The circadian clock gene *period* extends healthspan in aging

*Drosophila melanogaster*

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Abstract: There is increasing evidence that aging is affected by biological (circadian) clocks – the internal mechanisms that coordinate daily changes in gene expression, physiological functions and behavior with external day/night cycles. Recent data suggest that disruption of the mammalian circadian clock results in accelerated aging and increased age-related pathologies such as cancer; however, the links between loss of daily rhythms and aging are not understood. We sought to determine whether disruption of the circadian clock affects lifespan and healthspan in the model organism *Drosophila melanogaster*. We examined effects of a null mutation in the circadian clock gene *period* (per¹⁰⁶) on the fly healthspan by challenging aging flies with short-term oxidative stress (24h hyperoxia) and investigating their response in terms of mortality hazard, levels of oxidative damage, and functional senescence. Exposure to 24h hyperoxia during middle age significantly shortened the life expectancy in per¹⁰⁶ but not in control flies. This homeostatic challenge also led to significantly higher accumulation of oxidative damage in per¹⁰⁶ flies compared to controls. In addition, aging per¹⁰⁶ flies showed accelerated functional decline, such as lower climbing ability and increased neuronal degeneration compared to age-matched controls. Together, these data suggest that impaired stress defense pathways may contribute to accelerated aging in the per mutant. In addition, we show that the expression of *per* gene declines in old wild type flies, suggesting that the circadian regulatory network becomes impaired with age.

INTRODUCTION

Circadian clocks generate daily endogenous rhythms in behavior, physiological functions, and cellular activities, which are coordinated with external day/night cycles [1, 2]. Circadian rhythms become impaired with age as evidenced by the dampening of daily oscillations in melatonin and other hormones and the disruption of night-time sleep in aged rodents and humans [3, 4, 5]. Remarkably, age-associated sleep fragmentation was also reported in *Drosophila melanogaster* [6], suggesting that effects of aging on circadian systems may be evolutionarily conserved. While aging impairs the circadian systems, there is also evidence that loss of circadian rhythms may, in turn, contribute to aging. Genetic disruption of circadian rhythms by knockout of specific clock genes leads to various age related pathologies and visible signs of premature aging in mice [7, 8]. In addition, chronic jet-lag which disrupts the circadian clock, increases mortality in aged mice [9]. As extension of healthspan is of critical importance in aging human population, there is a need to elucidate how strong circadian clocks may support healthy aging.

The mechanisms linking circadian rhythms to the rate of aging and healthspan are not well understood. To
address these mechanisms, we investigated whether disruption of the circadian clock affects response to homeostatic challenge and aggravates selected aging biomarkers in the model organism *Drosophila melanogaster*. We used a null mutation in the circadian clock gene *period* (*per*<sup>01</sup>) [10]; this gene is one of the four core clock genes that act in a negative auto-regulatory feedback loop generating daily endogenous rhythms [11, 12]. The loss of *per* function disrupts behavioral and molecular rhythms in flies [10, 11, 13].

To compare lifespan and healthspan in flies with normal or disrupted circadian clock, we measured their ability to maintain ROS homeostasis during aging. We probed the health status of aging flies by exposing them to mild oxidative stress of 24h hyperoxia at increasing chronological ages, followed by assessment of the resulting oxidative damage and mortality hazards. Hyperoxia was chosen as a homeostatic challenge, because it directly leads to ROS production irrespective of age-related changes in food consumption and other physiological parameters [14].

We report that *per*<sup>01</sup> flies have shortened healthspan as evidenced by their increased mortality hazard in response to homeostatic challenge during aging. This conclusion is also supported by accelerated functional senescence, and increased signs of neurodegeneration in *per* mutants compared to age-matched controls with an intact circadian clock. In addition, we show that the expression of *per* gene declines with age leading to disruption of the circadian regulatory network in old wild type flies.

