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Aging and circadian dysfunction increase alcohol sensitivity and exacerbate mortality in *Drosophila melanogaster*

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Abstract

Alcohol abuse is a rising problem in middle-aged and older individuals resulting in serious health, family and economic consequences. Effective treatment necessitates the identification of factors influencing alcohol toxicity with aging. We investigated the interaction between aging, alcohol toxicity and circadian function using *Drosophila* as a model system. We found as wild type flies age, sensitivity to alcohol increases and circadian regulation of alcohol-induced behaviors weakens. Decreased circadian modulation is correlated with significantly greater alcohol sensitivity during the subjective day. The circadian clock modulates alcohol-induced mortality in younger flies with increased mortality following alcohol exposure at night. Older flies exhibit significantly longer recovery times following alcohol-induced sedation and increased mortality following binge-like or chronic alcohol exposure. Flies rendered arrhythmic either genetically or environmentally exhibit significantly increased alcohol sensitivity, longer recovery times and increased mortality. We hypothesize that the circadian clock phase specifically buffers behavioral and cellular alcohol sensitivity with this protection diminishing as the circadian clock weakens with age.

Keywords

Drosophila; circadian rhythms; aging; alcohol

1. Introduction

Chronic alcohol consumption and the incidence of binge drinking represent a growing problem in older individuals (Blazer and Wu, 2009, Kendler et al., 2016), with more than 75% of alcohol-induced poisoning deaths occurring in individuals aged 35-64 (Kanny et al., 2015). Older individuals are more sensitive to alcohol, alcohol-induced motor impairments and cognitive deficits (Heuberger, 2009, Novier et al., 2015). Surprisingly, little is known about endogenous factors influencing alcohol toxicity during middle and old age. The

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circadian clock influences a vast range of biological processes including sleep-wake cycles, hormone release, body temperature, cardiac function, and gene expression (Huang et al., 2011). Early research in rodents showed a circadian rhythm in alcohol lethality (Haus and Halberg, 1959) with recent studies identifying daily rhythms in the effects of alcohol on postural control and sedation (Perreau-Lenz et al., 2009). However, the impact of the aging process on circadian regulation of alcohol sensitivity and toxicity has not been well studied.

Aging weakens the circadian system resulting in dampened molecular and neuronal rhythms causing variable and fragmented behavioral rhythms (Banks et al., 2016, Nakamura et al., 2016). Circadian misalignment associated with aging has been implicated in the elevated risk of cancer (Reszka and Przybek, 2016, Smolensky et al., 2016b, Stevens, 2009), obesity (Gibson et al., 2009, Karatsoreos et al., 2011), diabetes (Kawakami et al., 2004, Spiegel et al., 2005), cardiovascular disease (Ha and Park, 2005, Kivimäki et al., 2006) and the onset of myocardial infarction and stroke (Malik et al., 1990, Scheer et al., 2009, Smolensky et al., 2016a, Tofler et al., 1987). Furthermore, circadian dysfunction accelerates cellular aging and mortality with period alterations shortening lifespan in animal models (Krishnan et al., 2012, Park et al., 2012). The decline in the robustness of the circadian system appears to arise earlier in peripheral tissues than the central brain (Gubin et al., 2016, Libert et al., 2012) underscoring the need for examination of circadian processes during middle age. Given the circadian modulation of alcohol-induced behaviors and toxicity, the need exists for a tractable model suitable for dual examination of the circadian clock and alcohol neurobiology across multiple age groups.

The fruit fly *Drosophila melanogaster* with its well-characterized circadian clock (Allada and Chung, 2010) is an excellent model for investigating age-related changes in circadian function (He and Jasper, 2014, Robertson and Keene, 2013). With conserved signaling pathways and clear parallels to mammalian physiology, *Drosophila* also presents a suitable model for dissecting molecular and neural interactions underlying alcohol sensitivity (Grotewiel and Bettinger, 2015, Guarnieri and Heberlein, 2003, Rodan and Rothenfluh, 2010). Stereotypical alcohol behaviors are highly conserved across species (Guarnieri and Heberlein, 2003, Rodan and Rothenfluh, 2010, Wolf et al., 2002). Moreover, *Drosophila* exhibit circadian rhythms in alcohol-induced loss of motor control, sedation, and recovery following a single binge-like alcohol exposure (De Nobrega and Lyons, 2016, van der Linde and Lyons, 2011) comparable to the circadian regulation of alcohol-induced behaviors observed in mammalian models.

In the current study, we investigated the effect of aging on the circadian regulation of alcohol sensitivity and mortality. We found that as flies age alcohol sensitivity increased and circadian regulation of alcohol sensitivity weakened. Circadian phase influenced aging related changes in alcohol sensitivity with the greatest change in sensitivity occurring during the subjective day under constant conditions. Furthermore, we found that flies with a dysfunctional circadian clock were more sensitive to alcohol-induced sedation and required significantly longer to recover. Alcohol-induced toxicity in young flies was also modulated by the circadian clock as mortality following binge-like or repeat binge alcohol exposure exhibited circadian rhythmicity with the greatest mortality observed during the night. In older flies and flies with a non-functional circadian clock, mortality was significantly

increased following acute and repeat exposures to alcohol. Based on these studies, we hypothesize that the circadian regulation of alcohol neurobiology provides a protective buffer against the toxic effects of alcohol.

2. Materials and Methods

2.1. Fly maintenance

Wild type Canton-S (CS) and *per*^{01} flies in a CS background reared on cornmeal-molasses food were maintained at 25 °C and 60-70% humidity on 12h-12h light – dark (LD) cycles. To control for stress induced by culture conditions, flies were collected following eclosion and distributed with approximately 25 – 30 flies per vial and subsequently transferred every 3-4 days to new vials. For experiments in which constant light (LL) was used to induce arrhythmicity, CS flies were collected on the day of eclosion and subsequently maintained in constant light (25 °C; 60-70% humidity) as previously (De Nobrega and Lyons, 2016, Lyons and Roman, 2009). Zeitgeber time (ZT) represents time under light-dark entrainment conditions with ZT 0 denoting lights on (dawn) and ZT 12 signifying lights off (dusk). Circadian time (CT) refers to the free-running time of the animal in constant darkness (DD) and reflects the entrainment to the previous light dark cycle. Circadian experiments were performed on the 2nd day of constant darkness (DD). All experiments were done in temperature and humidity controlled dark rooms using dim red light (<1 lux).

