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Dissociation between diurnal cycles in locomotor activity, feeding behavior and hepatic PERIOD2 expression in chronic alcohol-fed mice

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Abstract

Chronic alcohol consumption contributes to fatty liver disease. Our studies revealed that the hepatic circadian clock is disturbed in alcohol-induced hepatic steatosis, and effects of chronic alcohol administration upon the clock itself may contribute to steatosis. We extended these findings to explore the effects of chronic alcohol treatment on daily feeding and locomotor activity patterns. Mice were chronically pair-fed *ad libitum* for 4 weeks using the Lieber-DeCarli liquid diet, with calorie-controlled liquid and standard chow diets as control groups. Locomotor activity, feeding activity, and real-time bioluminescence recording of PERIOD2::LUCIFERASE expression in tissue explants were measured. Mice on liquid control and chow diets exhibited normal profiles of locomotor activity, with a ratio of 22:78% day/night activity and a peak during early night. This pattern was dramatically altered in alcohol-fed mice, marked by a 49:51% ratio and the absence of a distinct peak. While chow-diet fed mice had a normal 24:76% ratio of feeding activity, with a peak in the early night, this pattern was dramatically altered in both liquid-diet groups: mice had a 43:57% ratio, and an absence of a distinct peak. Temporal differences were also observed between the two liquid-diet groups during late day. Cosinor analysis revealed a ~4-h and ~6-h shift in the alcohol-fed group feeding and locomotor activity rhythms, respectively. Analysis of hepatic PER2 expression revealed that the molecular clock in alcohol-fed and control liquid-diet mice was shifted by ~11 h and ~6 h, respectively. No differences were observed in suprachiasmatic nucleus explants, suggesting that changes in circadian phase in the liver were generated independently from the central clock. These results suggest that chronic alcohol consumption and a liquid diet can differentially modulate the daily rhythmicity of locomotor and

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feeding behaviors, aspects that might contribute to disturbances in the circadian timing system and development of hepatic steatosis.

Keywords

circadian rhythm; alcoholic liver disease; hepatic steatosis; Lieber-DeCarli liquid diet; clock gene; feeding behavior

Introduction

The endogenous circadian timing system, or clock, regulates behavior and physiologic activity in concert with the light-dark cycle and ensures that daily rhythms in metabolism are temporally coordinated with rest, activity, and feeding cycles. In mammals, the master circadian oscillator resides in the hypothalamic suprachiasmatic nucleus (SCN), which synchronizes and phase-resets the cell autonomous clocks in peripheral tissue through humoral signals, body temperature cycles, and feeding-related cues (Mohawk, Green, & Takahashi, 2012). A cell-autonomous circadian clock controls local hepatic physiology by driving rhythmic gene and protein expression, and these in turn impact time-of-day specific regulation of many processes including lipid and glucose metabolism, and bile acid (BA) synthesis (Bailey, Udoh, & Young, 2014; Marcheva et al., 2013).

Hepatic steatosis, the accumulation of triglyceride (TG) droplets in the hepatocytes, is the earliest response of the liver to excessive alcohol consumption (Sozio, Liangpunsakul, & Crabb, 2010). Alcohol metabolism reduces the nicotinamide adenine dinucleotide/reduced nicotinamide adenine dinucleotide (NAD/NADH) ratio, resulting in interrupted mitochondrial fatty acid (FA) β oxidation and thus leading to hepatic steatosis (Sozio et al., 2010). FA, cholesterol, and BA synthesis fluctuate over the diurnal cycle with a circadian periodicity, as does the expression of genes associated with their regulation (Zhou, Ross, Pywell, Liangpunsakul, & Duffield, 2014). Feeding mice with Lieber-DeCarli alcohol-containing diet for 4 weeks results in both hepatic steatosis as well as disturbances to the hepatic circadian clock (Zhou et al., 2014).

There is emerging evidence that the time of day at which meals are consumed influences behavioral and physiological systems. Reverse feeding of nocturnally active rodents during the light phase of the light:dark (LD) cycle results in increased weight gain (Arble, Bass, Laposky, Vitaterna, & Turek, 2009). Human studies suggest that shift workers who have altered meal times are at a higher risk of developing obesity and metabolic syndrome (Wang, Armstrong, Cairns, Key, & Travis, 2011). Human participants who confined their energy intake to the early day lost more weight than those who consumed a similar diet later in the day (Garaulet et al., 2013).

Given the drawbacks of the ethanol-in-drinking-water procedure, which does not result in alteration in hepatic pathology as observed in humans with heavy drinking, the Lieber-DeCarli ethanol liquid diet was developed in response to the need to develop an animal model with an alcohol consumption of clinical relevance (Lieber, DeCarli, & Sorrell, 1989; Matson et al., 2013). The liquid diet results in high ethanol intake, high blood alcohol level,

and evidence of hepatic steatosis after 24 days of access (DeCarli & Lieber, 1967; Lieber & DeCarli, 1970), and has become the standard method of inducing liver steatosis in rodents. Three standardized basic formulas are used widely: 1) an all-purpose (35% fat kcal) Lieber-DeCarli diet, which is appropriate for most experiment applications, 2) a high-protein formula diet (25% protein kcal) useful in pregnancy and lactation, and 3) a low-fat formula diet (12% fat kcal), which is intended to minimize the hepatic changes by fat consumption (Lieber et al., 1989; Matson et al., 2013).

