action remains to be elucidated.

The rhythmic generation of mitochondrial H₂O₂ in CSP coinciding with carbonylation rhythms may be linked to physical activity since flies are day-active. Enhanced H₂O₂ generation in arrhythmic *per*⁰¹ flies suggest that the circadian system is involved in maintaining ROS

homeostasis. Consistent with this hypothesis, the elevation of ROS was reported in various organs of mice lacking clock protein BMAL1 [23].

Conceivably, rhythmic H₂O₂ levels could be related to changes in catalase expression as circadian rhythms in *cat* mRNA were suggested in fly bodies [4], rat blood cells [24], and plants [25]. However, no such rhythm was observed in our study. Interestingly, CAT activity was significantly decreased in *per*⁰¹ flies compared to CS^p at most time points, in agreement with a previous report [26]. CAT was not induced by exposure to H₂O₂, consistent with previous reports in flies [27].

We determined that the likely reason for decreased CAT activity is higher carbonylation of CAT in *per*⁰¹ than in CS^p flies. CAT, while participating in oxidative metabolic reactions may itself be vulnerable to oxidative damage as in case of mitochondrial and peroxisomal proteins [28], which may lead to their decreased activity [29].

Taken together our data suggests that circadian clock gene *period* plays important roles in effective organismal defense against exogenous ROS and in maintaining the balance between endogenous ROS production and removal. It remains to be determined whether PER protein in flies act as an element of the circadian clock or via non-circadian roles and warrants further studies on the role of this gene in organismal oxidative stress defense.

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References

1. Hardin PE. The circadian timekeeping system of *Drosophila*. *Curr. Biol* 2005;15:R714–R722. [PubMed: 16139204]
2. Giebultowicz JM. Peripheral clocks and their role in circadian timing: insights from insects. *Phil. Trans. R. Soc. B* 2001;356:1791–1799. [PubMed: 11710986]
3. Hall JC. Genetics and molecular biology of rhythms in *Drosophila* and other insects. *Adv. Genet* 2003;48:1–280. [PubMed: 12593455]
4. Ceriani MF, Hogenesch JB, Yanovsky M, Panda S, Straume M, Kay SA. Genome-wide expression analysis in *Drosophila* reveals genes controlling circadian behavior. *J. Neurosci* 2002;22:9305–9319. [PubMed: 12417656]
5. McDonald MJ, Rosbash M. Microarray analysis and organization of circadian gene expression in *Drosophila*. *Cell* 2001;107:567–578. [PubMed: 11733057]
6. Miller BH, McDearmon EL, Panda S, Hayes KR, Zhang J, Andrews JL, Antoch MP, Walker JR, Esser KA, Hogenesch JB, Takahashi JS. Circadian and CLOCK-controlled regulation of the mouse transcriptome and cell proliferation. *Proc. Natl. Acad. Sci. U S A* 2007;104:3342–3347. [PubMed: 17360649]
7. Gorbacheva VY, Kondratov RV, Zhang R, Cherukuri S, Gudkov AV, Takahashi JS, Antoch MP. Circadian sensitivity to the chemotherapeutic agent cyclophosphamide depends on the functional status of the CLOCK/BMAL1 transactivation complex. *Proc. Natl. Acad. Sci. U S A* 2005;102:3407–3412. [PubMed: 15689397]
8. Gachon F, Olela FF, Schaad O, Descombes P, Schibler U. The circadian PAR-domain basic leucine zipper transcription factors DBP, TEF, and HLF modulate basal and inducible xenobiotic detoxification. *Cell Metab* 2006;4:25–36. [PubMed: 16814730]
9. Lee JE, Edery I. Circadian regulation in the ability of *Drosophila* to combat pathogenic infections. *Curr. Biol* 2008;18:195–199. [PubMed: 18261909]