2.2. Alcohol exposure

2.2.1. Binge-like Alcohol Exposure—Alcohol delivery occurred using an alcohol vapor system in the dark as previously described (De Nobrega and Lyons, 2016, van der Linde et al., 2014, van der Linde and Lyons, 2011). Briefly, four tubes of flies (approximately 30 flies per tube) were exposed to a mixture of alcohol vapor and humidified air at a predetermined ratio of 95% alcohol and de-ionized water (Koptec, Declon Labs, Inc. King of Prussia, PA). The percent of alcohol vapor to which flies were exposed was dependent upon the age of the flies and whether flies were subjected to a single or repeat alcohol exposure paradigm. Flow rates for airstreams were monitored during the experiment to maintain predetermined flow rates and ensure a steady concentration of ethanol vapor. We used a single exposure of 1 h alcohol vapor (or until 100% of flies were sedated for recovery experiments) on DD day 2 to assess behavioral alcohol sensitivity (the loss-of-righting reflex, sedation and recovery from alcohol-induced sedation). A repetitive alcohol exposure protocol was used to assess alcohol-induced mortality with flies exposed for 3 days to 1 h alcohol vapor at the same circadian time (exposures separated by 24 h) starting on DD day 2.

2.2.2. Chronic Alcohol Exposure—Chronic alcohol exposure occurred for a 10 d period with flies transferred to alcohol containing food (varying percentages) at either 10 d, 20 d or 30 d of age. Control food contains 2.5% alcohol. Mortality was assessed daily.

2.3. Behavioral Assays

For all experiments, separate groups of flies were tested at each time point.

2.3.1 The Loss-of-Righting Reflex—The loss-of-righting reflex assay was done as described previously (van der Linde and Lyons, 2011). Briefly, flies were habituated to the behavioral room conditions for 1 h prior to alcohol exposure. Flies for all age groups were exposed to 30% alcohol vapor, with the LORR scored every five minutes (number of flies that could not right themselves) following a gentle tap to the vial. Linear extrapolation was used to determine the time it took 50% of the flies to express LORR.

2.3.2 Sedation—Sedation was performed as previously (De Nobrega and Lyons, 2016, van der Linde et al., 2014). Flies were exposed to 40% alcohol vapor on the second day of DD, except for 3 d flies that were exposed to 50% alcohol vapor to avoid tolerance development within the time period to sedation. Every five minutes during alcohol exposure the vials were gently tapped and flies were scored as sedated if no coordinated leg movement was observed. Linear extrapolation was used to determine time to 50% sedation.

2.3.3 Recovery—Recovery was defined as the restoration of the righting reflex after a gentle tap to the vial following sedation. Upon 100% of the flies reaching sedation (assessed independently for each vial), flies were transferred to clean food vials positioned horizontally. The number of flies that recovered their righting reflex following a gentle tap to the vial was scored every 5 minutes for an hour. Linear extrapolation was used to determine the time at which 50% of the flies recovered (RT50).

2.3.4 Mortality—Following alcohol exposure, flies were transferred to fresh vials positioned horizontally to prevent the flies sticking to the food while sedated, and allowed to recover prior to vials being uprighted. Mortality was determined 24 h and 48 h following a single alcohol exposure. For the repeat alcohol exposure paradigm, mortality was assessed daily for 7 d following the last alcohol exposure.

2.4. Statistics

Statistics were performed using GraphPad Prism Version 6.0. Differences between circadian time points were assessed using analysis of variance (ANOVA), with 50% LORR, 50% ST, 50% RT or % Mortality as the dependent variable and CT as categorical independent variable. Post hoc analyses were performed using Bonferroni correction for multiple comparisons. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Alcohol sensitivity increases with age

A weakening of the circadian system occurs as a consequence of aging (Banks et al., 2016, Duffy et al., 2015, Pandi-Perumal et al., 2005) and is implicated in the aggravation of age-related diseases (Ha and Park, 2005, Karatsoreos et al., 2011, Kivimäki et al., 2006, Reszka and Przybek, 2016). As the question of whether aging affects circadian regulation of alcohol sensitivity remains unanswered, we investigated whether circadian regulation of alcohol-induced behaviors changed as a function of age. For comparison, we replicated previous circadian alcohol studies with mixed sex populations of young (3 d old) wild-type CS flies finding a robust rhythm in LORR following exposure to 30% alcohol vapor with the greatest

sensitivity observed during the subjective night in constant dark conditions (Fig. 1A and B). We then measured the LORR with alcohol exposure in 10 d, 20 d and 30 d old flies CS flies (Figure 1). Initially, we analyzed the predicted peak CT 9 and trough CT 21 time points for each age group finding significant differences between age groups ($F_{7, 148}$= 167.3, $p <$ 0.0001 with Bonferroni post-hoc analyses indicating significant differences in the 50% LoRR between the 3 d and 10 d flies at both time points and in comparison of either 3 d or 10 d flies with either of the older age groups). 10 d CS flies exhibit greater alcohol sensitivity than 3 d old flies with a damped, albeit significant, rhythm in comparison (Fig. 1C and D). To determine whether phase specific differences occurred in alcohol responses between 3 d and 10 d flies, we analyzed the magnitude of the difference in the 50% LoRR between age groups at each time point as previously done (De Nobrega and Lyons 2016). The largest factor contributing to the decreased amplitude of the rhythm appeared to be a phase specific increase in alcohol sensitivity during the late subjective day (ANOVA: $F_{5, 102}$ = 7.92, $p < 0.001$). In older flies (20 d and 30 d), alcohol sensitivity was no longer altered as a function of time of day (Fig. 1E-H) revealing decreased circadian regulation of alcohol sensitivity with aging. Middle aged and older CS flies (20 d and 30 d flies respectively) also reach 50% LORR in significantly less time than younger flies (3 d and 10 d) signifying increased alcohol sensitivity in older flies (ANOVA of 50% LoRR time across age groups at CT 9: $F_{3, 74}$ = 287.4, $p < 0.0001$ with Bonferroni post-hoc analyses indicating significant differences in the 50% LoRR between all age groups except between 20 d and 30 d flies). Two way ANOVA analysis of all time points for alcohol-induced LoRR revealed significant age and time interactions (Interaction $F_{15, 408}$ = 19.01, $p < 0.0001$; Time of Day $F_{5, 408}$ = 64.58, $p < 0.0001$; Age $F_{4, 408}$ = 571.5, $p < 0.0001$). Thus, alcohol sensitivity increases and circadian modulation of behavioral sensitivity weakens with aging.