Our previous study using the low-fat Lieber-DeCarli diet (Zhou et al., 2014) revealed that chronic alcohol feeding (CAF) interferes with the normal operation of the hepatic circadian clock, leading to loss of temporal coordination of liver-associated metabolic function at both the gene expression and physiological levels. Furthermore, another group reported differences in the diurnal expression of clock genes in the liver of chronically alcohol-fed mice (Filiano et al., 2013), a finding broadly consistent with our studies. Furthermore, it is known that adaptation of peripheral clocks is dependent on the time of feeding, meal frequency pattern, and metabolic state (Kuroda et al., 2012). However, currently there are few studies systematically characterizing the feeding and locomotor activity patterns in mice chronically fed with the Lieber-DeCarli diet. As such, the data presented here build upon our previous studies by elucidating the effects of chronic alcohol treatment on daily feeding and locomotor activity patterns, and on the circadian phase of the liver clock in CAF mice.

Materials and methods

Animals and diets

C57BL/6 male mice from Jackson Laboratory (Bar Harbor, ME) and PER2::LUCIFERASE mice (PER2::LUC; C57BL/6J background) generated from in-house breeding at University of Notre Dame (UND) (Yoo et al., 2004) were housed individually in a room with controlled temperature (20–22 °C) and humidity (55–65%). Mice were first entrained to a 12:12 light:dark (LD) cycle for 4 weeks and maintained on a regular chow diet. The experimental design consisted of three dietary groups: 1) liquid control diet (LCD) (Calories – 10:18:72; by % calculation as fat: protein: carbohydrate; Lieber-DeCarli liquid diet, Dyets Inc., Bethlehem, PA), 2) ethanol-containing diet (ED) (identical to the LCD, except that ethanol was added to account for 27.5% of total calories and the caloric equivalent of carbohydrate [maltose-dextrin] removed), and 3) regular chow diet (RD) (Calories – 22:23:55; Teklad Global diet 2919) (Mathew et al., 2013; Zhou et al., 2014). Alcohol was introduced gradually into the diet during the first 5 days of feeding starting from 9% alcohol (of the total calories) for 2 days, 18% for the next 3 days, and then finally 27.5% until the end of the experiment (Liangpunsakul et al., 2012; Zhou et al., 2014). Ethanol-containing diet or LCD were available *ad libitum* and dispensed using a standard calibrated 50-mL liquid diet feeding tube (Bio-Serv, Flemington, NJ) attached to the end of the cage (Bertola, Mathews, Ki, Wang, & Gao, 2013). A separate water source was also provided using a standard water bottle. Fresh food was prepared daily by homogenization in a blender and routinely changed between Zeitgeber Time (ZT) 2 and ZT3 (ZT0 = lights on [morning], ZT12 = lights off [evening]). Ethanol-containing diet or LCD was provided in equal volumes (25 mL each) at the time of food change. Feeding activity was measured (see details below) during the 2-h

span (one hour before and one hour after) after the food was changed. Mice were weighed weekly and food consumption (volume) was measured every day for 4 weeks. Experiments were approved by the UND IACUC and performed in accordance with NIH Guidelines for the Care and Use of Laboratory Animals.

Feeding and locomotor activity recording

Mice (aged 9–12 weeks) were housed individually with a 12:12 LD cycle, and chronically pair-fed *ad libitum* for 4 weeks using ED (not including the first 5 days of alcohol introduction), or with LCD or RD as control groups. Feeding activity was assessed by either video monitoring (SONY HDR-PJ790 Digital HD video camera recorder) and behavior scoring as duration (seconds) feeding at the liquid feeder (ED and LCD; see supplemental video SV1), or by visits to the food hopper in specialized cages (RD) and as previously described (Mathew et al., 2013). The Smart Cutter program (SWREG, Inc., Minnetonka, MN, USA) was used for video editing. Locomotor activity was assessed by passive infrared (PIR) motion detection (Mathew et al., 2013). RD feeding activity and three groups of PIR activity were monitored using *Clocklab* hardware and software (Actimetrics, Wilmette, IL). For RD feeding and PIR locomotor activity, individual animal data were selected for analysis: Minute-to-minute data were recorded over 5 consecutive days and occurring within 25 days of the onset of experimentation. Liquid diet (ED and LCD) video footage and PIR activity were recorded for individual animals for 24 h. All data from each mouse were averaged into hourly bins and converted to percentage values, with the maximum values as 100% for each mouse.

Biochemical measurements

Whole blood was collected at 4-h intervals (ZT0, 4, 8, 12, 16, and 20, n = 1 [ZT0–12]; n = 2 at ZT16 and ZT20) by an intracardiac puncture and clotted in Microtainer tubes (BD, Franklin Lakes, NJ, USA) for 45 min, and serum was extracted. Blood alcohol levels were determined using the Alcohol Reagent Set (Pointe Scientific, Inc., Canton, Michigan) (Filiano et al., 2013).

Real-time luminescence recording and analysis

PER2::LUC mice were pair-fed with ED, LCD, or RD for 4 weeks. Mice were sacrificed at ZT4 or ZT8. Explant cultures were prepared as previously described (van der Veen, Shao, Xi, Li, & Duffield, 2012; Zhou et al., 2014). Tissues were placed in a light-tight box at 36 °C, and biolumine scence was measured using a photomultiplier tube (LumiCycle, ActiMetrics, IL, USA) every 10 min over 4 days. Bioluminescence data from PER2::LUC mouse SCN and liver explants were de-trended by subtracting the 24-h running average from the raw data. Explants expressing only one circadian peak were considered arrhythmic. The peak of the circadian oscillation in culture was determined by measuring the highest point in the first complete cycle *in vitro* (van der Veen et al., 2012; Yoo et al., 2004; Zhou et al., 2014).

