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Mutations in the Circadian Gene *period* Alter Behavioral and Biochemical Responses to Ethanol in *Drosophila*

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Abstract

Clock genes, such as *period*, which maintain an organism's circadian rhythm, can have profound effects on metabolic activity, including ethanol metabolism. In turn, ethanol exposure has been shown in *Drosophila* and mammals to cause disruptions of the circadian rhythm. Previous studies from our labs have shown that larval ethanol exposure disrupted the free-running period and *period* expression of *Drosophila*. In addition, a recent study has shown that arrhythmic flies show no tolerance to ethanol exposure. As such, *Drosophila period* mutants, which have either a shorter than wild-type free-running period (*perS*) or a longer one (*perL*), may also exhibit altered responses to ethanol due to their intrinsic circadian differences. In this study, we tested the initial sensitivity and tolerance of ethanol exposure on *Canton-S*, *perS*, and *perL*, and then measured their Alcohol Dehydrogenase (ADH) and body ethanol levels. We showed that *perL* flies had slower sedation rate, longer recovery from ethanol sedation, and generated higher tolerance for sedation upon repeated ethanol exposure compared to *Canton-S* wild-type flies. Furthermore, *perL* flies had lower ADH activity and had a slower ethanol clearance compared to wild-type flies. The findings of this study suggest that *period* mutations influence ethanol induced behavior and ethanol metabolism in *Drosophila* and that flies with longer circadian periods are more sensitive to ethanol exposure.

Keywords

alcoholism; circadian rhythm; *Drosophila*; ethanol; *period*

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INTRODUCTION

Alcohol is one of the most commonly abused drugs in the world. Consequently, alcohol use disorders (AUD) are extremely common. Therefore, it is important to understand the biological effects of ethanol and mechanisms behind ethanol metabolism. Ethanol intoxication exhibits a biphasic effect, inducing initial hyperactivity at lower doses followed by depressed activity resulting in sedation at higher doses. Alcoholism and alcohol consumption in humans are known to affect a multitude of molecules and pathways including biological rhythms e.g. melatonin [1], temperature rhythms [2], and cycling of circadian genes [3]. Interestingly, human genetics studies have linked mutations to the *per* genes and increased alcohol consumption [4].

Furthermore, numerous mammal studies have shown that alcohol exposure can affect the period [5-7] and phase [7-10] of the free-running locomotor activity rhythm. Additionally, alcohol can affect transcript and protein levels of core clock genes (including *period* (*per*)) [11, 12]. Alternatively, previous studies have suggested that ethanol intake and drug addictions are strongly associated with mutations in clock genes, including *per*. Mice with mutated *per* genes, including *per1* and *per2*, exhibit increased alcohol consumption and alcohol seeking behavior [13, 14]. In turn, animals selectively bred for high or low alcohol consumption have also shown aberrations in their circadian clock function [15-17]. Taken together, these studies demonstrate clear connections between circadian mutations and alcohol exposure by showing profound effects of alcohol on the molecular rhythms and behavioral and physiological outputs of the circadian clock.

The focus of this study was to explore sedation and tolerance to high-dose acute ethanol exposure in adult *Drosophila per* mutants, in order to investigate how ethanol affects animals with rhythmic, but atypical circadian periods. *Drosophila* can be a powerful tool to investigate the physiological and behavioral effects of alcohol intoxication for the following reasons. First, *Drosophila* is amenable to efficient genetic and behavioral analysis. Second, the ethanol-induced biphasic behavior in humans is mirrored in *Drosophila*; during initial stages of exposure, hyperactivity and increased locomotion was observed, but after greater amounts of ethanol had accumulated, the flies were sedated [18]. Third, there exists a high degree of homology between the *Drosophila* and mammalian clock rhythm [19]. Therefore, fruit flies have been extensively used to investigate molecular and behavioral aspects of the circadian rhythm.

Work on the effects of ethanol on *Drosophila* has demonstrated that, similar to mammals, ethanol can disrupt the biological clock and has the capability to modulate the expression of circadian genes [20, 21]. In addition, a recent study showed that *Drosophila* arrhythmic mutants (including *per* null (per^0)) do not exhibit ethanol tolerance, indicating that a circadian clock is necessary for alcohol tolerance [22]. Although such studies have found strong correlations between circadian rhythm defects and ethanol exposure, few have directly investigated the effect of acute ethanol exposure on *Drosophila* with altered free-running circadian periods - *period Short (perS)* and *period Long (perL)* mutants. The *perS* and *perL* flies have a stable entrainment to a LD cycle, but they have free-running circadian periods of approximately 19.5 h and 28.5 h, respectively [23]. As alcohol consumption may

have differing affects on the circadian rhythm depending upon free-running rhythm length [6, 20], there may be a connection between period length and alcohol related behaviors. Here we show that the *perS* and *perL* mutations have altered sedation rates, recovery from sedation, and ethanol tolerance and demonstrate that these effects may be the result of interplay between circadian rhythms and ethanol metabolism.