![Figure 1](image-url)

**Figure 1. Lifespan of *per*<sup>01</sup> and CS<sup>0</sup> *D. melanogaster* in normoxia and following 24h hyperoxia at different ages (marked by arrow in B-D).** (A) In normoxia, there was no significant difference in mean survival curves (p=0.23) (B) Hyperoxia on day 5 did not significantly affect longevity or survival curves (p=0.12) (C) Hyperoxia on day 20 resulted in a significant reduction (p<0.05) in average survival of *per*<sup>01</sup> flies compared to CS<sup>0</sup> with significant (p<0.0001) difference in survival curves. (D) Hyperoxia on day 35 resulted in more significant reduction (p<0.001) in average lifespan in *per*<sup>01</sup> flies compared to CS<sup>0</sup> and significant difference in survival curves (p<0.0001). Males with rescued *per* function (*per*<sup>01</sup> {per<sup>+</sup>}) treated with hyperoxia on day 35 had average lifespan similar to CS<sup>0</sup> but significantly different (p<0.001) from *per*<sup>01</sup> mutants.
RESULTS

Short-term oxidative stress shortens the lifespan in per01 mutants

To determine how loss of per affects lifespan and healthspan, per01 were backcrossed for 6 generations to Canton S strain, and this control stock was designated as CSp. Under normal laboratory conditions, the longevity of per01 males was similar to CS p controls (Figure 1A, Table 1). However, lifespan was significantly reduced in per01 flies exposed to 24 h hyperoxia in middle age. Hyperoxia on day 20 shortened the average lifespan in per01 mutants by 12% while hyperoxia on day 35 decreased average lifespan of per01 flies by 20% compared to CS p males (Table 1); survival curves were significantly different in both ages (Figure 1C-D). We also calculated age specific mortality trajectories, and showed that mortality hazard significantly increased after exposure to 24 h hyperoxia on day 20 or 35 in per01 but remained unchanged in CS p males (see Supplemental Information Figure S1 and Table S1). To verify that these effects are indeed linked to the lack of per gene function, we tested the lifespan of per01 flies transformed with a wild type copy of per, designated as per01 {per+}. When flies with rescued per function were exposed to hyperoxia on day 35, their average survival (59 ± 2.0 days) and mortality trajectories were similar to CS p controls, but significantly different from per01 mutants (Figure 1D, S1D, and Table S1). This verified that shortened lifespan and increased death-risk in per mutants are due to the loss of per gene. Importantly, exposure to hyperoxia on day 5 did not affect the average lifespan or mortality trajectories of per01 mutants (Figure 1B and S1B), demonstrating that hyperoxia sensitivity in these mutants is an age dependent phenotype.

per01 mutants accumulate more oxidative damage in response to stress and during normal aging

Given the increased mortality hazard in response to hyperoxia in per01 mutants, we next assessed the levels of oxidative damage incurred after 24 h hyperoxia exposure at the age of 5, 20, 35 and 50 days in both genotypes. Levels of protein carbonyls (PC) and the lipid peroxidation product 4-HNE were measured separately in heads and bodies. Exposure to hyperoxia induced significantly higher (p<0.001) PC levels in per01 than in CSp heads at all ages except day 5 (Figure 2A and Table S2). Similar as in heads hyperoxia on day 35 or 50 led to moderate PC increase in CS p bodies and dramatic increase in the bodies of per01 flies (Figure 2B and Table S2). Restoring per+ function in a per01 background resulted in PC content similar as in CS p and significantly lower than in per01 males (Table S2). Thus, the loss of per function leads to dramatically higher accumulation of PC in per01 flies faced with oxidative challenge. Similar as in the case of mortality hazard this deleterious phenotype is age dependent occurring in middle aged and old flies but not young per01 mutants (Figure 1-2 and S1).

Table 1. Average lifespan of CS p and per01 males exposed to 24h hyperoxia at indicated ages

| Treatment         | Genotypes | CS p         | per01        |
|-------------------|-----------|--------------|--------------|
|                   |           | Normoxia     | Hyperoxia day 5 | Hyperoxia day 20 | Hyperoxia day 35 |
|                   |           | 61.5 ± 1.8a  | 60.4 ± 0.8a  | 58.4 ± 0.93a     | 59.5 ± 1.03a     |
|                   |           | (n = 596)    | (n = 447)    | (n = 415)        | (n = 328)        |
|                   |           | 59.0 ± 1.02a | 56.9 ± 0.93b | 51.35 ± 1.07**c  | 47.8 ± 1.68**c  |
|                   |           | (n = 640)    | (n = 480)    | (n = 385)        | (n = 350)        |