Statistics

Data were analyzed using SigmaPlot 12.0 software (Chicago, IL) and Prism 5.0 (La Jolla, CA) to run a two-factor repeated-measures (RM) ANOVA or two-factor ANOVA. Tukey's *post hoc* tests were performed when significant ANOVA results between factors were revealed. When the Shapiro-Wilk normality test failed ($p < 0.05$), data were rank transformed to correct for non-normal distributions (specifically for feeding and PIR CG analyses, PIR activity analysis, and liver explants PER2::LUC peak phase analysis). CircWave v1.4 software (www.huttlab.nl, www.euclock.org; an extension of the cosinor analysis, courtesy of Dr. Roelof Hut) (van der Veen, Mulder, Oster, Gerkema, & Hut, 2008) was used to analyze the rhythmicity of feeding and locomotor activity by fitting a Fourier curve (one sine wave with up to two additional harmonics) to the data, and producing R^2 and center of gravity values (Zhou et al., 2014). The *p* values reported are the result of an *F* test from the software. Two-tailed Student's *t* tests were used to compare two groups. The value of alpha was set at 0.05 for total feeding and PIR activity. For individual animal feeding and PIR activity plots, user-defined $\alpha = 0.5$ was used (Gilbert, Douris, Tongjai, & Green, 2011; Zhou et al., 2014). Graphs marked with ‡ represent data sets to which no curve could be fit. Bartlett's test and *F* test were conducted to test for differences in variance between experimental groups.

Results

Daily feeding and locomotor activity profile of mice on alcohol, liquid control, and chow diet

Given that the hepatic clock is affected by CAF (Zhou et al., 2014), the daily patterns of feeding and locomotor activity of mice pair-fed with Lieber-DeCarli diet (ED/LCD) were evaluated compared to those fed with RD. The feeding activities of the ED and LCD groups were altered compared to those of the RD group [RM-ANOVA, ED vs. RD: Time (Ti), $p < 0.001$; Treatment (Tr), $p < 0.001$; Interaction (I), n.s.; LCD vs. RD: Ti, $p < 0.001$, Tr, $p < 0.01$; Interaction (I), n.s.] (Fig. 1A, B.) We also observed a difference between ED and LCD mice in the level of feeding activity during the light phase immediately prior to lights-off (ZT12) (RM-ANOVA, ED vs. LCD: Ti, $p < 0.001$; Tr, n.s.; I, $p < 0.05$) (Fig. 1C). Note that RM-ANOVA inclusive of all three experimental groups revealed similar time-of-day specific results (Ti, $p < 0.001$; Tr, $p < 0.01$; I, $p < 0.05$). When we considered the individual data plots for each treatment group generated from the CircWave analysis, RD mice showed a normal expected 24:76% ratio of feeding with a peak in the early night (center of gravity [CG] ZT14.2; $R^2 = 0.55$; *F* test, $p < 0.001$) (Fig. 1D). However, the pattern of feeding was dramatically altered in both ED and LCD groups, in which mice had a 43:57% ratio, and there was an absence of a distinct peak (ED: CG, ZT18.0; $R^2 = 0.06$; *F* test, $p < 0.001$; LCD: CG, ZT13.3; $R^2 = 0.14$; *F* test, $p < 0.001$) (Fig. 1D). Notably, cosinor analysis revealed a ∼4–h shift in the ED feeding rhythm compared to LCD and RD rhythms. Representative individual animal feeding CircWave plots and individual activity profiles are shown in Supplemental Fig. 1 and 2, respectively. The CG of individual animal feeding activity is shown in Fig. 1E, and significant differences among three group CGs were identified

(ANOVA, $F[2,26] = 4.8$, $p < 0.05$). No difference in variance of CG among three groups was found (Bartlett's test, n.s.).