MATERIALS AND METHODS

2.1 *Drosophila* stocks

Drosophila fly stocks *Canton-S* (*CS*, stock number 1), and adh^{null} (stock number 3976), were obtained from the Bloomington *Drosophila* Stock Center. *perS* and *perL* mutants were generated in *CS* flies [23] (generously provided by Dr. Michael Rosbash, Brandeis University). To rule out effects of genetic background differences, similar behavioral effects were observed in stocks independently maintained at Colby College and Bridgewater State University. The flies were raised on standard Nutri-Fly™ Bloomington Formulation (Genesee Scientific) at 25°C under 12-hour light/12-hour dark conditions, unless otherwise stated.

2.2 Ethanol Sedation, recovery, and tolerance assays

To determine the effect of *period* mutations on *Drosophila* behavioral responses to ethanol, a sedation assay was carried out according to previous assays outlined by Maples and Rothenfluh, with minor modifications [24]. This study used male flies due to gender-related differences in ethanol-induced behavior and their stronger tolerance to ethanol exposure [18]. *CS*, *perS*, and *perL* male flies of different ages (one-day, one-week, and two-week-old – typical experiments with adult flies are done on 3-7-day old flies) were collected and sorted into standard food vials of 8 flies each approximately 18 hours before sedation in order to prevent confounding effects of the CO_2 sedation on ethanol-induced sedation. Flies were transferred to new empty vials at ZT4 on the day of sedation and allowed to acclimate to the new environment for 15 minutes. Sedation was carried out by dispensing 500 μL ethanol (200-proof) (Sigma-Aldrich) onto a new cotton plug, and then immediately replacing the original plug with the ethanol infused one, forming an ethanol chamber. Vials were deliberately tapped 5 times after each minute to determine the number of sedated flies. Sedation is defined as the inability of the flies to move, right themselves, or uncontrollable beating of the wings after a ten-second observational period. ST50 is defined as the time until 50% of the flies have been sedated. The flies were left in the ethanol chamber for a total of 15 minutes regardless of sedation time.

Recovery began after the flies have been removed from the ethanol chamber. The flies were returned to their original food vials with the vial laid horizontally to prevent non-sedation-related mortality. Every 30 minutes, the vials were gently rotated and observed to determine if the flies had recovered, which is indicated by the regaining of their ability to right themselves. RT50 is defined as the time required for 50% of the sedated flies to recover. Flies that have not recovered at the beginning of tolerance were excluded from the total number of flies for RT50 calculations. Tolerance after repeated exposure was tested 24 hours after the initial sedation at ZT4. The flies were once again transferred into a new

empty food vial and the sedation process was repeated to determine the ST50 for tolerance measurement. For each genotype, tolerance was calculated by determining the percent change in sedation times on consecutive days (%T - to normalize tolerance between genotypes).

Statistical analyses on independent biological replicates (n=3-5) for sedation, recovery, and tolerance were performed in STATA using ANOVA; post hoc Scheffe pair-wise comparison test.

2.3 Alcohol dehydrogenase enzyme activity assay

Alcohol dehydrogenase (ADH) enzyme activity was measured for both fluctuations throughout the circadian cycle and age-dependent differences according to a previously described protocol [25, 26]. Twenty-four-hour ADH cycling differences between *CS, perS and perL* were measured at time points ZT0, ZT4, ZT8, ZT12, ZT16, ZT20, and ZT24, with adh^{null} as the control. One to three-day old flies were kept at 25°C under 12-hour light/12-hour dark standard conditions until the appropriate time point. To determine age-dependent differences, 1-day, 1-week, and 2-week-old flies of each genotype were collected at ZT4.

Five flies were homogenized from each genotype at each time point and age in 150 μL of 20 mM phosphate buffer. 100 mM potassium phosphate buffer was made with 19.2: 90.8, monobasic potassium phosphate (100 mM): dibasic potassium phosphate (100 mM) (Sigma-Aldrich). The homogenate was centrifuged at 12500 rpm in 4°C for 5 minutes, and then the supernatant collected and stored at −20°C until all extracts had been collected. ADH assay solution, 940 μL, was prepared for each sample, composed of 500 μL potassium phosphate buffer (100 mM), 40μL NAD+ (50 mM), 100 μL absolute ethanol (200-proof), and 300 μL dH_2O. Directly before absorbance measurements were made, 60 μL of the sample extract was added to the assay solution to initiate the reaction. The absorbance over time of each sample produced by colorimetric changes was measured at room temperature and a wavelength of 340 nm for 10 minutes (Beckman Coulter DU530 UV/Vis Spectrophotometer). Measurements were made every 2 minutes for a total of 6 optical density readings. The data points were plotted and fitted with a regression line to determine the change in optical density (ΔOD).

In order to account for differences in protein concentration of each sample, we normalized samples for protein content using a bicinchoninic acid assay (BCA assay), also known as a Smith assay. The assay was carried out with a Pierce™ BCA Protein Assay Kit. For each sample, 200 μL of assay solution was prepared, consisting of 196 μL reagent A and 4 μL reagent B. 25 μL of sample extract was added to the assay solution and allowed to react in an incubator at 37°C for 30 minutes. After the incubation, 2 μL of the reacted sample was measured on a Thermo Scientific Nanodrop (ND-1000) spectrophotometer for the number milligrams of protein in the sample at room temperature (approximately 25°C) and a wavelength of 562 nm. The ADH activities were plotted as the normalized absorbance units (NAU - ΔOD/mg protein).

