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Short communication

Larval ethanol exposure alters free-running circadian rhythm and *per Locus* transcription in adult *D. melanogaster period* mutants

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HIGHLIGHTS

- Larval ethanol exposure alters circadian rhythm in *D. melanogaster period* mutants.
- Changes in circadian period and *per* mRNA persist in adults after removal of ethanol.
- Larval ethanol shortens period and decreases *per* mRNA levels in *perL* mutants.
- Larval ethanol lengthens period and increases *per* mRNA levels in *perS* mutants.

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ABSTRACT

Alcohol consumption causes disruptions in a variety of daily rhythms, including the circadian free-running rhythm. A previous study conducted in our laboratories has shown that larval ethanol exposure alters the free-running period in adult *Canton-S Drosophila melanogaster*. Few studies, however, have explored the effect of alcohol exposure on organisms exhibiting circadian periods radically different than (normal) 24-h. We reared *Canton-S*, *period* long, and *period* short *Drosophila melanogaster* larvae on 10%-ethanol supplemented food, and assessed their adult free-running locomotor activity and *period* transcript at ZT 12. We demonstrate that in *Canton-S* larval ethanol exposure shortens the adult free-running locomotor activity but does not significantly alter *period* mRNA levels at ZT 12. *Period* long mutants exposed to larval ethanol had significantly shortened adult free-running locomotor activity rhythms and decreased *period* mRNA levels, while *period* short mutants lengthened their free-running rhythm and showed increased *period* mRNA levels at ZT 12 after being exposed to larval ethanol. These results indicate that the effects of ethanol on the circadian clock might depend upon the baseline circadian period of the organism or that *period mutant* gene expression is sensitive to developmental ethanol treatment.

Alcohol intake can lead to disruptions of daily behavioral and molecular rhythms in both humans and animal models [1]. Previous investigations using rodent models illustrate that chronic alcohol treatment can alter the free-running rhythm, a fundamental parameter of circadian clocks [2–5]. Chronic ethanol can alter molecular rhythms as well, including *period* (*per*) mRNA expression within the Suprachiasmatic Nucleus (SCN), the location of the mammalian light-entrainable circadian clock [6]. Not only does ethanol intake affect the adult circadian clock, perinatal or neonatal ethanol exposure also causes disruptions of *per* mRNA expression rhythms [7,8], and free-running rhythms [7,9]. These studies show clear connections between ethanol consumption and alterations of the circadian clock, at both behavioral and molecular levels.

While *Drosophila melanogaster* has been a widely used model in studying the behavior and genetics of both the circadian clock [10] and ethanol exposure [11], few studies have investigated effects of ethanol on the free-running rhythm in fruit flies. A recent study from our laboratories [12] shows that chronic developmental ethanol exposure alters the adult circadian free-running locomotor rhythm in wild-type *Canton-S* (CS) fruit flies in a dose dependent manner, even after ethanol treatment has been suspended. These previous investigations (including the aforementioned mammalian studies) have used only "wild-type" animal models, all with free-running periods approximating 24 h. There appears to be a link

Abbreviations: CT, Circadian time; LD, light-dark; DD, dark-dark (i.e. constant darkness); ZT, Zeitgeber time.

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Table 1
Adult locomotor activity levels (average beam crosses per 10 min bin ± SEM) and bout analysis of *Canton-S*, *perS*, and *perL*, in LD and DD, under the differing ethanol treatment conditions. On average, *perS* exhibit higher locomotor activity than *perL*, manifesting itself in more beam crosses per bin and longer bouts in both LD and DD. There are no differences in the number of bouts between *perS* and *perL* in LD, but *perL* showed increased number of bouts per day in DD compared to *perS*. There is no effect of ethanol on the activity amongst individual genotypes for overall activity levels, length of bout, beam crosses per bout, or number of bouts per day in LD or DD, excepting that *perL* receiving larval 10% ethanol showed increased number of bouts per day compared to control *perL* in DD only. Values with differing letters (a,b,c) are significantly different ($p < 0.05$).

Genotype	EtOH %	N	Activity LD	Activity DD
Canton-S	0%	26	5.84 ± .36[a]	5.40 ± .36[a]
	10%	24	5.18 ± .31[a]	5.42 ± .31[a]
perL	0%	34	4.31 ± .30[a]	4.58 ± .25[a]
	10%	25	3.97 ± .27[a]	4.95 ± .34[a]
perS	0%	30	9.85 ± .89[b]	9.24 ± .71[b]
	10%	25	10.66 ± .86[b]	10.32 ± .86[b]

		LD			DD		
Genotype	EtOH %	Length of bout (min)	Beam crosses per bout	Bouts per day	Length of bout (min)	Beam crosses per bout	Bouts per day
Canton-S	0%	48.56 ± 5.82	73.86 ± 9.57	11.92 ± 1.04	44.89 ± 6.73	58.33 ± 9.63	14.36 ± 1.14
	10%	46.54 ± 4.17	69.75 ± 6.56	9.90 ± 0.83	40.57 ± 3.61	54.51 ± 6.37	14.07 ± 0.73
perL	0%	23.67 ± 1.70[a]	27.60 ± 2.43[a]	12.35 ± 0.92[a]	22.84 ± 2.56[a]	28.62 ± 4.59[a]	15.12 ± 1.00[a]
	10%	21.04 ± 1.49[a]	30.46 ± 2.48[a]	10.22 ± 0.91[a]	26.78 ± 2.40[a]	34.64 ± 3.74[a]	19.32 ± 1.15[b]
perS	0%	33.04 ± 4.94[b]	74.93 ± 13.98[b]	11.84 ± 0.84[a]	39.01 ± 6.11[b]	79.81 ± 16.10[b]	12.42 ± 1.05[c]
	10%	37.09 ± 4.52[b]	81.85 ± 11.90[b]	11.97 ± 0.68[a]	41.20 ± 7.43[b]	82.67 ± 16.62[b]	10.69 ± 0.85[c]