Consistent with the feeding activity patterns, the daily patterns of general locomotor activity were altered in ED mice when compared to those of LCD and RD (RM-ANOVA, ED vs. LCD: Ti, $p < 0.001$; Tr, $p < 0.05$; I, $p < 0.001$; ED vs. RD: Ti, $p < 0.001$; Tr, n.s.; I, $p < 0.001$) (Fig. 2A, C). No difference was found between LCD and RD groups (RM-ANOVA, Ti, $p < 0.001$; Tr, n.s.; I, n.s.) (Fig. 2B). Note that RM-ANOVA inclusive of all three experimental groups revealed similar time-of-day specific results (Ti, $p < 0.001$; Tr, $p < 0.05$; I, $p < 0.001$). CircWave analysis of individual data plots for each treatment group of LCD and RD mice showed a normal 22:78% day:night activity ratio, and a peak during the early night (LCD: CG, ZT15.5; $R^2 = 0.41$; *F* test, $p < 0.001$; RD: CG, ZT16.9; $R^2 = 0.59$; *F* test, $p < 0.001$) (Fig. 2D). However, the pattern of locomotor activity was dramatically altered in the ED group, marked by a 49:51% ratio and absence of a distinct peak (CG, ZT22.8; $R^2 = 0.05$; *F* test, $p < 0.001$) (Fig. 2D). Moreover, the CG of the ED mice was shifted ∼6 h compared to the profile of the RD mice. Representative individual animal PIR CircWave plots and individual activity profiles are shown in Supplemental Fig. 1A and Supplemental Fig. 2, respectively. When the mean values of the daily counts of locomotor activity were analyzed, no significant differences were observed between treatment groups (Fig. 2E). Similar to feeding activity, there are significant differences among the three groups in the CG of individual animal PIR locomotor activity profiles (ANOVA, $F[2,28] = 29.2$, $p < 0.0001$) (Fig. 2F). Further, the variance of CG for the ED group was significantly greater than LCD and RD (Bartlett's test: $p < 0.01$; *F* test, ED vs RD, $p < 0.05$; ED vs. LCD, $p < 0.01$; RD vs. LCD, n.s.). Next, we examined the R^2 values of feeding and locomotor activity of individual animals (Supplemental Fig. 1B). The R^2 values are a measure of fit to the cosine wave, the cosinor being a standard tool in circadian biology for testing for the presence of a 24-h rhythmic profile. Significant differences of R^2 values among the three groups were found for feeding behavior (ANOVA, $F[2,25] = 4.7$, $p < 0.05$) but not locomotor activity (ANOVA, $F[2,28] = 2.3$, n.s.). Specifically, the R^2 values of the LCD group were lower than the RD for feeding, and thereby indicated a weaker rhythmic profile for LCD animals. Furthermore, the proportions of mice deemed to not show rhythmic profiles according to our criteria in either feeding or locomotor activity behavior, were highest for the ED animals, followed by the LCD group: For feeding activity, 69% of ED-treated animals and 85% of LCD-treated animals expressed rhythmicity. For locomotor activity, 75% of ED-treated animals and 92% of LCD-treated animals were scored as rhythmic. All (100%) chow-control mice exhibited rhythms in both feeding and locomotor activity.

Biochemical and physiological measurements of mice fed with alcohol and liquid control diet

Representative blood alcohol concentration (BAC) was measured in ED and LCD animals (Fig. 3A). As expected, the BAC level was higher during the night phase than during the day phase of the LD cycle ($t_6 = 4.8$, $p < 0.01$). Moreover, we observed an increased feeding activity in the LCD mice following the changing of food ($t_{24} = 2.3$, $p < 0.05$), a feature absent in ED-fed mice (Fig. 3B). Overall food consumption was decreased in the ED group

compared to that of the LCD group ($t_{61} = 4.5$, $p < 0.001$) (Fig. 3C). Moreover, while the average body weight of ED mice was higher compared to LCD mice in the first week of the feeding experiment ($t_{60} = 2.2$, $p < 0.05$), it was lower when measured at the end of the study in the fifth week ($t_{60} = 2.2$, $p < 0.05$) (Fig. 3C).

Bioluminescence analysis of suprachiasmatic nucleus (SCN) and hepatic PER2 protein expression of mice fed with alcohol, liquid control diet, and chow diet

Our previous study showed that the hepatic PER2 rhythm of alcohol-treated mice was phase-shifted and that the SCN clock was unaffected by the CAF (Zhou et al., 2014). In the current study and using a new cohort of mice, we extended our study by integrating the RD group and assessing whether there was any difference in the profiles of PER2 rhythms among ED, LCD, and RD groups examined simultaneously. PER2 is a canonical rhythmic clock component and is used widely as a marker for the phase of the circadian clock. Representative baseline subtracted traces for PER2 protein-driven bioluminescence in the SCN and liver explants from ED, LCD, and RD are shown in Fig. 4A. Consistent with our previous study (Zhou et al., 2014), we observed a reduced peak to trough amplitude in PER2:LUC rhythm in the ED group. Using the peak of circadian oscillation in luminescence within the first 24 h of isolation, the phase distribution maps for SCN and liver were constructed (Fig. 4B, C). As expected, no difference in PER2::LUC expression of SCN was detected between the three diet groups, which is identical to previous observations (Filiano et al., 2013; Zhou et al., 2014). However, significant differences in the mean phase were found between liver explants derived from the three groups (individual explants: ANOVA: $F[2,87] = 47.1$, $p < 0.001$; Average for individual animals: ANOVA: $F[2,16] = 9.2$, $p < 0.01$). In addition, the mean peak phase of PER2 expression between ED (ZT4.6) and RD-treated mice (ZT17.9) was found to be in ~12-h anti-phase. Large variances in peak phases were also found among the three groups, both in analysis of individual liver explants and analysis of averaged values for liver that represent individual animals (Explants, Bartlett's test $p < 0.001$; F test: ED vs RD, $p < 0.001$; LCD vs. RD, $p < 0.001$; ED vs. LCD $p < 0.05$; Average for individual animals, Bartlett's test $p < 0.01$; F test: ED vs. RD $p < 0.05$; LCD vs. RD, $p < 0.001$; ED vs. LCD, n.s.). Notably, the peaks of PER2 expression in liver explants of the LCD group had a particularly large variance that could be divided into two groups: one group peaking at ZT8.2 ± 0.7 (mean ± SEM, n = 13), which is significantly different from the other group, peaking at ZT18.4 ± 0.3, n = 9 ($t_{20} = 11.7$, $p < 0.001$). No large variances were observed between explants derived from an individual animal.