3.2. Circadian regulation of alcohol-induced sedation dampens with age

Multiple brain regions and signaling pathways regulate alcohol-induced behaviors in *Drosophila* (McClure and Heberlein, 2013, Rodan and Rothenfluh, 2010). To better understand how the circadian regulation of alcohol sensitivity changes with age, we examined a second alcohol behavior, alcohol-induced sedation, across age groups. Sedation is the induction of a sleep state characterized by immobility (De Nobrega and Lyons, 2016, van der Linde et al., 2014). We observed a robust rhythm in alcohol-induced sedation in young (3 d old) flies (Fig. 2A and B) and a depressed but significant circadian rhythm in 10 d old flies (Fig. 2C and D), with flies of both ages sedating faster during the subjective night. While middle-aged (20 d old) flies sedated faster in comparison to younger flies, a low amplitude rhythm persisted in this age group (Fig. 2E and F). However, by 30 d of age flies no longer exhibited any circadian variation in alcohol sedation (Fig. G and H). Although 20 d and 30 d flies sedated more quickly than younger flies at all time points tested, the difference in the time to sedation between age groups varied significantly with time of day. Two Way ANOVA analysis of 50% sedation for 10 d, 20 d and 30 d flies revealed significant interaction effects (Interaction $F_{10, 270}$ = 10.53, $p < 0.0001$; Time of Day $F_{5, 270}$ = 39.55, $p <$ 0.0001; Age $F_{15, 408}$ = 342.3, $p < 0.0001$). The greatest difference in the time to sedation between young and older flies occurred phase specifically during the late subjective day based upon analysis of the magnitude of the difference in 50% sedation at each time point (10 d vs 20 d CS: ANOVA: $F_{5,90}$ = 6.53, $p < 0.0001$; 10 vs 30 d CS: ANOVA: $F_{5,90}$ = 16.84,

$p < 0.0001$). Changes in circadian regulation of alcohol sensitivity with aging appear to result in significantly greater increases in alcohol sensitivity during the subjective day compared to the night.

3.3. A functional circadian clock appears to buffer the sensitivity to alcohol-induced sedation

The above results outline an inverse relationship between circadian regulation of alcohol-induced behaviors and increasing alcohol sensitivity with aging suggesting that a robust circadian clock may function as a protective buffer against alcohol sensitivity. To test the hypothesis that the lack of a functional circadian clock increases alcohol sensitivity, we performed similar experiments using flies with a mutation in the *period* gene (per^{01} mutants), a core circadian clock gene in central and peripheral oscillators in *Drosophila*. Sedation was assessed at 6 times on the second day of DD in 10 d old per^{01} flies. As expected, no time of day differences were observed in sedation for the per^{01} mutant flies. In comparison to age-matched CS flies, the duration of alcohol exposure necessary for sedation in per^{01} flies was significantly decreased during the subjective day (Fig. 3A and B). To confirm these results, we performed a second set of experiments using wild type flies in which an environmental manipulation (constant light) was used to render the circadian clock arrhythmic. In *Drosophila*, constant light exposure is sufficient to dampen molecular circadian oscillations and abolish circadian rhythms in locomotor activity, memory formation, and the rhythm in alcohol-induced LoRR (Ewer et al., 1992, Lyons and Roman, 2009, Power et al., 1995, Price et al., 1995, van der Linde and Lyons, 2011, Yoshii et al., 2005). Under LL conditions, the free-running circadian rhythm in alcohol-induced sedation was eliminated in 10 d flies (Fig. 3C and D). Flies housed in LL also demonstrated increased sensitivity to alcohol. Analysis of the magnitude of difference in sedation between rhythmic 10 d CS flies (data presented in Figure 2C and D) and 10 d CS flies housed in LL found that alcohol sensitivity was significantly increased during the subjective day in the flies housed in LL (ANOVA: $F_{5, 66} = 16.94$, $p < 0.0001$). Thus, an intact and functional circadian clock appears to serve a vital role in attenuating the sensitivity to alcohol-induced sedation during the subjective day.

3.4. Circadian rhythms in recovery from alcohol are absent in older flies with longer recovery times needed

Given the differences in alcohol sensitivity we observed with aging, we also investigated whether recovery following alcohol exposure was affected with aging. Recovery from the sedating effects of alcohol is actively regulated by the circadian clock (De Nobrega and Lyons, 2016). We found a robust rhythm in the recovery from alcohol sedation measuring the recovery of postural control in 3 d and 10 d flies. Recovery occurred faster following alcohol exposure at CT 9 compared to CT 21 despite longer alcohol exposures necessary to achieve sedation at CT 9. No apparent differences were observed in either the amplitude of the rhythm or the times necessary for recovery between 3 d and 10 d flies (Fig. 4 A-D). In older flies (20 d and 30 d), the rhythm in alcohol recovery was absent and longer times were required for recovery at all time points (Fig. 4 E-H). Two Way ANOVA analysis of the time necessary for 50% of the flies to recover the LoRR for 10 d, 20 d and 30 d flies revealed significant interaction effects (Interaction $F_{10, 270} = 69.49$, $p < 0.0001$; Time of Day $F_{5, 270} =$

104.1, $p < 0.0001$; Age $F_{15, 408} = 151.6$, $p < 0.0001$). These results suggest that the ability to recover from alcohol decreases as a function of aging such that older animals take longer to recover from the effects of alcohol even with reduced alcohol exposures.

3.5. A non-functional circadian clock significantly increases the time needed to recover from the sedating effects of alcohol

To determine whether a functional circadian clock contributed to the ability to recover from alcohol-induced sedation, we analyzed recovery in 10 d old per^{01} mutants and flies housed in LL conditions. As expected, recovery from alcohol-induced sedation in per^{01} flies did not show any time-of-day differences (Fig. 5A and B). per^{01} flies needed considerably longer times to recover from sedation compared to age-matched wild-type flies despite significantly shorter alcohol exposure times. The mean time to recovery across all time points for 10 d per^{01} flies was even longer than 30 d old CS flies (10 d per^{01} 47.14 ± 0.49 min; 30 d CS 42.06 ± 0.31 min, $t_{(71)} = 7.95$, $p < 0.0001$). Similarly, flies in LL conditions failed to exhibit any circadian variation in recovery (Fig. 5C and D) and had significantly longer recovery times than age-matched flies with a functional circadian clock shown in Figure 4C. Thus, the lack of a functional circadian clock diminishes the ability of flies to recover from alcohol-induced impairments.

3.6. The circadian clock regulates alcohol-induced mortality

Concomitant with the circadian regulation of alcohol-induced behaviors, the circadian clock may regulate alcohol toxicity. Early research in mice found that the toxicity of injected ethanol varies with time of day (Haus and Halberg, 1959). However, little is known about circadian regulation of alcohol toxicity with aging. To test whether the circadian clock regulated alcohol toxicity, 10 d CS flies were exposed to 40% alcohol vapor for 1 h, an acute binge-like model of alcohol exposure (Fig 6A), during either the late subjective day (CT 9) or the late subjective night (CT 21). Mortality was assessed 24 hours later. We found that the circadian clock strongly modulated alcohol-induced mortality with approximately 12.83% lethality in the flies exposed to alcohol vapor at CT 21 compared to approximately 6.37% lethality in those exposed to alcohol vapor at CT 9 (Fig. 6B). Significant circadian differences in alcohol-induced toxicity were also observed when mortality was assessed 48 h following alcohol exposure (~14.25% at CT 9; ~20.5% at CT 21, data not shown). These results suggest that circadian modulation of alcohol toxicity is also conserved across phyla.