Statistical analyses on independent biological replicates (n=4-6) for the ADH activity at ZT4 were performed in STATA using ANOVA; post hoc Scheffe pair-wise comparison test. To

calculate the ADH activity during lights-on and lights-off phases of the 24-hour cycle (n=3), data from ZT4 and ZT8 (lights-on) were averaged, and data from ZT16 and ZT20 (lights-off) were averaged. Lights-on and lights-off data analyses were performed in STATA using T-tests.

2.4 Ethanol pharmacokinetics assay

The ethanol accumulation assay was performed to determine the amount of ethanol accumulation that occurs after ethanol exposure treatment. *CS*, *perS*, and *perL* flies were exposed to ethanol vapor (200-proof) (Sigma-Aldrich) for 15 minutes, and then allowed to recover for an hour on standard Nutri-Fly™ Bloomington Formulation at room temperature (approximately 25°C). Methods to create the ethanol chamber can be found in the "Ethanol sedation, recovery, and tolerance" section.

Colorimetric determination of ethanol concentration was carried out using manufacturer protocol described in the EnzyChrom™ Ethanol Assay Kit. Four flies from each appropriate genotype were homogenized in 40 μL of 100 mM potassium phosphate buffer and centrifuged at 12500 rpm and 4°C for 5 minutes. The supernatants were collected and stored at −20°C. The assay solution was prepared by mixing 80 μL Assay Buffer, 1 μL Enzyme A, 1 μL Enzyme B, 2.5 μL NAD, and 14 μL MTT. 90 μL of the assay solution and 10 μL of sample extract were added and mixed in a 96-well plate. The mixtures were allowed to react at room temperature (approximately 25°C) for 30 minutes undisturbed, and then 100 μL of stop solution was added and mixed into each well. The absorbance was measured at room temperature at 570 nm (BioTek ELx808 spectrophotometer). The relative ethanol concentration values were expressed as absorbance units (AU).

The measurement of the protein concentration of each sample was carried out with a Pierce™ BCA Protein Assay Kit, with slight modifications compared to the BCA assay performed for the ADH normalization, due to the *Drosophila* sample size variation between the ADH assay and the ethanol accumulation assay. For each sample, 200μL of assay solution was prepared, consisting of 196μL reagent A and 4μL reagent B. 10μL of sample extract and assay solution were then distributed into a 96-well plate and allowed to react in an incubator at 37°C for 30 minutes. After the incubation, the reacted samples were measured for the number of milligrams of protein in the sample at room temperature (approximately 25°C) and a wavelength of 562 nm (BioTek ELx808 spectrophotometer). The ethanol concentration values were plotted as the normalized absorbance units (NAU), which refers to ΔOD/mg protein.

Statistical analyses on independent biological replicates (n=3) for the ADH activity at ZT4 were performed in STATA using ANOVA; post hoc Scheffe pair-wise comparison test.

2.5 Ethanol Viability Assay

An ethanol viability assay was performed (n=4-5), where *CS*, *perS*, and *perL* flies were placed on 10% ethanol-supplemented food and the percentage of flies surviving after five days was calculated, as previously described [21].

RESULTS

3.1 Ethanol-induced behavior in *period* mutants

To determine the effect of *period* mutations on ethanol-induced behavior, we performed sedation, recovery, and tolerance assays at ZT4 on 1-day-old, 1-week-old, and 2-week-old *CS*, *perS*, and *perL* flies.

3.1.1 Sedation—The ST50 of 1-week-old *perL* flies (5.5 minutes) was significantly higher than the ST50 of 1-week-old *CS* flies (4.75 minutes; p=0.03) (Figure 1A). All other comparisons of genotypes of the same age showed similar sedation time (ST50), which was between 6.5 minutes and 6.75 minutes for 1-day-old flies and between 4.33 minutes and 4.83 minutes for 2-week-old flies (Figure 1A). Additionally, all genotypes showed a significant age-dependent decrease in ST50 in 1-week-old and 2-week-old flies compared to 1-day-old flies ($p \leq 0.046$) (Figure 1B).

3.1.2 Recovery—The recovery time (RT50) of *CS* flies and *perS* flies was not significantly different within the same age groups. The RT50 of *CS* flies and *perS* flies was 45 minutes and 37.5 minutes in 1-day-old, 63.75 minutes and 52.5 minutes in 1-week-old, and 80 minutes and 75 minutes in 2-week-old flies, respectively (Figure 2A). In contrast, *perL* flies showed a significantly longer RT50 compared to both *CS* flies and *perS* flies of the same age (p=0.0001 for all pairwise comparisons). The RT50 of *perL* flies was 105 minutes, 138.75 minutes, and 170 minutes in 1-day-old, 1-week-old, and 2-week-old flies, respectively. All genotypes showed a trend of age-dependent increase in RT50 (Figure 2B). The RT50 of *CS* flies and *perS* flies showed statistically significant increase between 1-day-old and 2-week-old flies (p=0.03 and p=0.005), while *perL* flies showed significant increase between 1-day-old and 1-week-old flies (p=0.015), 1-day-old and 2-week-old flies (p=0.0001), and 1-week-old and 2-week-old flies (p=0.032).