A dissociation of hepatic PER2 rhythmicity, feeding activity, and locomotor activity in mice fed with alcohol or liquid control diet

Based on the feeding and locomotor CG, and hepatic PER2 peak expression, distribution maps were constructed for each diet type (Fig. 5). For the RD group, the mean CG of feeding and of locomotor activity, and PER2 peak expression fell at night at ZT14.2, ZT16.9, and ZT17.9, respectively. For ED groups, means of feeding and locomotor activity occurred at ZT18.0 and ZT22.8, and PER2 peak at ZT4.6 (daytime). Notably, in the LCD group, the mean PER2 peak occurs at ZT12.3, which contains two separate clusters shown as dash-lined arrows (Fig. 5). The CG of feeding and locomotor activity in the LCD group fell at ZT13.3 and ZT15.5, respectively.

Discussion

Our previous study revealed that CAF results in the disturbance of the hepatic circadian clock, resulting in profound changes in the rhythms in TG, BA, in the phase and amplitude of expression profiles of canonical clock genes and in expression of rhythmic clock-controlled genes (Zhou et al., 2014). These findings were also broadly consistent with the results of the Filiano group in a similar study, although conducted using the *all-purpose* Lieber-DeCarli liquid diet (36% fat kcal) (Filiano et al., 2013). The Filiano et al. (2013) study also found that the circadian clock phase of the liver, but not the SCN, was shifted following CAF, although the magnitude of the response in their investigation was smaller. In this current study, we extend our previous findings by characterizing diurnal feeding activity, locomotor activity, and hepatic PER2 protein expression of mice fed with the Lieber-DeCarli diet compared to a normal chow diet.

Our studies reveal that mice exhibit weaker diurnal feeding rhythmicity (a lower cosinor R^2 value) and a ~4-h phase shift in its CG when fed the ED compared to RD. Similarly, it has been reported that mice fed with an alcohol liquid diet versus alcohol in the drinking water or compared with water/lab chow diet have a dampened diurnal amplitude in rhythmic fluid consumption (Freund, 1970; Goldstein, 1977). Moreover, under CAF, the diurnal locomotor rhythmicity was dampened, characterized by a decreased nighttime and increased daytime activity pattern, which has a ~6-h phase shift in its CG. Changes in the diurnal pattern of locomotor activity have also been reported in mice forced to consume ethanol in drinking water and maintained in a skeleton photoperiod, specifically, increased sporadic activity with reduced duration during the ‘active phase’ of the cycle, and a reduction in the 3-h duration of intense activity starting at lights-off (Brager, Ruby, Prosser, & Glass, 2010). This reduction in normal peak activity following lights-off at ZT12 is a shared feature of the Brager et al. (2010) study and our current findings. In addition, previous studies have shown a decrease in locomotor activity during the active phase of the LD cycle (i.e., night) in CAF rats, which is broadly consistent with our results (Taylor, Tio, Bando, Romeo, & Prolo, 2006). This decrease in nighttime activity and in the strength of rhythm (lower R^2 value of the cosinor fit) might be due to an inhibitory effect of ethanol drinking on activity, and is likely related to drinking-induced hypothermia known to suppress locomotor activity (Taylor et al., 2006; Wasielewski & Holloway, 2001). We also observed that mice fed with LCD showed increased daytime feeding activity and dampened rhythmicity (lower cosinor R^2 value) compared to RD. The behavioral analysis showed locomotor activity to be closely aligned with feeding activity in the ED and RD groups, but there was dissociation between behaviors under the LCD.

Under ED, blood alcohol levels were elevated during the night, showing that mice consumed more food during this phase of the LD cycle. Further, we observed that mice under ED showed less food intake and weight gain. It has been shown that rodents decrease their overall food intake and show reduced body weight when ethanol is introduced to the liquid diet (Lieber et al., 1989; Saville & Lieber, 1965; Shorey, Terranella, & Shive, 1977). This appears not to be due to a deficiency or inadequate nutrients in the diet, but rather due to the fact that alcohol decreases food intake and depresses growth (Lieber et al., 1989).

Real-time bioluminescence recording of PER2::LUC expression in cultured liver explants revealed that the molecular clock in ED and LCD mice was shifted by ~11 h and ~6 h, respectively. No differences were observed in SCN explants, which is consistent with previous studies showing that, under CAF, an internal desynchrony exists between liver and SCN clocks (Filiano et al., 2013; Zhou et al., 2014). Interestingly, in the LCD group, the peak phases of PER2 expression showed a large variance, which were in agreement with the dissociation of behaviors we observed between feeding with locomotor activity.

The differences observed in the LCD mice in behavior and the phase of liver PER2 expression might be partly due to the higher sugar content of the Lieber-DeCarli diet: 30% sugar by weight, and mostly monosaccharides and disaccharides, as the chow diet contains much lower sugar content (2–5% sugar by weight, and the carbohydrate portion is mostly starch). It has been documented that animals with *ad libitum* access to sugar solution tend to drink throughout the day, including during their inactive period (daytime) (Avena, Rada, & Hoebel, 2008), which is consistent with what we observed in LCD feeding patterns. Also, the early rise in feeding activity observed in LCD mice at ZT9–ZT11 is somewhat consistent with a study of rats maintained under a ‘delayed pair-feeding system’ and with a morning food change (Israel, Oporto, & Macdonald, 1984): LCD rats exhibited an earlier pattern of consumption occurring during the light phase of the LD cycle, while ED rats fed progressively over the 24 h and with elevated consumption during the dark phase. However, comparison of this CAF work with the current study is limited. Israel et al. (1984) used a different feeding paradigm, where the analysis was not of feeding activity at the hourly resolution but of progressive diet consumption over the 24 h, and where LCD rats had reached 100% accumulated consumption by 8 h after introduction of food, and were thus forced to fast during the remaining 16 h of the LD cycle. Furthermore, in the current study, despite being fed *ad libitum*, fresh food was made available daily at ZT2–3, and we observed an increase in feeding activity after food changing in LCD mice. This is similar to the finding that rats fed with sugar intermittently increase their sugar intake during the first hour of daily access (Colantuoni et al., 2001).