Values shown with SEM, n denotes the sample size. One-Way ANOVA with Tukey-Kramer multiple comparisons test. Statistical comparison across genotypes * p<0.05, ** p<0.001; within genotype, values with different superscripts are significantly different at p<0.05.
The second indicator of oxidative damage, the lipid peroxidation product 4-HNE, was also measured in heads and bodies of CS<sup>p</sup> and per<sup>01</sup> flies. Exposure to hyperoxia on day 35 and 50 significantly increased HNE in per<sup>01</sup> heads compared to respective CS<sup>p</sup> controls (p<0.001) while exposure on day 5 or 20 had no significant effect (Figure 2C and Table S3). Similar as in heads, hyperoxia administered on day 35 and 50 induced significantly more HNE in per<sup>01</sup> than in CS<sup>p</sup> bodies, however, the increase was less pronounced than in fly heads (Figure 2C-D). These effects depend on the per gene as males with restored per function exhibited significantly lower HNE profiles than per<sup>01</sup> males, and similar as those observed in CS<sup>p</sup> flies (Table S3).

**Aging per<sup>01</sup> mutants show greater mobility impairment and neurodegeneration**

Our data show significantly higher accumulation of oxidative damage even in unchallenged per<sup>01</sup> mutants under normoxia compared to age matched controls (Figure 2, Tables S2-S3). As oxidative damage is one of the important biomarkers of aging, we asked whether other signs of aging are advanced in per<sup>01</sup> mutants. First, we compared age-related locomotor performance between mutant and control flies. We used the RING assay, which utilizes negative geotaxis in Drosophila to assess climbing performance (15, 16). We measured climbing ability of per<sup>01</sup> and CS<sup>p</sup> flies aged to day 5, 20,
35 or 50. Surprisingly, 5 day old per\textsuperscript{01} flies showed significantly higher climbing ability than control flies. In contrast, middle-aged and older per\textsuperscript{01} males showed significantly impaired climbing ability compared to age-matched controls (Figure 3). The difference was especially dramatic on day 50; at this age the average climbing ability of per\textsuperscript{01} males was approximately 4 fold lower than in CS\textsuperscript{0} controls. This was partly caused by lack of vertical movement in many per\textsuperscript{01} flies at this age. The fact that young per\textsuperscript{01} mutant flies did not show impaired climbing demonstrate that the period gene does not affect fly geotaxis per se, but rather contributes to impaired climbing ability in an age-dependent fashion.

Another indicator of aging that we tested in per\textsuperscript{01} flies was the health of their nervous system. As aging is associated with degenerative morphological changes in the central nervous system, we examined brain sections from 50 day old per\textsuperscript{01}, CS\textsuperscript{0}, and per\textsuperscript{01} (per\textsuperscript{+}) males. We evaluated number of vacuoles, as they reflect the level of neurodegenerative damage in the brain [17]. Brains of per\textsuperscript{01} males showed significantly (p<0.05) greater number of vacuoles than control CS\textsuperscript{0} and per\textsuperscript{01} (per\textsuperscript{+}) flies with restored per function (Figure 4). These vacuoles, which were found mainly in the neuropils of the optic lobes and the central brain, lead to disrupted neuronal connections. Increased vacuolization in 50 day old per\textsuperscript{01} flies is consistent with their severely impaired mobility (Figure 3).

**Expression of per gene declines significantly with age**

Since age related functional decline is accelerated in per\textsuperscript{01} flies compared to flies with normal clock, it was of interest to investigate daily profiles of per expression during aging in control CS\textsuperscript{0} flies. Therefore, we used qRT-PCR to measure the expression levels of per mRNA extracted from flies collected every 4h for 24h at age 5, 35 and 50 days. As expected [11], per mRNA levels showed daily cycling with lowest levels in the morning and a peak at early night in the heads of young flies (Figure 5A). The levels of per between peak and trough changed with a 12-fold amplitude. This amplitude dampened significantly in 35 day old flies; however, there was still pronounced cycling of per mRNA with 8-fold amplitude. A dramatic dampening of per oscillation was observed on day 50 with the amplitude reduced to 2-fold. Comparison of the relative per mRNA levels at the peak showed significant reduction by ca 70% in 50 day old flies relative to peak expression levels in young flies. Since per encodes an essential component of circadian clock, our data suggest that the circadian network is severely impaired in old flies.