between ethanol consumption and genes regulating the circadian clock, as mutations in *per2* lead to significantly increased ethanol drinking in mice and are associated with human alcoholics [13]. *Per2* mutant mice exhibit increased drinking bouts compared to wild-type mice, and acamprosate (a drug known to reduce ethanol drinking) reduces the number of drinking bouts in an LD cycle [14]. It is currently unknown, however, how chronic ethanol affects the free-running rhythm in circadian mutants which do not exhibit wild-type length circadian periods. Thus, the present experiments aim to uncover effects of chronic ethanol ingestion on circadian behavior (locomotor activity rhythm) and its molecular mechanism (*per* mRNA) in *Drosophila melanogaster*, using the Long (*perL*) and Short (*perS*) variants of the *period* mutants, which have circadian periods of approximately 28.5 and 19.5 h, respectively [10].

For the behavioral assays, *CS*, *perS*, and *perL Drosophila melanogaster*, were reared and the activity and free-running periods were calculated using the protocols as previously described [12]. Flies were considered entrained only with LD periods of 24.00 ± 0.05 h. Composite actograms were generated by copying the raw numbers within each individual channel file (produced by FileScan) into a single column within a spreadsheet. Each individual fly received its own column. As each row constitutes the activity during a single 10-min bin for each fly, the numbers were averaged across each row and copied into a blank text file. The newly formed averaged activity file was imported into ClockLab to generate the actogram. A bout analysis for both LD and DD was conducted for all genotypes and ethanol treatments. The mean length of time (minutes), beam crosses per bout, and number of bouts per day were analyzed. An activity bout was defined as being greater than or equal to the average size of activity counts across the day, separated by at least 10-min of inactivity.

Six separate groups of these fly strains were also raised on either 0%- or 10%-ethanol supplemented food (same as in the behavioral analyses), and after eclosion, the flies were tested to determine the relative *per* mRNA level at ZT 12, using quantitative real time-Polymerase Chain Reaction (qRT-PCR). Total RNA (with DNAse treatment) from 25–30 heads of appropriate genotype and treatment was isolated using RNeasy miniprep kit (Qiagen, Valencia, CA). RNA quality and yield was measured using nanodrop spectrometer (Thermo Scientific, Wilmington, DE). qRT-PCR was performed in duplicates on 50–100 ng total RNA using one step Quantifast SYBR Green RT-PCR kit (Qiagen) on StepONE Real-Time PCR system (Applied Biosystems, Foster City, CA). Dissociation curve was analyzed to ensure primer specificity. Relative normalized transcript level was determined by delta-delta Ct method. *RP49* was used as the normalizing gene. Mean ± SEM *per* transcript was calculated from six independent experiments. Welch's *t*-tests with the Bonferroni correction were performed to determine differences between ethanol receiving flies and controls, among the three genotypes. *Per* primers were 5′-GACCGAATCCCTGCTCAATA-3′and 5′-GTGTCATTGGCGGACTTCTT-3′, and *RP49* primers were 5′-CGGTTACGGATCGAACAAGC-3′ and 5′-CTTGCGCTTCTTGGAGGAGA-3′.

The mean activity level (number of beam crosses per 10 min bin) in LD and DD across all genotypes and ethanol treatments are listed in Table 1. *CS* receiving larval 10%-ethanol were not significantly different from *CS* control receiving 0%-ethanol, regarding the mean activity level in LD ($t_{1,48} = 1.378$=; $p = 0.18$) or DD ($t_{1,48} = 0.048$; $p = 0.96$). Regardless of ethanol treatment, *perS* flies had significantly greater mean activity than *perL*, in both LD (Two-way ANOVA; $F_{1,110} = 88.99$; $p < 0.001$) and DD ($F_{1,110} = 66.82$; $p < 0.001$). In addition, there was no genotype by ethanol interaction for mean activity for *perS* and *perL*, in LD ($p = 0.37$) or DD ($p = 0.57$). These results are consistent with our previous study in flies [12], where no differences in activity level in DD were found between larval-ethanol treated animals versus controls.

The bout analysis showed that *perS*, regardless of ethanol treatment, had increased bout length (Two-way ANOVA; $p = 0.001$) and beam crosses per bout ($p < 0.001$), but not number of bouts per day ($p = 0.64$), compared to *perL* in LD. In DD, *perS* had increased bout length ($p = 0.005$) and beam crosses per bout ($p < 0.001$), but had decreased number of bouts per day ($p < 0.001$) compared to *perL*. There was no genotype by ethanol interaction for the bout duration, number of counts per bout, and number of bouts per day in LD or DD (all $p > 0.10$), except that *perL* exposed to larval-ethanol showed more bouts per day than control *perL* in DD ($p = 0.028$). This result suggests that *perL* may be more sensitive to the effects of ethanol on activity than *perS*. There were no differences between *CS* receiving ethanol and controls for the bout analysis (t-tests, all $p > 0.10$). The results from the bout analyses are different from a previous report using mice, where mean bout duration and quantity was altered during ethanol exposure and withdrawal in a 1-min