Given the fact that meal frequency patterns determine the phase of mice peripheral clocks (Kuroda et al., 2012), it is plausible that altered feeding patterns due to high sugar in the LCD mice contributed to the observed phase differences within the mouse hepatic clock. Additionally, the shift in the ED mice feeding profile might be due to the addictive property of alcohol. It has been documented that with increased alcohol consumption in humans, normal drinking patterns are perturbed and extended into previously non-preferred times (Danel, Jeanson, & Touitou, 2003; Room et al., 2012). CAF rats show a reduction in nocturnal locomotor activity and withdrawal-induced hypothermia (Taylor et al., 2006). Moreover, core body temperature and locomotor activity are decreased in subjective night during repeated sessions of alcohol vapor intoxication in mice, and there was a trend toward greater hypothermia in mice commencing intoxication during the daytime (Damaggio & Gorman, 2014). In addition, CAF rats show a shift in sleep propensity from the light to dark phase of the LD cycle (Mukherjee & Simasko, 2009). Mice exposed to a rotational shift-work schedule to induce circadian desynchrony differentially altered free-access alcohol consumption behavior (Gamsby & Gulick, 2015) – the average alcohol feeding bout length

was reduced while the number of bouts per day increased, but bout activity characteristics of locomotor activity and of water consumption remained unaffected.

As shown in Fig. 5, RD-treated animals exhibit a tightly clustered temporal association between the CG of feeding and of locomotor activity, and the hepatic PER2 expression peak. The mean PER2::LUC peak phase of ZT17.9 is as predicted from published studies (van der Veen et al., 2012; Yoo et al., 2004). There was only a 2–4-h interval between these three events. For the ED group, a ∼4–h delay in feeding CG is associated with a ~6-h delay in locomotor activity CG, and a large ∼11–h delay in hepatic PER2 expression peak. However, the temporal sequence of events remained unchanged, with the phase marker for feeding occurring first, followed by locomotor activity, and then by PER2. Although there was no statistical difference found between the individual CG measures of LCD animals compared to RD animals in feeding and locomotor activity, a dissociation between overall activities was observed, and the effect of liquid diet on the phase of LCD hepatic PER2 expression was profound – the PER2 peak expression of LCD group fell into two distinct groups, leading to the mean phase of the PER2 rhythms falling at a time preceding the CG for both feeding and locomotor activity.

Circadian gene expression in the liver can be driven by both local and systemic signals, including changes in temperature and glucocorticoid (GC) signaling (Kornmann, Schaad, Bujard, Takahashi, & Schibler, 2007; Le Minh, Damiola, Tronche, Schütz, & Schibler, 2001; Saini, Morf, Stratmann, Gos, & Schibler, 2012; van der Veen et al., 2012). We speculate that altered rhythmicity in body temperature, humoral signaling, and the sleep-wake cycle may contribute to the phase entrainment of hepatic clock observed in ED-treated mice.

Several possible mechanisms could explain the altered locomotor and feeding rhythms in mice under CAF. Chronic alcohol consumption could possibly impact the master clock in the SCN (Prosser & Glass, 2014), thereby altering feeding and locomotor activity rhythms. For example, in rats a year-long alcohol exposure resulted in the depression of key SCN neuropeptides (Madeira et al., 1997), and a 2-week alcohol liquid diet altered the SCN rhythmic expression profiles of the clock genes *per2* and *per3* (Chen, Kuhn, Advis, & Sarkar, 2004). However, we observed no differences in SCN PER2 expression between ED and RD or LCD groups, suggesting that the phase of the SCN clock under entrained conditions is unaffected by the liquid diet regime or alcohol exposure (Filiano et al., 2013; Zhou et al., 2014). The SCN clock phase is insensitive to feeding cues, and in SCN-lesioned mice the liver clock entrains more rapidly to feeding cycles (Saini et al., 2013). It is therefore plausible that in our alcohol-feeding paradigm the SCN contributes through competing signals to provide a reduced response of the liver clock to changes in the feeding cycle. Alternatively, the observed alcohol effects upon the liver in the current study are generated independently of the SCN clock. As discussed above, chronic alcohol-induced body temperature change and fragmented sleeping patterns appear to shift behaviors, which likely contribute to the phase shift in the hepatic clock. Interestingly, the clock genes *per1*, *per2*, and *NPAS2*, that exhibit altered expression patterns in the steatotic liver under CAF (Zhou et al., 2014), have also been shown to contribute to the regulation of behaviors associated with feeding or alcohol preference: *per2* mutant mice exhibit enhanced alcohol

preference/intake (Spanagel et al., 2005), including increased free-choice ethanol drinking bouts during both the dark and light phases of the LD cycle (Prosser & Glass, 2014); *per1* mutant mice display an altered feeding rhythm (Liu et al., 2014); and *NPAS2* null mice exhibit delayed anticipatory locomotor activity in response to restricted feeding (Dudley et al., 2003). Lastly, a dampened plasma cortisol rhythmicity has been reported in chronic-alcohol liver disease patients (Rosman et al., 1982). Given the fact that GC signaling is a major Zeitgeber of peripheral tissues (Chung, Son, & Kim, 2011; van der Veen et al., 2012), it is possible that CAF might result in a shift or amplitude change in the GC rhythm through its modulation of the hypothalamic-pituitary-adrenal axis (Koob & Bloom, 1988; Uhart & Wand, 2009). This in turn might contribute to hepatic clock adjustment. These multiple factors (locomotor activity, temperature, feeding and nutrient signals, and GC signaling) could contribute, at least in part, to setting the liver clock phase. However, they are unlikely to fully explain the dramatic change in phase relationship in alcohol-fed mice that we observe between the liver and the SCN pacemaker and the environmental LD cycle.