3.7. Alcohol-induced mortality increases with aging or the lack of a functional circadian oscillator

As aging increases behavioral sensitivity to alcohol, we investigated whether alcohol toxicity was also affected by aging and the interaction with the circadian clock. We assessed mortality in 20 and 30 d flies following a 1 h exposure to 40% alcohol vapor. Aging significantly increased mortality following a single alcohol exposure with mortality greater than 25% in 20 d flies and 35% in 30 d flies 24 h following alcohol exposure (Fig. 6B). Additional increases in lethality were observed 48 h after exposure (20d CS: 37% mortality at CT 9 and 40.5% mortality at CT 21; 30 d CS: 36% mortality at CT 9and 39.5% mortality at CT 21; data not shown). Similar to the lack of circadian modulation of behavioral sensitivity to alcohol in older flies, there was no apparent circadian regulation of alcohol-

induced mortality in 20 d and 30 d old flies (Fig. 6B). Alcohol toxicity significantly increased between 10 and 20 day old flies corresponding to the diminution of circadian regulation of alcohol-induced behavioral sensitivity. Based on these results, we hypothesized that the circadian clock buffers alcohol-induced toxicity as well as alcohol-induced behavioral impairments. To test this hypothesis, we assessed mortality following alcohol exposure in flies with a non-functional circadian clock (per^{01} flies and flies housed in LL). We observed significantly increased mortality in both 10 d per^{01} flies and 10 d flies housed in LL compared to age matched CS flies with an intact clock (Fig. 6C).

3.8. Alcohol-induced mortality following repeated binge-like alcohol exposure is regulated by the circadian clock and increases with circadian dysfunction

Little research has been done investigating the effects of repeated alcohol exposure during aging despite the increased frequency of binge alcohol drinking reported in older individuals (Kanny et al., 2015). We investigated whether the circadian clock modulated alcohol-induced toxicity following repeat alcohol exposures using a repeat binge-like alcohol exposure model in which flies were exposed to alcohol vapor (30% alcohol vapor) for 1 h at either CT 9 or CT 21 for three consecutive days (Fig. 6D). We found that the circadian clock strongly modulated alcohol-induced mortality in 10 d flies following repeated alcohol exposures (Fig. 6E). As with a single alcohol exposure, older flies and flies lacking a functional circadian clock had significantly greater mortality following three alcohol exposures than flies with robust circadian rhythms (Fig. 6E and 6F). These results suggest that even with heavy alcohol exposure, the circadian clock modulates alcohol toxicity and the absence of robust circadian function contributes to alcohol-induced mortality. The greatest change in mortality in older flies and flies with non-functional circadian clocks was observed during the late subjective day at CT 9. These results suggest that while aging is a risk factor for increased alcohol sensitivity and mortality, an intact clock may be necessary for protection against alcohol toxicity during the subjective day.

3.9. Mortality with chronic alcohol exposure increases with circadian dysfunction or aging

Although ~ 2.5% of alcohol-related deaths are due to acute alcohol poisoning (Kanny et al., 2015), deaths attributed to long-term alcohol exposure account for 43.8% of all alcohol-related deaths, with more than 85% of these occurring in middle-aged and older adults (Centers for Disease Control, 2013). To determine if alcohol toxicity increased proportionally as a function of aging using a 10 day exposure paradigm, flies were transferred at different ages to alcohol containing food. As expected, 20 d and 30 d flies demonstrated significantly greater alcohol-induced mortality compared to 10 d flies (Fig. 7A). However, increased mortality did not linearly increase with age as there were no significant differences between 20 d and 30 d flies at any time during the exposure period (Fig. 7B). These studies suggest that increasing alcohol toxicity is not a linear function of aging but rather a function of changes occurring in middle age at a time period when decreased circadian regulation of alcohol sensitivity is also observed. To test whether circadian dysfunction affected mortality occurring with chronic alcohol exposure, 10 d per^{01} flies were placed on varying percentages of alcohol containing media. per^{01} flies exhibited significantly increased mortality than age-matched controls with a functional circadian clock (Fig. 7C). Taken together, the results suggest that the mortality observed in response to

alcohol exposure may occur as a function of circadian disruption in addition to increased aging.

4. Discussion

Alcohol consumption and its physiological consequences during middle age and in older adults represent a rapidly growing health and economic problem for individuals and society (Substance Abuse and Mental Health Services Administration, 2014). Individuals aged 65 and older constitute approximately 13% of the US population in 2014, but this number is expected to double by 2050 (Wan et al., 2015). Within this age group, individuals that abuse alcohol report binge drinking more frequently than individuals in other age groups, approximately 5-6 times per month (Kanny et al., 2015). While much attention has been focused upon binge-drinking and alcohol abuse in adolescents and young adults, more than 75% of alcohol-induced poisoning deaths occur in middle-aged adults (Kanny et al., 2015) and more than 85% of alcohol-induced liver disease deaths occur in individuals 35 years and older (Centers for Disease Control, 2013). However, compared to other age groups relatively little research has been done to determine the endogenous factors or mechanisms affecting alcohol toxicity during middle and old age presumably due to the difficulties and costs associated with aging studies.

Aging is accompanied by the breakdown of circadian rhythmicity at the cellular, metabolic and physiological levels (Arellanes-Licea et al., 2014, Gibson et al., 2009, Rakshit et al., 2012, Tevy et al., 2013). The comparatively short lifespan of *Drosophila* with age-related changes in metabolism and physiology makes *Drosophila* an excellent model for aging and circadian studies (He and Jasper, 2014, Sun et al., 2013). Aging related issues in *Drosophila* surface by 20-30 days of age, analogous to the middle age in humans (Dambroise et al., 2016, Horiuchi et al., 2003, Vaccaro et al., 2017). Thirty day old flies display significantly reduced cardiac dysfunction (Paternostro et al., 2001), locomotor decline, changes in muscle composition (Beramendi et al., 2007, Gargano et al., 2005), decreased immune system responses (Eleftherianos and Castillo, 2012), as well as changes in sleep patterns and sleep quality (Pace-Schott and Spencer, 2011, Seugnet et al., 2008). We found that as flies age, circadian regulation of alcohol sensitivity observed at the behavioral level is weakened. Although 10 d flies are reproductively active, high in cardiac muscle strength and performance and display little signs of locomotor or cognitive impairments (Gargano et al., 2005, Miller et al., 2008, Miller et al., 2014, Paternostro et al., 2001), we found that dampening circadian rhythmicity of alcohol sensitivity may be observed in these flies. The loss of circadian rhythmicity in LORR and sedation was exacerbated with increased age. Aging also increased behavioral sensitivity to alcohol, although smaller magnitude changes in alcohol sensitivity were observed between 20 and 30 d flies than between 10 and 20 d flies.