3.1.3 Tolerance—The tolerance to sedation after repeated exposure to ethanol (%T) of *CS* flies and *perS* flies was not significantly different within the same age groups. The %T of *CS* flies and *perS* flies was 25.8% and 23.5% in 1-day-old, 34.5% and 46.6% in 1-week-old, and 58.8% and 40.7% in 2-week-old flies, respectively (Figure 3A). Among the genotypes, *perL* flies generated the highest tolerance. The %T of 1-day-old *perL* flies (41.0%) was significantly higher than %T in 1-day-old *perS* flies (p=0.038). Furthermore, the %T in 1-week-old (69.8%) and 2-week-old (77.4%) *perL* flies was significantly higher than %T in 1-week-old *CS* flies and *perS* flies (p=0.001 (1wk) and 0.02 (2wk) and p=0.014 (1wk) and . 009 (2wk) respectively). All genotypes showed a trend of age-dependent increase in %T (Figure 3B). The %T in *CS* flies and *perS* flies showed statistically significant increase between 1-day-old and 2-week-old flies (p=0.02 and p=0.03), while *perL* flies showed significant increase between 1-week-old and 2-week-old flies (p=0.05).

3.2 Circadian rhythmicity of ADH activity

To determine if ADH activity follows a circadian pattern and the effect of *period* mutations on ethanol metabolism, ADH assays were performed on *CS*, *perS*, and *perL* flies every four hours at ZT0, ZT4, ZT8, ZT12, ZT16, and ZT20. All genotypes appeared to show an

oscillating trend in ADH activity under 12-hour light/12-hour dark standard conditions (Figure 4). ADH activity appeared to decrease after ZT0 and increase after ZT12. ADH activity during lights-on (ZT4 and ZT8) and lights-off (ZT16 and ZT20) time points was averaged (Figure 5). For all genotypes, ADH activity increased in light condition compared to dark condition, although the results were not statistically significant (Figure 5). Among genotypes, *perS* flies showed the highest ADH activity throughout the 24-hour cycle, whereas *perL* flies were the lowest ADH activity at all time points (Figure 4).

3.3 Alcohol dehydrogenase (ADH) activity in *period* mutants

To further analyze the effect of *period* mutations on ethanol metabolism, ADH activity was measured at the same time points as ethanol-induced behavior assays i.e. at ZT4 in 1-day-old, 1-week-old, and 2-week-old *CS*, *perS*, and *perL* flies. Adh^{null} flies were used as control. ADH activity was not significantly different between genotypes at all ages except between 1-week-old *perS* flies and *perL* flies (p=0.01) (Figure 6A). Nevertheless, certain genotype-related trends in ADH activity were observed. *perL* flies showed lower ADH activity compared to *CS* flies and *perS* flies in all time points (Figure 6A). Age-dependent trends in ADH activity were also observed. *CS* flies appear to show an increase in ADH activity with age, while *perS* flies and *perL* flies appear to show a decline in ADH activity with age (Figure 6B).

3.4 Ethanol pharmacokinetics in *period* mutants

To determine if higher RT50 and low ADH activity in *perL* flies correlates with ethanol clearance, ethanol content was measured in 1-week-old *CS*, *perS*, and *perL* flies 1 hour after exposure to ethanol for 15 minutes (ethanol exposure at ZT4 followed by ethanol content assay at ZT5). *perL* flies showed significantly higher ethanol content compared to *CS* flies (*CS*: 0.92 NAU and *perL* 1.43 NAU; p-value=0.04) (Figure 7).

3.5 Ethanol-induced mortality in *period* mutants

After five days on 10% ethanol-supplemented food, average mortality rates for each genotype were 29.3 ± 3.9, 10.0 ± 5.0, and 50.6 ± 8.8 percent for *CS*, *perS*, and *perL* flies respectively. After five days on control food, no mortality was observed in any genotypes. *perL* flies showed significantly increased mortality on the ethanol food compared to *perS* flies (p=0.003) and marginally more compared to *CS* flies (p=.080). No differences were present between *CS* flies and *perS* flies in terms of viability rates (p=0.14).