10. Langmesser S, Albrecht U. Life time-circadian clocks, mitochondria and metabolism. *Chronobiol. Int* 2006;23:151–157. [PubMed: 16687289]
11. Hardeland R, Coto-Montes A, Poeggeler B. Circadian rhythms, oxidative stress, and antioxidative defense mechanisms. *Chronobiol. Int* 2003;20:921–962. [PubMed: 14680136]
12. Kondratov RV. A role of the circadian system and circadian proteins in aging. *Ageing Res. Rev* 2007;6:12–27. [PubMed: 17369106]
13. Coto-Montes A, Hardeland R. Diurnal rhythm of protein carbonyl as an indicator of oxidative damage in *Drosophila melanogaster*: Influence of clock gene alleles and deficiencies in the formation of free radical scavengers. *Biol. Rhythm Res* 1999;30:383–391.
14. Konopka RJ, Benzer S. Clock mutants of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* 1971;68:2112–2116. [PubMed: 5002428]
15. Cheng Y, Gvakharia B, Hardin PE. Two alternatively spliced transcripts from the *Drosophila* period gene rescue rhythms having different molecular and behavioral characteristics. *Mol. Cell Biol* 1998;18:6505–6514. [PubMed: 9774666]
16. Rush B, Sandver S, Bruer J, Roche R, Wells M, Giebultowicz J. Mating increases starvation resistance and decreases oxidative stress resistance in *Drosophila melanogaster* females. *Aging Cell* 2007;6:723–726. [PubMed: 17875001]
17. Krishnan N, Vecera J, Kodrik D, Sehnal F. 20-Hydroxyecdysone prevents oxidative stress damage in adult *Pyrhocoris apterus*. *Arch. Insect Biochem. Physiol* 2007;65:114–124. [PubMed: 17570141]
18. Orr WC, Sohal RS. The effects of catalase gene overexpression on life span and resistance to oxidative stress in transgenic *Drosophila melanogaster*. *Arch. Biochem. Biophys* 1992;297:35–41. [PubMed: 1379030]
19. Radyuk SN, Klichko VI, Orr WC. Catalase expression in *Drosophila melanogaster* is responsive to ecdysone and exhibits both transcriptional and post-transcriptional regulation. *Arch. Insect Biochem. Physiol* 2000;45:79–93. [PubMed: 11093245]
20. Price JL, Dembinska ME, Young MW, Rosbash M. Suppression of PERIOD protein abundance and circadian cycling by the *Drosophila* clock mutation *timeless*. *EMBO J* 1995;14:4044–4049. [PubMed: 7664743]
21. Sohal RS, Sohal BH, Orr WC. Mitochondrial superoxide and hydrogen peroxide generation, protein oxidative damage, and longevity in different species of flies. *Free Radic. Biol. Med* 1995;19:499–504. [PubMed: 7590400]
22. Peberdy MA, Ornato JP, Larkin GL, Braithwaite RS, Kashner TM, Carey SM, Meaney PA, Cen L, Nadkarni VM, Praestgaard AH, Berg RA. Survival from in-hospital cardiac arrest during nights and weekends. *Jama* 2008;299:785–792. [PubMed: 18285590]
23. Kondratov RV, Kondratova AA, Gorbacheva VY, Vykhovanets OV, Antoch MP. Early aging and age-related pathologies in mice deficient in BMAL1, the core component of the circadian clock. *Genes Dev* 2006;20:1868–1873. [PubMed: 16847346]
24. Du Y-Z, Fan S-J, Meng Q-H, Wang G-Q, Tong J. Circadian expression of clock and screening of clock-controlled genes in peripheral lymphocytes of rat. *Biochem. Biophys. Res. Commun* 2005;336:1069–1073. [PubMed: 16168960]
25. McClung CR. Regulation of catalases in *Arabidopsis*. *Free Radic. Biol. Med* 1997;23:489–496. [PubMed: 9214587]
26. Dittrich, M.; Hardeland, R. Temporal patterns of antioxidative enzymes in the *Drosophila* clock mutant *per⁰* kept under a light-dark regimen.. In: Hardeland, R., editor. *Biological Rhythms and Antioxidative Protection*. Cuvillier; Gottingen: 1997. p. 131-134.
27. Klichko VI, Radyuk SN, Orr WC. Profiling catalase gene expression in *Drosophila melanogaster* during development and aging. *Arch. Insect Biochem. Physiol* 2004;56:34–50. [PubMed: 15101064]
28. Toroser D, Orr WC, Sohal RS. Carbonylation of mitochondrial proteins in *Drosophila melanogaster* during aging. *Biochem. Biophys. Res. Commun* 2007;363:418–424. [PubMed: 17884014]
29. Nguyen AT, Donaldson RP. Metal-catalyzed oxidation induces carbonylation of peroxisomal proteins and loss of enzymatic activities. *Arch. Biochem. Biophys* 2005;439:25–31. [PubMed: 15922287]