The circadian clock influences both the sensitivity to alcohol and the recovery from its sedative effects. Previously, we found that flies were more resistant to the sedative effects of alcohol and recovered more quickly during the late subjective day (De Nobrega and Lyons, 2016). We found that as flies age the time necessary to recover from sedation significantly increased particularly following alcohol exposure during the subjective day. These results

are consistent with previous research suggesting that the sensitivity to other drugs of abuse increase with aging (Cherry and Morton, 1989, Dowling et al., 2008). Older individuals metabolize benzodiazepines slower with greater CNS effects compared to younger people (Cherry and Morton, 1989). Aged animals exhibit significantly increased cortical and blood nicotine levels following acute nicotine exposure compared to young animals (Okamoto et al., 1994). Older animals have lower brain reward thresholds, are more sensitive to morphine-induced oral stereotypy and require increased morphine for anti-nociception effects than younger animals (Jha et al., 2004, Knapp et al., 2004).

Behavioral alcohol sensitivity appears to be a predictor of alcohol toxicity. We observed a rhythm in alcohol-induced mortality in young flies with increased mortality during the subjective night following single or repeat binge alcohol exposures. However, this rhythm in alcohol mortality was lost with aging and aged flies are more vulnerable to alcohol-induced mortality. Aged flies also demonstrated increased mortality compared to younger flies following long-term alcohol exposures. The susceptibility to alcohol toxicity does not appear to be a linear function of aging as the largest increase in alcohol-induced mortality occurred between 10 d and 20 d flies. The increases in alcohol-induced sensitivity and toxicity occur during the same time frame in which circadian regulation of alcohol-induced behaviors weakens raising the possibility that age-associated loss of temporally coordinated processes could aggravate the sensitivity to alcohol.

In flies, as in mammals, behavioral and molecular rhythms weaken with age (Duffy et al., 2015, Robertson and Keene, 2013, Vaccaro et al., 2017, Yu and Weaver, 2011). Older flies have significantly reduced amplitudes *of per, tim, pdp1ε and vri* mRNA oscillations compared to younger flies (Rakshit et al., 2012). Studies have shown age-related declines in rest/activity rhythms with older flies exhibiting a lengthening of free running locomotor activity rhythms with a higher percent of flies becoming arrhythmic by 35 days old (Rakshit et al., 2012). However, whether these disrupted rhythms occur in the central or peripheral oscillators or both remain unresolved as contrasting results have been reported (Luo et al., 2012, Rakshit et al., 2012).

In our studies, genetic (per^{01} mutation) or environmental (LL) disruption of the circadian clock preferentially increased the sensitivity to alcohol during the subjective day. The absence of a functional circadian clock also significantly increased the time necessary to recover from the sedative effects of alcohol. Thus, the circadian clock appears to function as an important mediator of alcohol-induced behaviors. The circadian clock also appears to be a critical factor in modulating the toxic effects of alcohol. Young flies with a disrupted circadian clock (10 d per^{01} or flies housed in LL) exhibited mortality rates more similar to older flies following binge-like alcohol exposures or long-term alcohol exposure suggesting that alcohol-induced mortality may not be strictly a consequence of aging-related processes alone but may be tied to the presence of a functional clock. This hypothesis is consistent with other research on the circadian clock and aging. Expression of the *per* gene is robustly rhythmic in the heads of young flies but significantly declines in older flies (Krishnan et al., 2009). In *Drosophila*, per^{01} mutations exhibit accelerated aging (Krishnan et al., 2009, Krishnan et al., 2012, Rakshit et al., 2012) and in mammals, disruption in clock gene expression results in faster aging (Bonaconsa et al., 2014, Kolker et al., 2003, Musiek et al.,

2013) decreased lifespan (Davidson et al., 2006, Dubrovsky et al., 2010, Kondratov et al., 2006, Yu and Weaver, 2011). Disruption of the circadian clock through jet lag paradigms also increases mortality in aged mice (Davidson et al., 2006).

Circadian clock genes also have been shown to be affect alcohol-mediated behaviors across species (Gu et al., 2009, Kovanen et al., 2010, Spanagel et al., 2005, Udoh et al., 2015). Mutations in the clock genes *per1* and *per2* increase alcohol intake in mice following social defeat (Dong et al., 2011, Gamsby et al., 2013, Spanagel et al., 2005). Furthermore, *per1* and *per2* mutations in young adults are correlated with increased sensitivity to stress and heavy alcohol drinking (Blomeyer et al., 2013, Spanagel et al., 2005). Thus, phylogenetically conserved molecular mechanisms exist that could potentially link age-related changes in the circadian system and increased alcohol sensitivity.

Identification of the factors that mediate the effects of repetitive binge drinking on behavior and the processes through which alcohol induces tissue injury across age groups may facilitate development of novel therapies or enable improvements in currently available therapies. Our results highlight the circadian clock as a candidate mediating age-related increases in alcohol toxicity and demonstrates that disruption of circadian function exacerbates alcohol toxicity, similar to aging phenotypes. *Drosophila* has proven a valuable model for studies of aging (Giebultowicz and Long, 2015, He and Jasper, 2014, Jones and Grotewiel, 2011), the circadian clock (Allada and Chung, 2010) and alcohol neurobiology (Grotewiel and Bettinger, 2015, Guarnieri and Heberlein, 2003) and the current study demonstrates the value of *Drosophila* as a practical model for investigating the interplay among these factors. This research lays the groundwork for future studies investigating the cellular and physiological mechanisms through which the circadian clock and aging influence alcohol-induced toxicity.

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Highlights

- The circadian clock regulates alcohol sensitivity and alcohol toxicity in Drosophila
- Behavioral sensitivity to alcohol increases with age and circadian modulation weakens
- Circadian dysfunction increases alcohol sensitivity and lengthens recovery
- Alcohol-induced mortality significantly increases with aging or circadian dysfunction
- The circadian clock may phase specifically buffer alcohol sensitivity and toxicity