**DISCUSSION**

This study demonstrates healthspan extending role of the clock gene period and suggest that functional circadian clocks may prevent premature aging in flies. Research on Drosophila has demonstrated that different genetic manipulations and environmental interventions can extend fly lifespan [18]. Less attention has been paid to healthspan, despite that extension of healthspan is of critical importance in aging human population. Here, we show that healthspan can follow different trajectories in flies which have similar lifespan under stress-free laboratory conditions. Healthspan is an important but poorly defined concept, and there is an ongoing debate whether model organisms, such as Drosophila, can help to characterize parameters that could detect differences in healthspan [19]. We demonstrate that a relatively mild exogenous stress of 24 h hyperoxia, which revealed health impairment of per\textsuperscript{01} mutant, could be established as a convenient method to probe fly healthspan in a search for mechanisms supporting healthy aging.

Here, we show that healthspan, measured as the ability to respond to homeostatic challenge is reduced in per\textsuperscript{01} flies. Exposure to mild oxidative stress in middle age significantly shortened life expectancy in per\textsuperscript{01} flies but, importantly, not in control flies. The lower capacity of per\textsuperscript{01} mutants to buffer short-term oxidative challenge was linked to greatly increased accumulation of...
oxidative damage during hyperoxia exposure. Thus, it appears that increased mortality hazard in hyperoxia-exposed per01 mutants may be caused by their impaired ability to clear the oxidative damage which is suggested to be one of the major causes of aging [20].

The higher accrual of oxidative damage observed in per01 flies in normoxia and especially after hyperoxia could be influenced by a number of factors, with the primary suspect being higher production of endogenous ROS, which has been reported to increase in clock-disrupted flies [21] and mice [7]. Whether higher ROS is associated with decreased activity of ROS scavenging enzymes remains to be determined. While microarray studies suggested that expression of superoxide dismutase and catalase may be controlled by the circadian clock in flies [22], qRT-PCR did not confirm such rhythm for catalase, but demonstrated that catalase activity is significantly lower in young clock-deficient flies [21]. It is currently unknown whether enzymes involved in protein repair are controlled by the circadian clock in animals, although such control was reported in plants [23]. Finally, excessive agglomeration of oxidatively damaged proteins in per01 flies could be related to impaired degradation as proteasome activity has been shown to decline with age in flies, and may be inhibited by PC and HNE [24, 25].

Figure 4. Neuronal degeneration is accelerated in per01 mutants compared to CS0 and flies with restored per function (per01 {per+}) on day 50. (A) Mean number of vacuoles (with SEM) representing neuronal degeneration was significantly higher in per01 mutants compared with wild type CS0 and flies with rescued per. Bars with different superscripts are significantly different at p<0.05, data based on 10-15 heads for each genotype. (B-D) Photomicrographs of representative brain sections of CS0, per01, and per01{per+} males. Arrows point to vacuolization.
As in humans, age-related functional declines such as disrupted sleep and decreased mobility are observed in Drosophila [6, 26]. The negative geotaxis assay revealed significant impairment in climbing ability in aging per01 flies relative to age-matched controls suggesting that lack of per impairs physical performance during aging. Importantly, exacerbated mobility decline in per01 flies was associated with increased neuronal degeneration in the brain. Neurodegenerative effects in the form of vacuoles in the neuropil region were observed with higher frequency in 50-day old per01 mutants than in CSp or per01(per+)} flies with restored per function. The formation of vacuoles was previously linked to oxidative damage and accelerated aging in Drosophila with impaired carbonyl reductase gene [27], and in flies with Alzheimer-like phenotypes [28].

Our study suggests that functional circadian rhythms support healthy aging in flies. PER protein is the essential element of circadian clock and its absence disrupts molecular and cellular rhythms. We reported previously that young wild type flies have daily rhythms in ROS and PC levels, while in per01 flies levels of these deleterious compounds are significantly higher and arrhythmic [21]. We hypothesize that the circadian clock slows down the accumulation of oxidative damage in aging organisms by synchronizing the activities of enzymes involved in protein homeostasis. For example, microarray studies reported synchronous upregulation of several GST enzymes in flies [29], and it is known that glutathione participates in the conjugation of oxidized proteins [30]. In the absence of circadian clock, enzymes working in a specific pathway may become dysregulated leading to impaired removal of oxidative damage. However, we cannot exclude the possibility that per could affect efficiency of anti-oxidative defense systems independent of its role as a clock component, by acting in a pleiotropic non-circadian manner.