DISCUSSION

In this study, we demonstrated a strong link between mutations in the circadian rhythm gene, *period*, and altered behavioral and biochemical responses to acute ethanol exposure. All genotypes showed similar sedation times at each age tested except for 1-week-old *perL* flies, which exhibited a longer sedation time than either wild-type or *perS* flies. These results with rhythmic *per* mutants mirrors what was recently found in arrhythmic circadian mutants (including *per0*), which also showed no differences in sedation time to alcohol after one exposure [22]. Still, the role of the circadian clock in ethanol sedation cannot be ruled out entirely because there seems to be circadian variation in ethanol sensitivity in both wild-

type flies [27] and mice [28]. The current and previous study [22] conducted these assays during the subjective day (ZT 4-9), so it is currently unknown whether circadian mutants show the same day vs. night variation in sensitivity. It is worth noting that *per2*^{Bdrm1} mutant mice (which may be arrhythmic) lack circadian variation in ethanol sensitivity, while *per1*^{Bdrm1} mice (which are rhythmic) do not [28], indicating a functional circadian clock is necessary for temporal variation in ethanol sensitivity. It may be likely that arrhythmic fly mutants, as well as wild-type flies made arrhythmic through constant light exposure, also might lack the circadian variation of sensitivity [27]. Additionally, all genotypes show a similar pattern to age effects of ethanol exposure as both one-week and two-week old flies exhibited increased sensitivity to ethanol exposure compared to one-day old flies, which has been previously well characterized (initially by Pearl et al., 1929 [29]). The lack of significant differences in sedation times may also be because sedation during acute exposure is governed by the amount of ethanol vapors absorbed [30]. *Adh*^{null} flies, which are unable to process ethanol, still maintained comparable (slightly less) sedation times to wild-type, suggesting that sedation does not depend on ADH enzymatic activity (data not shown and [31]).

While all flies showed similar patterns of sedation to ethanol exposure, *perL* flies did show longer recovery times and increased mortality compared to *CS* flies and *perS* flies, indicating that *perL* flies may be more sensitive to ethanol because of slower ethanol metabolism. *perL* flies exhibited increased levels of alcohol after exposure compared to *CS* flies and *perS* flies, indicating slower ethanol metabolism. Additionally, correlations have been found between internal ethanol levels and sedation and recovery time [32]. Accordingly, *Adh*^{null} flies have significantly longer recovery times after sedation with many not recovering resulting in high ethanol exposure-induced mortality (data not shown), indicating a role in ethanol exposure recovery but not sedation by ADH. Therefore, lower ADH enzymatic activity accompanied by higher amounts of residual ethanol during recovery would be associated with longer recovery times. While *perL* flies' slow recovery time and differences between the alcohol levels of *perS* flies and *perL* flies can be explained by differences in ADH levels, it does not explain why *CS* flies had significantly faster recovery compared to *perL* flies. This result suggests that *perL* flies might be deficient in other enzymes that regulate ethanol metabolism and tolerance, and further studies will be needed to determine what else will be implicated in alcohol tolerance.

To the best of our knowledge, circadian oscillations of ADH enzymatic activity have not been previously characterized in *Drosophila*. Our study has found that ADH enzymatic activity follow a circadian pattern; ADH activity was observed to be moderately higher during the lights-off phases compared to lights-on. The similarity in day ADH activity fluctuation patterns between *CS*, *perS*, and *perL* flies suggest that the PER-mediated regulation of ADH enzymatic activity is an alternative function of PER, which is separate from the CLK/CYC and TIM/PER circadian negative feedback loop oscillator. Additionally, the ADH daily cycle in *perL* flies is severely blunted compared to *perS* flies (which was extremely robust) and *CS* flies. Flattened-out or blunted rhythms are indicators of impaired physiological function, which can lead to poor health and disease progression. For example,

in humans, blunted melatonin rhythms are known to exacerbate alcoholism, insomnia and depression [33, 34], Parkinson's Disease [35], and diabetes [36].

In the current study, *perS* flies had significantly more ADH, a more robust rhythm of ADH, and lower mortality on ethanol-containing food than *perL* flies. These results may indicate that individuals with longer circadian periods might be more sensitive to ethanol exposure compared to shorter period individuals. Ethanol (in most, but not all cases) tends to lead to period shortening in a wide variety of species including *Drosophila* [21] including *perL* [20], mice [7], rats [5], and humans [37]. Additionally, $per2^{Bdrm1}$ mutants, which initially exhibit shorter circadian periods in DD (before becoming arrhythmic), also are less sensitive to ethanol exposure and in fact have increased preference for it [8, 13, 14]. In addition, alcohol consumption also shortens REM cycles and decreases sleep time [38]. Furthermore, humans with a mutation PER2, which leads to increased alcohol drinking similar to what is seen in $per2^{Bdrm1}$ mutant mice, exhibit less sleep due to earlier awaking times [39]. These studies all indicate there are possible connections among alcohol consumption, *period* mutations, and shortened circadian/sleep rhythms.

With this study, we add to a body of knowledge that strongly links mutations in the circadian rhythm gene, *period*, and altered behavioral and biochemical responses to acute ethanol exposure. Taken together, we conclude that recovery from acute ethanol exposure is largely dependent on ADH enzymatic activity and that *perL* flies exhibit poorer ethanol sensitivity and recovery from exposure than flies with normal rhythms due to blunted ADH activity.

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Highlights

Drosophila show faster ethanol-induced sedation and slower recovery with age.

perL flies have longer recovery time from ethanol sedation compared to wild-type.

perL flies have higher residual ethanol post-sedation compared to wild-type.

Alcohol dehydrogenase activity appears to fluctuate in a circadian pattern and influence ethanol response.