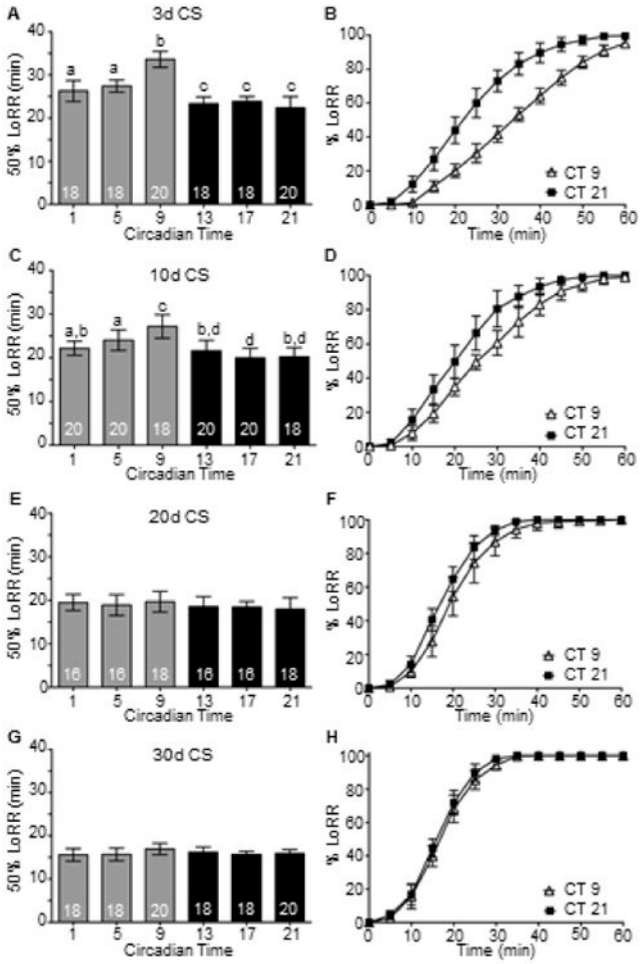

Figure 1. Alcohol sensitivity increases with age while circadian modulation decreases

(A) Loss of Righting Reflex (LoRR) using 30% alcohol vapor was measured at 6 times during the second day of DD in age-matched mixed populations of male and female CS flies. The length of alcohol exposure necessary for 50% of wild-type flies (3 d old) to lose their righting reflex is modulated by the circadian clock (ANOVA $F_{5, 106} = 91.76$, $p < 0.0001$). Mean time necessary for 50% of flies to lose their righting reflex during alcohol exposure and standard error of the mean plotted for all experiments. N shown on bars for each group is the number of vials of flies tested for each time point with 25 – 30 flies per vial. Post-hoc analyses performed using Bonferroni corrections for multiple comparisons. Different letters above columns denote statistically different responses at time points ($p < 0.05$), while shared letters between groups indicate no significant difference. (C) A significant but weakened rhythm in alcohol-induced LoRR exists in 10 d CS flies (ANOVA: $F_{5, 110} = 26.94$, $p < 0.0001$). (E) = 1.48, $p = 0.2036$) or (G) 30 d flies (ANOVA: $F_{5, 102} = 3.734$, $p = 0.38$). Complete time course of alcohol exposure showing percent of flies exhibiting for CT 9 and 21 alcohol exposure are shown for 3 d (B), 10 d (D), 20 d (F) and 30 d flies (H).

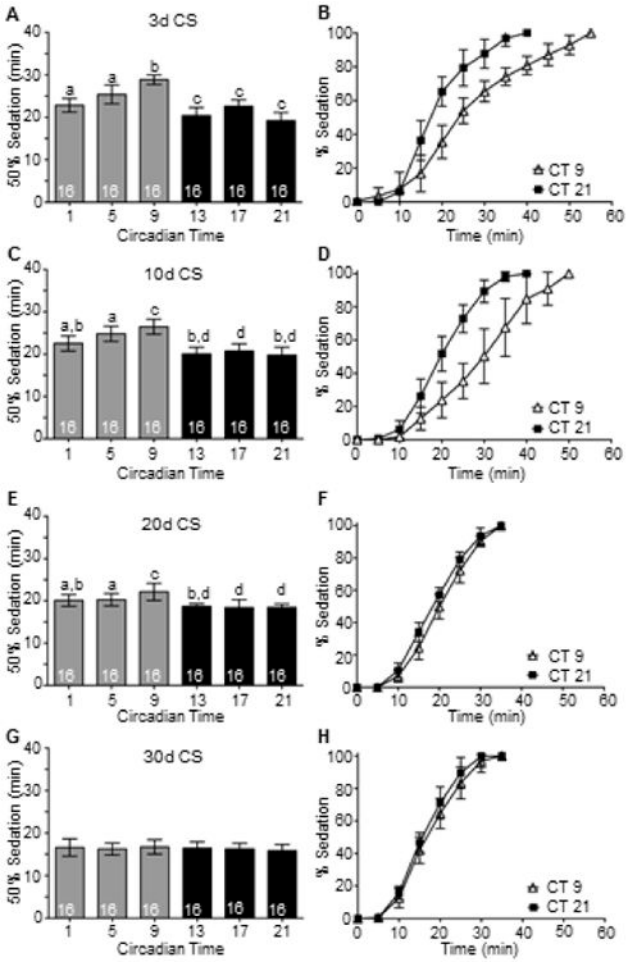

Figure 2. Circadian regulation of alcohol-induced sedation decreases with age

(A). Sedation was measured at 6 times on the second day of DD in age-matched CS flies (ANOVA $F_{5, 90} = 64.01$, $p < 0.0001$). (C) 10 d flies exhibit increased alcohol sensitivity with a lower amplitude circadian rhythm (ANOVA: $F_{5, 90} = 39.03$, $p < 0.0001$). (E) The circadian rhythm in alcohol sedation was damped in 20 d flies (ANOVA: $F_{5, 90} = 16.08$, $p < 0.0001$) (G) and absent in 30 day flies (ANOVA: $F_{5, 90} = 0.62$, $p = 0.68$). Complete sedation time courses for CT 9 and 21 are shown for 3 d (B), 10 d (D), 20 d (F) and 30 d (H) flies.