While loss of the circadian rhythms by disruption of the gene period accelerates aging, organisms with normal clocks also age. Our data demonstrate that at middle age per01 mutant shows aging phenotypes normally observed in chronologically older wild type flies, suggesting that clock gene activities may decline with age. Indeed, we demonstrate the amplitude of per mRNA oscillation is severely dampened in 50 day old flies and levels of per mRNA are significantly reduced at late night, when PER acts as essential element of clock negative feedback loop [11]. This suggests that circadian clocks and, consequently circadian rhythms are severely impaired in individuals of advanced age, which is consistent with declining strength of behavioral rhythms reported in aging flies [6]. While factors contributing to the decline of circadian rhythms in flies remain to be elucidated, oxidative stress is likely to be involved. We show here that oxidative damage accumulates to high levels even in wild type aging flies, and a previous report demonstrated that paraquat-induced oxidative stress, or decrease in FOXO expression, led to dampened per expression in Drosophila [31]. Decline in clock genes with age has been reported in zebrafish [32], rats [33] and most recently in rhesus monkey [34]. The intriguing similarities in the behavior of clock genes during aging...
between mammals, zebrafish, and flies warrants investigations of the mechanisms causing disruption of the circadian networks. Understanding these mechanisms will help to determine in future whether strong circadian clocks add water to the fountain of youth.

**EXPERIMENTAL PROCEDURES**

Fly rearing and life span analysis *Drosophila melanogaster* were reared on yeast-cornmeal-molasses-agar diet (35g yeast/l) at 25°C in a 12-hour light/12-hour dark cycles; all experiments were performed 4-8 h after lights-on. The per^{63} mutant flies [10] were backcrossed 6 times to the Canton-S (CS) flies designated as CS². To rescue per-function, we used transgenic flies carrying a wild-type copy of per (designated as per^{CS}) in a per^{63} background [35]. Males with two copies of per^{CS} (y w per^{63}; per^{CS}:32.1) were crossed with per^{63};+;+ females, and F1 males containing one copy of rescue construct designated per^{63}per^{CS} were used. We confirmed their rhythmic locomotor activity indicating rescue of circadian clock function.

To determine lifespan, 3-4 cohorts of 100 flies of each genotype were housed in 16 oz transparent plastic bottles inverted over 60 mm Petri-dishes containing 15 ml of diet. Diet was replaced on alternate days without anesthesia, and mortality was recorded daily. For hyperoxia exposure, males were transferred from cages to narrow vials with diet in groups of 25, and placed in a Plexiglas chamber filled with oxygen (100% medical grade) flowing at a constant rate (300ml/min) for 24 h. Control flies were transferred to narrow vials as above and kept under normoxia. Hyperoxia-treated and control flies were then either frozen for oxidative damage analysis or returned to cages and monitored for mortality.

**Oxidative damage assays** The amount of protein carbonyls was assayed separately in 25 heads and bodies. Carbonyls were quantified after reaction with 2,4-dinitrophenylhydrazine (DNPH) as described previously [21] at 370 nm in a BioTek Synergy 2 plate reader. Results were expressed as nmol.mg⁻¹ protein using an extinction coefficient of 22,000 M⁻¹.cm⁻¹. The lipid peroxidation product 4-hydroxy-2-nonenal (4-HNE) was assayed in heads and bodies by competitive enzyme-linked immunosorbent assay (ELISA) as described [36, 37]. Briefly, free HNE (Alpha Diagnostic, San Antonio, TX, USA) was conjugated to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein [38]. Wells in a 96-well plate were coated with 500 ng of HNE-GAPDH protein for 24h at 4°C, washed in PBS-Tween, and blocked with 1% BSA. A standard dose-response curve was developed from serial dilutions of HNE-GAPDH with polyclonal anti-HNE antibody (1:1000; Alpha Diagnostic). For samples, 10 µg of protein lysate was mixed with 1:1000 polyclonal rabbit anti-HNE antibody and added to wells in triplicate. Plates were incubated for 1 h, washed with buffer, incubated with 1:5000 secondary anti-rabbit antibody conjugated with horseradish peroxidase, washed, mixed with detection buffer TMB (Alpha Diagnostic), and read at OD 450nm in a BioTek plate reader.