In conclusion, in this study we reveal a clear effect of alcohol treatment upon feeding, locomotor activity, and hepatic PER2 expression. These three aspects in CAF animals are notably different from those of animals treated with *either* a normal chow diet or a liquid control diet. Moreover, the LCD (high in sugar content) -fed animals are also different from the RD-fed animals in these parameters. Notably, and compared to RD-fed mice, we observed two distinct patterns of behavior based upon our three parameters. For ED-treated mice, it is characterized by 1) a lower amplitude hepatic PER2 rhythm (Zhou et al., 2014), 2) a daytime peak of PER2 with large variance, that suggests that the hepatic clock is in anti-phase compared to normal animals, 3) increased variance in circadian phase of PER2 rhythms, 4) higher incidence of weak rhythmicity for feeding and locomotor activity with an absence of a distinct peak in activity when group analyzed, an increase in daytime feeding/locomotor activity which is associated with a delayed CG, and 5) very different phases for feeding activity, locomotor activity, and hepatic PER2 rhythm. For LCD-treated mice, it is characterized by 1) increased variance in circadian phase of hepatic PER2 rhythms, with PER2 peak profiles falling into two distinct groups, one set preceding and one delayed compared to the CG of feeding and locomotor activity, 2) a higher incidence of weak rhythms in feeding and locomotor activity, and 3) an increased occurrence of daytime feeding activity.

In summary, liquid diets, both *with* and *without* alcohol, are capable of altering diurnal patterns of feeding. However, highlighting the direct effect that alcohol has on hepatic circadian clock function is challenging. The observed effect, in this case the large hepatic phase differences that occur between the alcohol and control diet groups, could be a result of the alcohol alone or an interaction between the alcohol *and* exposure to the liquid diet that is its vehicle. Such a large change in phase relationship between central and peripheral oscillators, and the behaviors and physiological parameters they regulate, could have dramatic health implications. These data highlight the importance of careful interpretation and experimental design in animal studies using liquid diets in general and the Lieber-DeCarli diet in particular. Especially important are the implications to human alcohol

consumption and potential disturbances to internal circadian timing and to alcohol-induced liver damage/disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BAC	blood alcohol concentration
CAF	chronic alcohol feeding
ED	ethanol-containing diet
LCD	liquid control diet
RD	regular chow diet
LD	light:dark
ZT	Zeitgeber time
SCN	suprachiasmatic nucleus
BA	bile acid
TG	total cholesterol
FA	fatty acid
PIR	passive infrared
PER2::LUC	PERIOD2::LUCIFERASE
CG	center of gravity

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- Diurnal feeding pattern was found altered in alcohol and liquid control diet fed mice
- The diurnal pattern of locomotor activity was found altered in the alcohol fed mice
- Liver PER2, feeding and locomotor activity were dissociated in liquid diet fed mice
- Highlights the importance of interpretation and experiment design using liquid diet

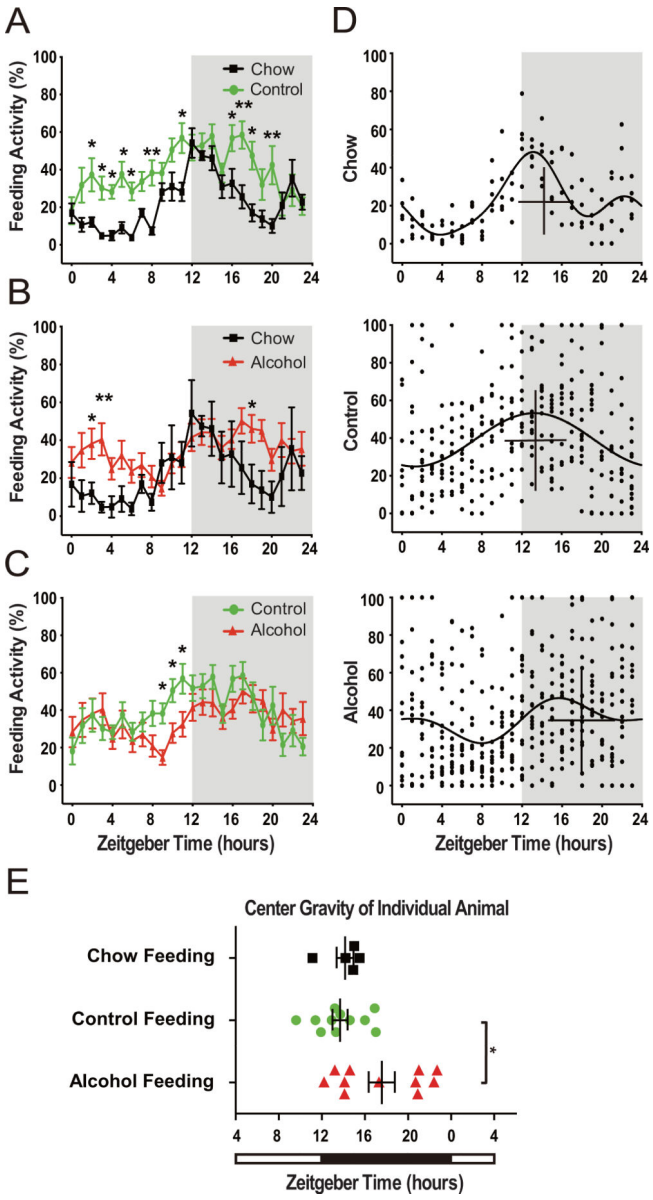

Fig. 1. Daily feeding activity profile of mice fed an alcohol, liquid control, or chow diet