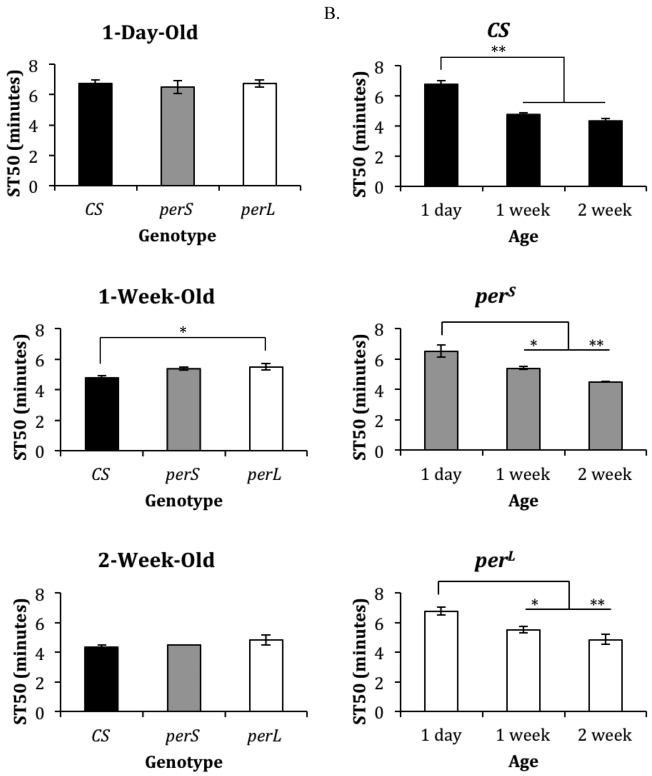

Figure 1. Ethanol-induced sedation in *period* mutants.

(A) Comparison of ST50 (duration of exposure to vapors generated by100% ethanol to sedate 50% of the flies) in different genotypes (wild type (*CS*), *perS* and *perL*) at the same age (1-day-old, 1-week-old, and 2-week-old). (B) Comparison of ST50 in each genotype with age. Columns represent mean ST50 +/− standard error of 3-4 biological replicates. For each ST50 eight male flies were used. *p<0.05 **p<0.01; ANOVA, *post hoc* Scheffe.

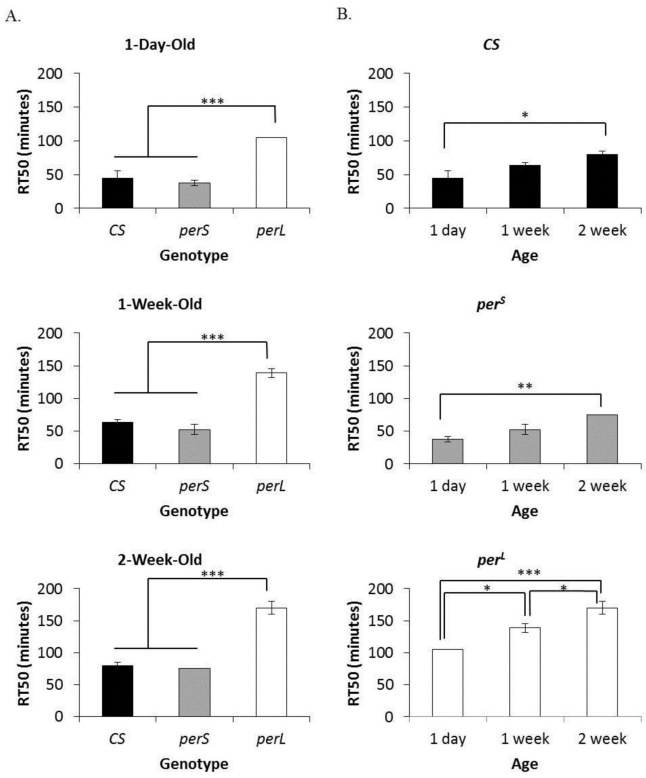

Figure 2. Recovery from ethanol-induced sedation in *period* mutants.

(A) Comparison of RT50 (duration for 50% flies to recover after exposure to 100% ethanol for 15 minutes) in different genotypes (wild type (*CS*), *perS* and *perL*) at the same age (1-day-old, 1-week-old, and 2-week-old). (B) Comparison of RT50 in each genotype with age. Columns represent mean RT50 +/− standard error of 3-4 biological replicates. For each RT50 eight male flies were used. *p<0.05 **p<0.01 ***p<0.001; ANOVA, *post hoc* Scheffe.