**Rapid iterative negative geotaxis (RING) assay** Vertical mobility was assayed using the RING method [15]. Briefly, 3 groups of 25 CSP or per^{63} flies were transferred into empty narrow vials, which were loaded into the RING apparatus. After 3 minutes rest, the apparatus was rapped sharply on the table three times in rapid succession to initiate a negative geotaxis response. The flies’ movements in tubes were videotaped and digital images captured 4 s after initiating the behavior. Five consecutive trials were interspersed with a 30s rest. The climbing performance was calculated and expressed as average height climbed in the 4 s interval. The performance of flies in a single vial was calculated as the average of 5 consecutive trials to generate n = 1.

**Neuronal degeneration** Paraffin-embedded sections of heads were used to examine neurodegenerative defects. Fly heads of all genotypes were processed and sectioned in parallel, and microscopic pictures taken at the same level of the brain and the number and volume of vacuoles counted in double-blind experiments using described methods [39, 40].

**Quantitative Real-Time PCR** 25 male heads were collected for each time point in triplicate, homogenized in TriReagent (Sigma), and RNA was isolated following manufacturer protocol. Samples were purified using the RNeasy mini kit (Qiagen) with on-column DNase digestion (Qiagen). Synthesis of cDNA was achieved with Sprint RT Complete kit (Clontech) or iScript cDNA synthesis kit (Biorad). Real-time PCR was performed on Step-One Plus real-time machine (Applied Biosystems) in triplicate under default thermal cycling conditions with a dissociation curve step. Each reaction contained iTaq SYBR Green Supermix with ROX (Biorad), 0.6-1ng cDNA, 80nM primers (IDT Technologies). Primers sequences are available upon request. Data were analyzed using the standard 2ΔΔCT method normalized to the gene rp49 and expressed relative to control samples at ZT0.

**Statistical analyses** Life span and survival curves were plotted following Kaplan Meier survival analysis and
statistical significance of curves assessed using the Log-Rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon test (GraphPad Prism v 5.0). Age-specific mortality was calculated using the Gompertz’s model of population aging. Ln values of instantaneous mortality ($\mu_x$) were plotted against chronological time. Mortality calculations and Gompertz-Makeham maximum likelihood estimates were done using WinModest V1.0.2 [41] and plotted on GraphPad Prism. For statistical analysis of biochemical results three-way ANOVA with post-hoc tests were performed using OpenStat (William G. Miller © 2009). Statistical analysis of locomotor assays was done with one and two-way ANOVA for comparison between ages and genotypes.

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**CONFLICT OF INTERESTS STATEMENT**

The authors have no conflict of interests to declare.

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SUPPLEMENTARY INFORMATION

Table S1. Mortality parameters derived from fitted Gompertz-Makeham model and maximum likelihood estimates (MLE)

| Treatment | Gompertz-Makeham parameters | Actual lifespan | Fitted lifespan | % Error in lifespan |
|-----------|-------------------------------|-----------------|-----------------|---------------------|
|           | a (intercept) | b (slope) | c (constant) | MLE value | MLE value | MLE value | MLE value | MLE value | MLE value | MLE value | MLE value | MLE value | MLE value | MLE value |
| Normoxia  | CS<sup>p</sup> | 1.0 (10<sup>-4</sup>) | 0.1096 | 1.0 (10<sup>-3</sup>) | 61.5295 | 61.3032 | 0.2 |
| CS<sup>per</sup> | 2.0 (10<sup>-3</sup>) | 0.1225 | 1.0 (10<sup>-3</sup>) | 59.0313 | 59.1527 | 0.4 |
| Hyperoxia | CS<sup>p</sup> | 5.2 (10<sup>-4</sup>) | 0.2061 | 2.1 (10<sup>-3</sup>) | 60.4421 | 60.8754 | 0.05 |
| Day 5     | CS<sup>per</sup> | 5.5 (10<sup>-5</sup>) | 0.2387 | 5.0 (10<sup>-4</sup>) | 56.9486 | 56.479 | 0.29 |
| Hyperoxia | CS<sup>p</sup> | 1.0 (10<sup>-4</sup>) | 0.1366 | 2.1 (10<sup>-3</sup>) | 58.3614 | 58.2499 | 0.19 |
| Day 20    | CS<sup>per</sup> | 1.0 (10<sup>-4</sup>) | 0.1480 | 2.1 (10<sup>-3</sup>) | 51.3507 | 58.2382 | 0.22 |
| Hyperoxia | CS<sup>p</sup> | 2.8 (10<sup>-5</sup>) | 0.1770 | 2.1 (10<sup>-3</sup>) | 59.5641 | 59.3094 | 0.43 |
| Day 35    | CS<sup>per</sup>/<sup>per</sup> | 6.4 (10<sup>-5</sup>) | 0.1897 | 2.4 (10<sup>-3</sup>) | 47.8511 | 47.6659 | 0.39 |
|           | CS<sup>p</sup> | 2.6 (10<sup>-5</sup>) | 0.1710 | 2.1 (10<sup>-3</sup>) | 57.7429 | 57.3871 | 0.61 |