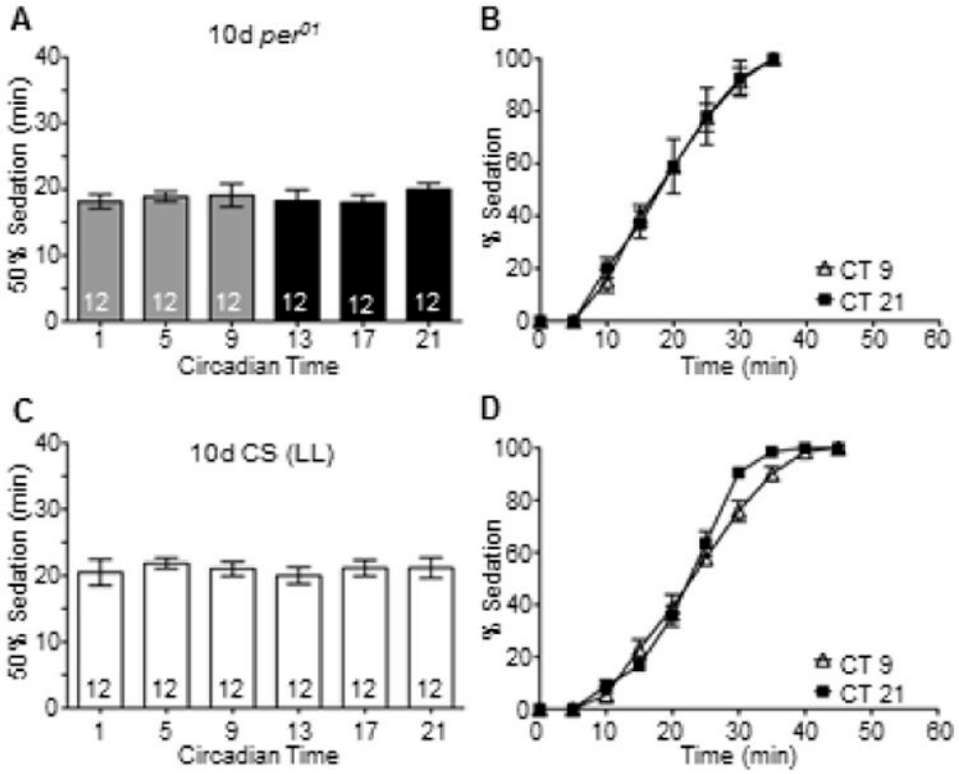

Figure 3. The absence of a circadian clock significantly increases alcohol sensitivity

(A) *per*^{01} flies exhibited no significant time-of-day differences in sedation (ANOVA $F_{5, 66}$ = 1.95, p = 0.09). (C) There was no significant time-of day difference in the time necessary for 50% of flies housed in LL conditions to become sedated (ANOVA: $F_{5, 66}$ = 2.03, p = 0.08). Complete time courses shown for alcohol-induced sedation in *per*^{01} flies (B) and CS LL flies (D) for CT 9 and CT 21.

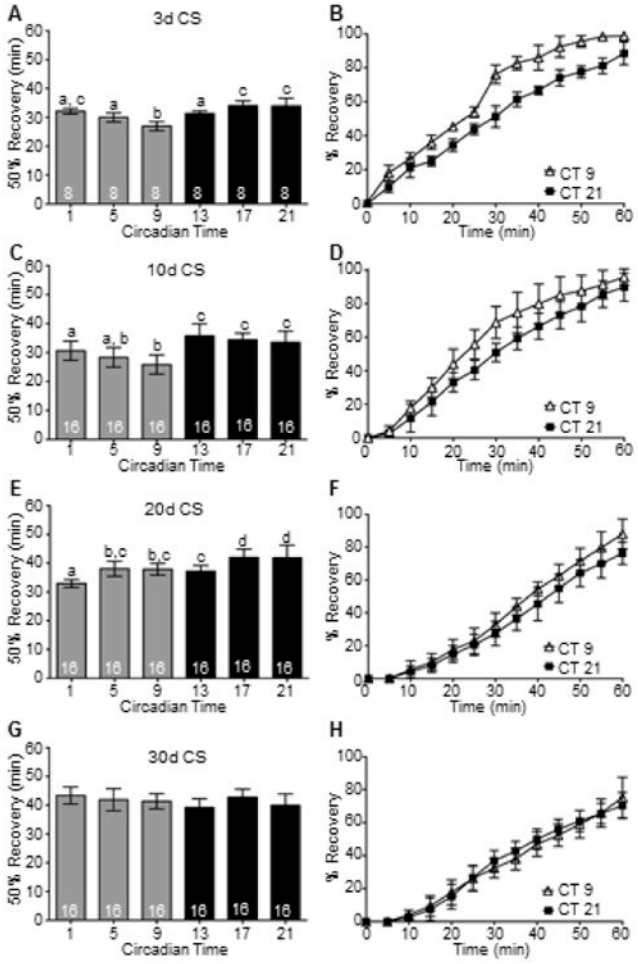

Figure 4. The time necessary for recovery from alcohol-induced sedation increases with age

(A) The time necessary for 50% of 3 d (young) wild type flies to recover the righting reflex following sedation in DD (ANOVA $F_{5, 66} = 35.05$, $p < 0.0001$). (C) 10 d flies exhibit a circadian rhythm in recovery from sedation (ANOVA: $F_{5, 90} = 20.13$, $p < 0.0001$) similar to 3 d flies. (E) The circadian rhythm in recovery from sedation is damped in 20 day old flies (ANOVA: $F_{5, 90} = 25.10$, $p < 0.0001$) with longer recovery times needed during the last half of the night. (G) No circadian rhythm in recovery was apparent in 30 d flies (ANOVA: $F_{5, 90} = 1.75$, $p = 0.13$). The complete recovery curves for 3 d (B), 10 d (D), 20 d (F) and 30 d (H) flies.