Figure 1.

(A) Wild type males of *Drosophila* (CS^P) exhibit circadian rhythm in susceptibility to oxidative stress caused by exposure to H₂O₂. Significantly higher ($p < 0.05$, $n = 6$, one-way ANOVA with Tukey's multiple comparison) mortality was recorded at ZT8 (light) than at ZT20 (dark). (B) The susceptibility to H₂O₂ in CS^P flies under constant light (LL) was equally high in subjective day (8) and subjective night (20). (C) Flies lacking *period* gene (*per*⁰¹) exhibited higher mortality with no apparent rhythm in LD. *per*-rescue flies exhibited similar rhythms in susceptibility as CS^P flies. Error bars represent $\pm$ SEM and columns with different superscripts differ significantly at $p < 0.001$, $n = 6-8$, two-way ANOVA with Bonferroni's post hoc test.

Figure 2.

Protein carbonylation and mitochondrial H₂O₂ production in heads of *Drosophila* males (**A**) Protein carbonylation was significantly higher at ZT8 (light phase) compared with ZT20 (dark phase) in CS^P. *per*⁰¹ flies showed no rhythmicity in protein carbonylation in heads but the levels were significantly higher at both time points. Error bars represent $\pm$ SEM and columns with different superscripts differ significantly at $p < 0.001$, $n = 9$, two-way ANOVA with Bonferroni's post hoc test. (**B**) Mitochondrial H₂O₂ production (Complex I and III) was elevated during daytime and lower during nighttime in wild type flies. In comparison, *per*⁰¹ fly heads showed arrhythmic and significantly increased H₂O₂ production ($p < 0.05$, $n = 6$, two-way ANOVA with Bonferroni's post-hoc test).

Figure 3.

(A) Catalase mRNA expression does not exhibit significant fluctuations in *Drosophila* CS^P or *per*⁰¹ heads. (B) Catalase enzymatic activity did not show significant rhythm in CS^P and *per*⁰¹ heads but was significantly lower ($p < 0.05$) in *per*⁰¹ flies compared with CS^P flies. Error bars represent $\pm$ SEM while asterisks (*) represent significant ($p < 0.05$, $n = 9$, two-way ANOVA with Bonferroni's post test) differences between activity in CS^P and *per*⁰¹ flies. (C) Challenge with H₂O₂ resulted in inhibition of CAT activity in both CS^P and *per*⁰¹ flies. The inhibition was sharp with a significant ($p < 0.05$) decrease in activity from 0–12 hours post exposure at ZT8. At ZT20, the decrease was less and not significant between 4–12 h post-exposure. In all

treatments, the inhibition was more severe in *per*⁰¹ than CSP. Error bars represent $\pm$ SEM and columns with different superscripts differ significantly at $p < 0.05$, unpaired Students t-test.

Figure 4.

Detection of carbonylated catalase in *Drosophila* heads extracts probed with anti-DNPH antibodies. The left lane shows carbonylated bovine catalase (control), the rightmost lane shows MW standards. Marked increase in carbonylated catalase was detected in heads of *per⁰¹* fly heads compared with *CS^P* flies.