Mortality at age x (μx) is given as μx = αe<sup>βx</sup> + c, where α is the baseline mortality rate (intercept), b is the age-dependent increase in mortality (slope), and c is the age-independent mortality.
Table S2. Protein carbonyl content (nmol.mg⁻¹ protein) in male heads and bodies

| Age (Days)/Tissue | Normoxia | Hyperoxia |
|-------------------|----------|-----------|
|                   | CS        | per<sup>a</sup> | per<sup>b</sup>{per<sup>c</sup>} | CS        | per<sup>a</sup> | per<sup>b</sup>{per<sup>c</sup>} |
| Heads             |           |           |           |           |           |           |
| 5                 | 5.8 ± 0.5<sup>a</sup> | 7.6 ± 0.9<sup>a</sup> | 14.5 ± 3.7<sup>a</sup> | 19.2 ± 2.9<sup>a</sup> |
| 20                | 15.6 ± 1.0<sup>b</sup> | 17.4 ± 3.3<sup>b</sup> | 36.6 ± 1.4<sup>b</sup> | 47.9 ± 2.1<sup>b<sup>**</sup></sup> |
| 35                | 41.0 ± 2.4<sup>c</sup> | 52.6 ± 0.5<sup>**</sup> | 38.6 ± 3.3<sup>c</sup> | 55.1 ± 3.5<sup>c</sup> | 72.1 ± 3.1<sup>**</sup> | 52.7 ± 3.2<sup>c</sup> |
| 50                | 45.2 ± 3.5<sup>c</sup> | 57.3 ± 4.1<sup>c</sup> | 41.3 ± 2.0<sup>c</sup> | 61.1 ± 5.3<sup>c</sup> | 87.6 ± 3.3<sup>**</sup> | 59.3 ± 3.0<sup>c</sup> |
| Bodies            |           |           |           |           |           |           |
| 5                 | 2.7 ± 0.3<sup>a</sup> | 4.3 ± 1.0<sup>a</sup> | 4.5 ± 1.2<sup>a</sup> | 5.7 ± 0.4<sup>a</sup> |
| 20                | 7.9 ± 0.3<sup>b</sup> | 9.6 ± 1.0<sup>b</sup> | 9.8 ± 1.0<sup>b</sup> | 12.6 ± 0.2<sup>b</sup> |
| 35                | 8.7 ± 1.4<sup>b</sup> | 12.3 ± 3.0<sup>b</sup> | 7.1 ± 2.0<sup>b</sup> | 19.0 ± 2.2<sup>b</sup> | 31.7 ± 4.2<sup>**</sup> | 18.3 ± 3.5<sup>b</sup> |
| 50                | 19.0 ± 3.0<sup>b</sup> | 28.4 ± 2.3<sup>b</sup> | 19.2 ± 1.5<sup>b</sup> | 29.1 ± 3.8<sup>b</sup> | 48.1 ± 5.1<sup>**</sup> | 32.1 ± 2.0<sup>b</sup> |

Values are Mean ± SEM of 3 separate bioreplicates. Three-way ANOVA with Bonferroni’s post-hoc tests was performed for each tissue. Values with different superscripts shown in columns are significantly different at p<0.01. For comparison between genotypes (rows) for each treatment, * = p<0.05 and ** = p<0.001, † = p<0.03 ‡ = p<0.01. Comparison between treatments for each genotype showed significance at p<0.001 in all ages for heads, and on day 35 and 50 for bodies.