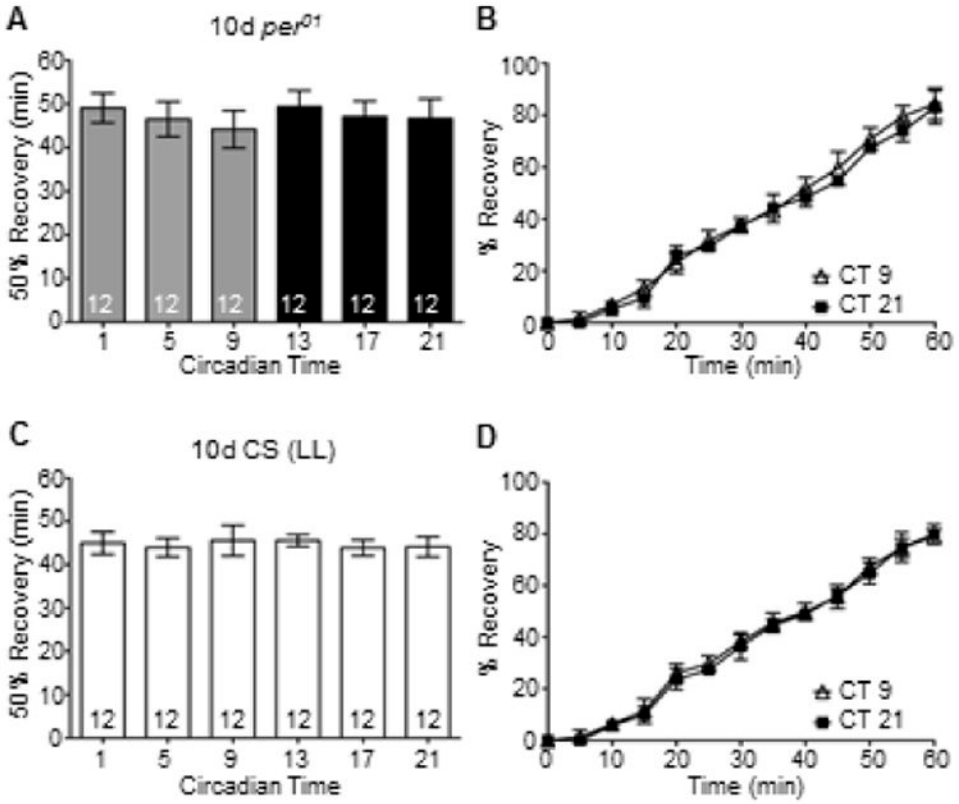

Figure 5. Time necessary for recovery increases with circadian dysfunction

(A) There was no significant time-of day difference in the time necessary for 50% of *per*^{01} flies to recover from alcohol-induced sedation as assessed by recovery of the righting reflex (ANOVA $F_{5, 66} = 1.86$, $p = 0.11$). *per*^{01} flies took significantly longer to recover at all times compared to age-matched CS flies shown in Figure 4C. (C) CS flies housed under LL conditions exhibited no circadian variation in recovery (ANOVA: $F_{5, 66} = 1.37$, $p = 0.26$) with longer recovery times needed than controls shown in Figure 4C. Complete time courses shown for recovery of the righting reflex following alcohol-induced sedation for CTs 9 and 21 in *per*^{01} flies (B) and CS flies in LL (D).

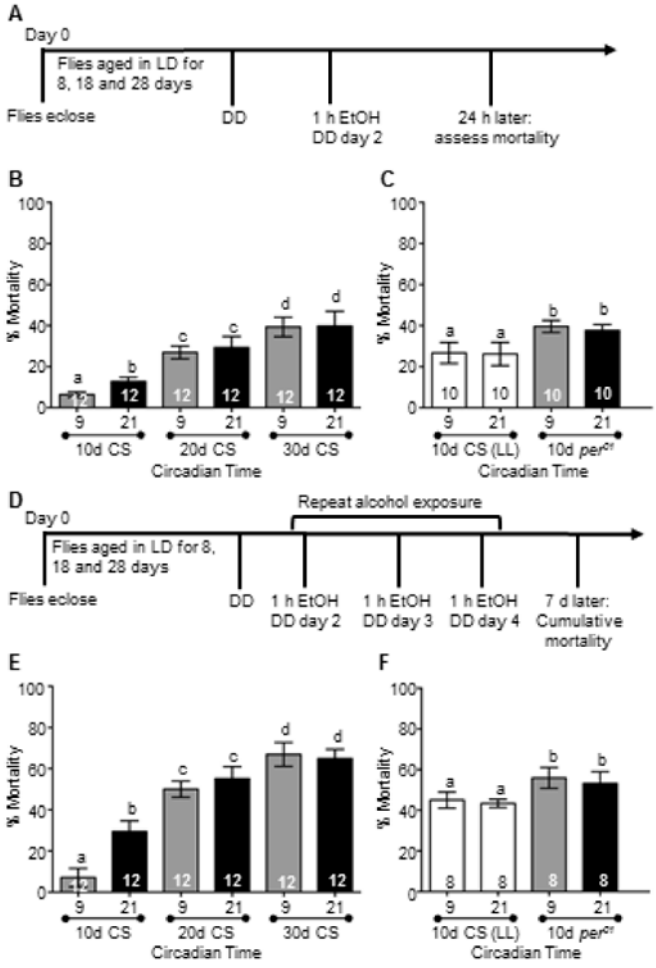

Figure 6. Alcohol-induced mortality is exacerbated by aging and circadian dysfunction

(A) Flies were exposed to a single 1 h alcohol exposure at either CT 9 or CT 21 on the 2nd day of DD. (B) The circadian clock regulates alcohol-induced mortality in young flies with significantly greater mortality occurring following alcohol exposure during the night. As flies age, increased mortality occurred following alcohol exposure with no circadian variation observed in 20 d or 30 d flies (ANOVA $F_{5, 62} = 112.6$, $p < 0.0001$). (C) per^{01} and arrhythmic flies in LL demonstrate higher levels of alcohol-induced mortality than age-matched controls with no time of day variation. (D) Mortality was assessed following a three exposure repeated binge-like alcohol paradigm with 1 h alcohol exposure (30% alcohol vapor) occurring at either CT 9 or CT 21 starting on the 2nd day of DD. (E) A significant rhythm in mortality occurs in 10 d flies following repeated binge-like alcohol exposure. Alcohol-induced mortality increased with age (ANOVA: $F_{5, 66} = 257.8$, $p < 0.0001$). (F) per^{01} flies and arrhythmic flies in LL have higher mortality levels during the subjective day and night following alcohol exposure than age-matched controls. As with a single alcohol exposure, 10 d per^{01} flies have higher mortality than CS LL flies (ANOVA: $F_{5, 28} = 15.01$, $p < 0.0001$).

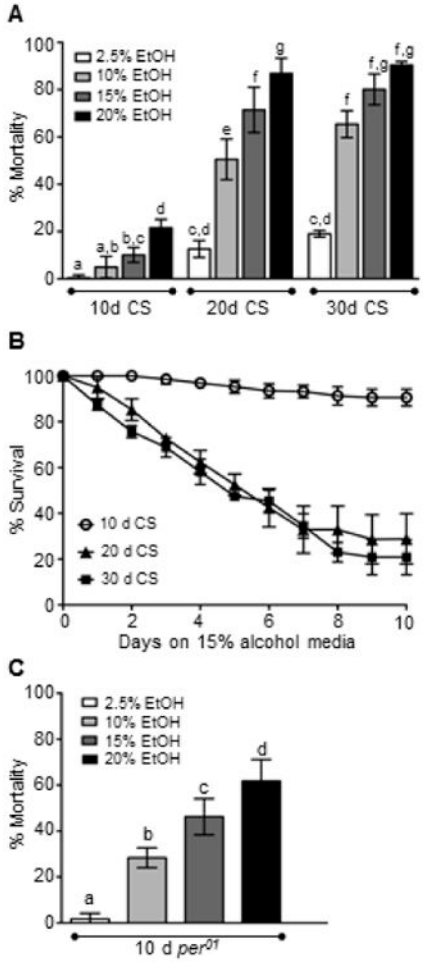

Figure 7. Mortality with long-term alcohol exposure is increased by aging or circadian dysfunction

(A) Flies were exposed to varying concentrations of alcohol in the food for 10 days starting at either 10, 20 or 30 days of age. Control media contains 2.5% alcohol in the food. Mortality with chronic alcohol exposure increased with age (ANOVA: $F_{11,64} = 252.0$, $p < 0.0001$). (B) Survival rates for flies on 15% alcohol containing food reveal no differences in the survival curves for 20 and 30 day old flies exposed to alcohol. (C) 10 d old per^{01} flies exhibit higher rates of mortality with long-term alcohol exposure in the food than age-matched CS flies shown in panel A (ANOVA: $F_{3,26} = 119.9$, $p < 0.0001$).