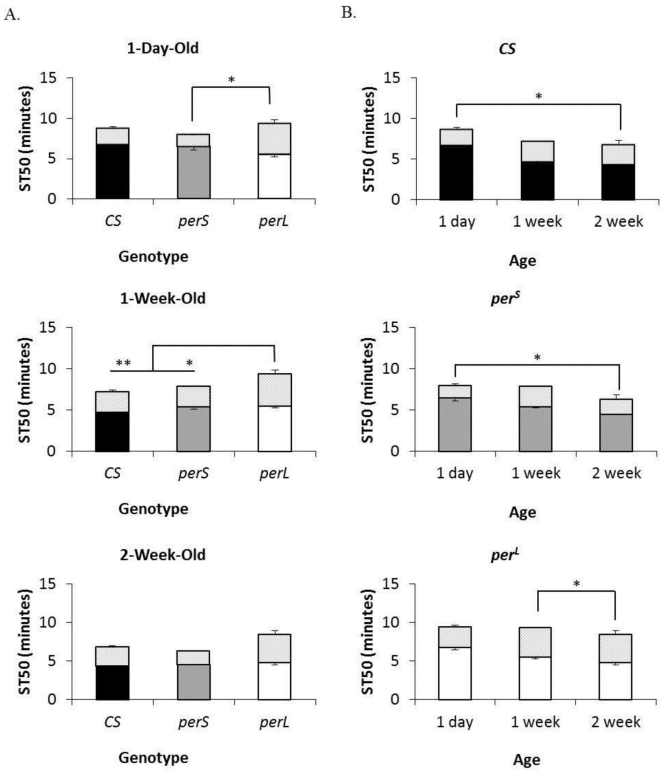

Figure 3. Tolerance to ethanol-induced sedation after repeated exposure in *period* mutants.

(A) Comparison of %T (percent change in ST50 upon ethanol exposure on consecutive days) in different genotypes (wild type (*CS*), *perS* and *perL*) at the same age (1-day-old, 1-week-old, and 2-week-old). (B) Comparison of %T in each genotype with age. Columns represent mean ST50 +/− standard error of second trial of 3-4 biological replicates. Shaded portion of each column depicts the difference in ST50 between trials. For each %T eight male flies were used. *p<0.05 **p<0.01; ANOVA, *post hoc* Scheffe.

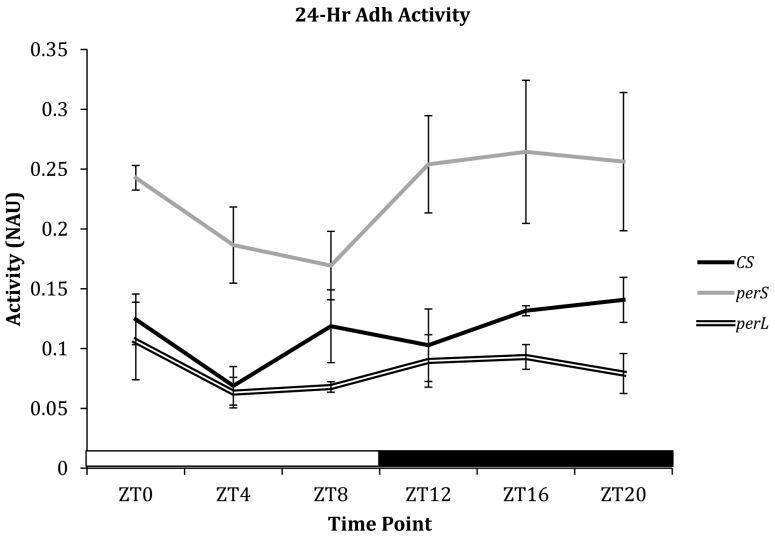

Figure 4. Circadian rhythm of alcohol dehydrogenase (ADH) activity in *period* mutants.

Comparison of ADH activity in 3-day-old wild type (*CS*), *perS* and *perL* flies at six time points in a 24-hour duration (ZT0, ZT4, ZT8, ZT12, ZT16, and ZT20). Each point on the line graph represents mean ADH activity +/− standard error of 3 biological replicates. ADH activity is represented as normalized absorbance units (NAU). NAU was calculated by normalizing the change in absorbance (ΔOD) due to accumulation of ADH-catalyzed NADH with the total protein concentration in each sample (BCA). X-axis is shaded clear and dark to depict light and dark conditions respectively. For each ADH activity assay five male flies were used.

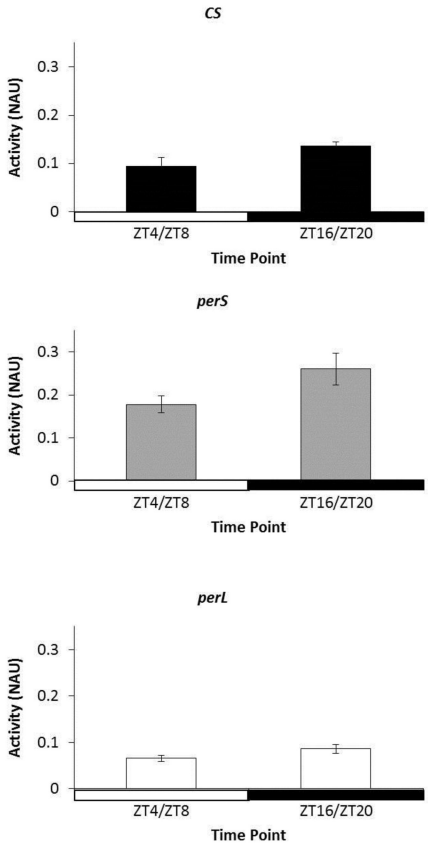

Figure 5. Day vs. Night alcohol dehydrogenase (ADH) activity in *period* mutants.