Table S3. 4-HNE content (nmol.mg⁻¹ protein) in male heads and bodies

| Age (Days)/Tissue | Normoxia | Hyperoxia |
|-------------------|----------|-----------|
|                   | CS        | per<sup>a</sup> | per<sup>b</sup>{per<sup>c</sup>} | CS        | per<sup>a</sup> | per<sup>b</sup>{per<sup>c</sup>} |
| Heads             |           |           |           |           |           |           |
| 5                 | 0.02 ± 0.0<sup>a</sup> | 0.02 ± 0.0<sup>a</sup> | 0.04 ± 0.01<sup>a</sup> | 0.05 ± 0.01<sup>a</sup> |
| 20                | 0.2 ± 0.03<sup>b</sup> | 0.3 ± 0.02<sup>b</sup> | 0.3 ± 0.01<sup>b</sup> | 0.4 ± 0.01<sup>b</sup> |
| 35                | 0.45 ± 0.03<sup>c</sup> | 0.5 ± 0.02<sup>c</sup> | 0.41 ±0.05<sup>a</sup> | 0.51 ± 0.04<sup>c</sup> | 0.8 ± 0.02<sup>c</sup> | 0.47 ± 0.2<sup>a</sup> |
| 50                | 0.6 ± 0.0<sup>d</sup> | 0.7 ± 0.02<sup>d</sup> | 0.65 ± 0.1<sup>d</sup> | 0.7 ± 0.02<sup>d</sup> | 0.9 ± 0.1<sup>d</sup> | 0.72 ±0.3<sup>d</sup> |
| Bodies            |           |           |           |           |           |           |
| 5                 | 0.14 ± 0.0<sup>c</sup> | 0.14 ± 0.0<sup>c</sup> | 0.14 ± 0.0<sup>c</sup> | 0.15 ± 0.0<sup>c</sup> |
| 20                | 0.24 ± 0.02<sup>c</sup> | 0.3 ± 0.02<sup>c</sup> | 0.32 ± 0.04<sup>c</sup> | 0.4 ± 0.02<sup>c</sup> |
| 35                | 0.52 ± 0.04<sup>c</sup> | 0.6 ± 0.03<sup>c</sup> | 0.58 ± 0.0<sup>c</sup> | 0.54 ± 0.03<sup>c</sup> | 0.7 ± 0.02<sup>c</sup> | 0.5 ± 0.5<sup>c</sup> |
| 50                | 0.84 ± 0.02<sup>d</sup> | 0.9 ± 0.04<sup>d</sup> | 0.79 ± 0.5<sup>d</sup> | 1.02 ± 0.04<sup>d</sup> | 1.2 ± 0.01<sup>d</sup> | 0.9 ± 2.0<sup>d</sup> |

Values are Mean ± SEM of 3 separate bioreplicates. Three-way ANOVA with Bonferroni’s post-hoc tests was performed for each tissue. Values in columns with different superscripts shown in columns are significantly different at p<0.001. For comparison between genotypes (rows) for each treatment, † = p<0.03, Ψ = p<0.05, ** = p<0.001, *** = p<0.0001. Comparison between treatments for heads showed significant difference (p<0.01) at all ages for per<sup>a</sup>{per<sup>c</sup>}, and on day 35 and 50 for CS<sup>c</sup>. In case of bodies, comparison between treatments showed significance at p<0.01 on day 35 and 50 for both genotypes.
Figure S1. Age-specific mortality trajectories (−ln μ_x) in normoxia and following 24h hyperoxia at different ages (marked by vertical dotted line) in CS^0 and per^{01} males. Mortality trajectories were plotted using Gompertz-Makeham mortality parameters and smoothed using 2^nd order smoothing of 5 neighbors. (A-B) Under normoxia and 24h hyperoxia on day 5 no significant difference in mortality trajectories was observed between CS^0 and per^{01} flies. (C) 24h hyperoxia on day 20 resulted in significantly different mortality trajectories (p<0.001), with mortality slope of per^{01} flies becoming steeper near day 40. (D) Hyperoxia on day 35 resulted in significantly steeper mortality trajectory in per^{01} males compared to CS^0 (p<0.001). Mortality trajectory in flies with restored per function ([per^{01} | per^+]) was indistinguishable from CS^0.