Comparison of ADH activity in 3-day-old wild type (*CS*), *perS* and *perL* flies during middle of light phase (ZT4 and ZT8) and middle of dark phase (ZT16, and ZT20). Each column represents mean ADH activity +/− standard error of at two time points in 3 biological replicates. ADH activity is represented as normalized absorbance units (NAU). NAU was calculated by normalizing the change in absorbance (ΔOD) due to accumulation of ADH-catalyzed NADH with the total protein concentration in each sample (BCA). X-axis is shaded clear and dark to depict light and dark conditions respectively. For each ADH activity assay five male flies were used.

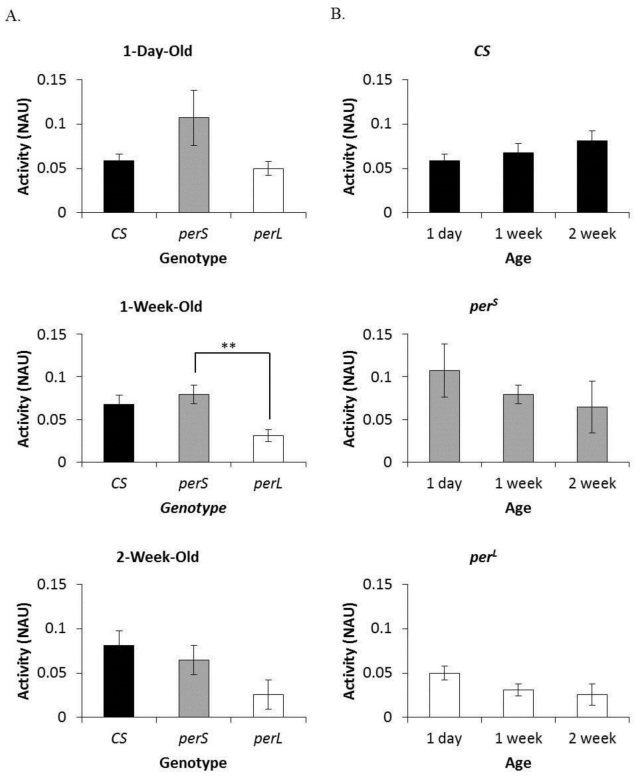

Figure 6. Alcohol dehydrogenase (ADH) activity in *period* mutants.

(A) Comparison of ADH activity in different genotypes (wild type (*CS*), *perS* and *perL*) at the same age (1-day-old, 1-week-old, and 2-week-old). (B) Comparison of ADH activity in each genotype with age. Columns represent mean ADH activity +/− standard error of 3-4 biological replicates. ADH activity is represented as normalized absorbance units (NAU). NAU was calculated by normalizing the change in absorbance (ΔOD) due to accumulation of ADH-catalyzed NADH with the total protein concentration in each sample (BCA). For each ADH activity assay five male flies were used. **p<0.01; ANOVA, *post hoc* Scheffe.

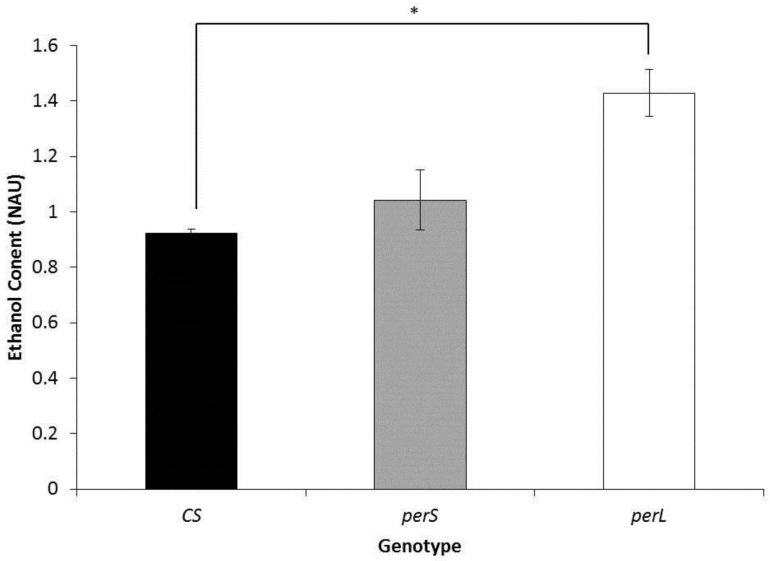

Figure 7. Ethanol pharmacokinetics in *period* mutants.

Comparison of ethanol content in 1-week-old wild type (*CS*), *perS* and *perL* flies. Flies were exposed to vapors generated by100% ethanol for 15 minutes at ZT4 followed by ethanol content assay at ZT5. Each column represents mean normalized ethanol content +/− standard error in 3 biological replicates. Ethanol content is represented as normalized absorbance units (NAU). NAU was calculated by normalizing the absorbance (OD) with the total protein concentration in each sample (BCA). For each ethanol content assay four male flies were used. *p<0.05; ANOVA, *post hoc* Scheffe.