Daily feeding activity profile **A)** of chow-fed (RD) (n = 5) and control-fed (LCD) (n = 13) mice; **B)** of RD and alcohol-fed (ED) (n = 16) mice; **C)** of LCD and ED mice. Values are mean ± S.E.M. RM-ANOVAs were performed followed by Tukey's *post hoc* tests, **p* < 0.05, ***p* < 0.01. **D)** Corresponding CircWave analysis of RD, LCD, and ED mice groups. In the graph, each dot represents the level of activity for each animal in time-specific groups, the curve represents the best-fit Fourier curve, the Center of Gravity is represented by the vertical bar, and the mean of the entire data set is represented by the cross. **E)** Center of gravity (CG) of individual animal feeding activity of RD (n = 5), LCD (n = 11) and ED (n = 11). CG was generated from CircWave, and only animals with rhythmic activity were used. Values are mean ± S.E.M. One-factor ANOVAs were performed followed by Tukey's *post hoc* tests, **p* < 0.05.

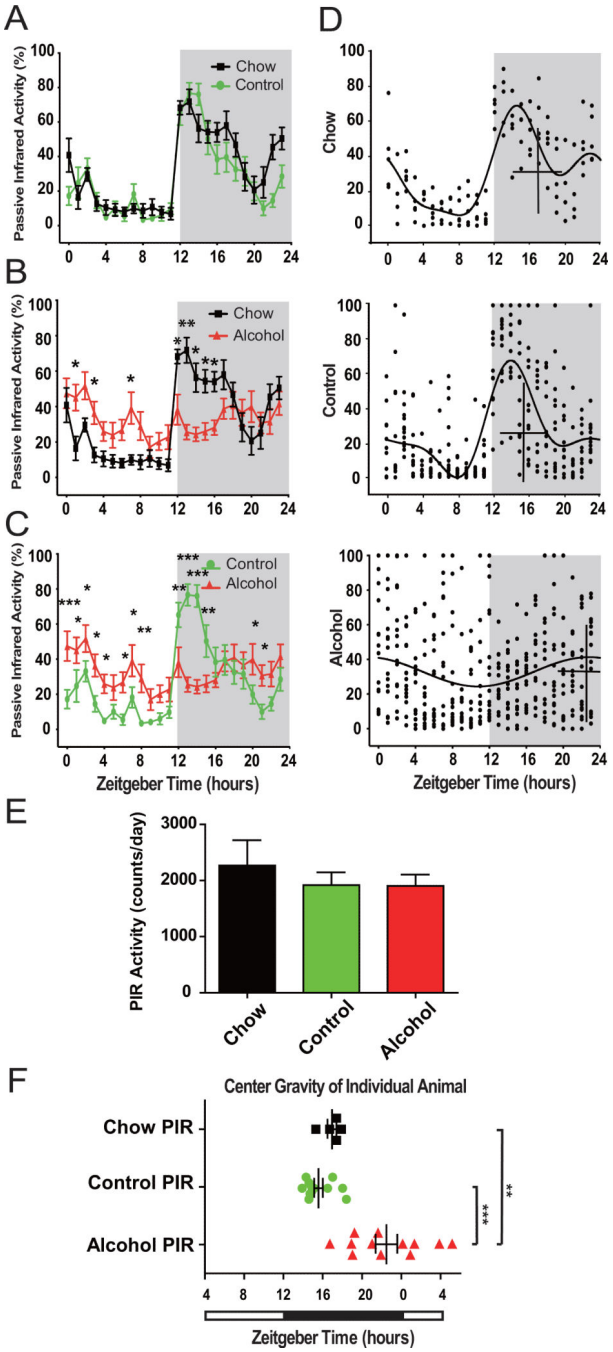

Fig. 2. Daily locomotor activity profile of mice fed an alcohol, liquid control, or chow diet

Daily locomotor activity profile **A)** of chow-fed (RD) (n = 5) and liquid control-fed (LCD) (n = 13) mice; **B)** of chow-fed (RD) and alcohol-fed (ED) (n = 16) mice; **C)** of LCD and ED mice. Values are mean ± SEM. RM-ANOVAs were performed followed by Tukey's *post hoc* tests, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. **D)** Corresponding CircWave analysis of chow-fed, liquid control-fed, and alcohol-fed mice groups. **E)** Total daily general activity counts in chow-fed, liquid control-fed, and alcohol-fed mice determined by passive infrared motion detectors (one-factor ANOVA; n.s.). **F)** CG of individual animal PIR activity of RD

(n = 5), LCD (n = 12) and ED (n = 12). Values are mean ± S.E.M. CG was generated from CircWave, and only animals with rhythmic activity were used. One-factor ANOVAs were performed followed by Tukey's *post hoc* tests, $^{**}p < 0.01$, $^{***}p < 0.001$.

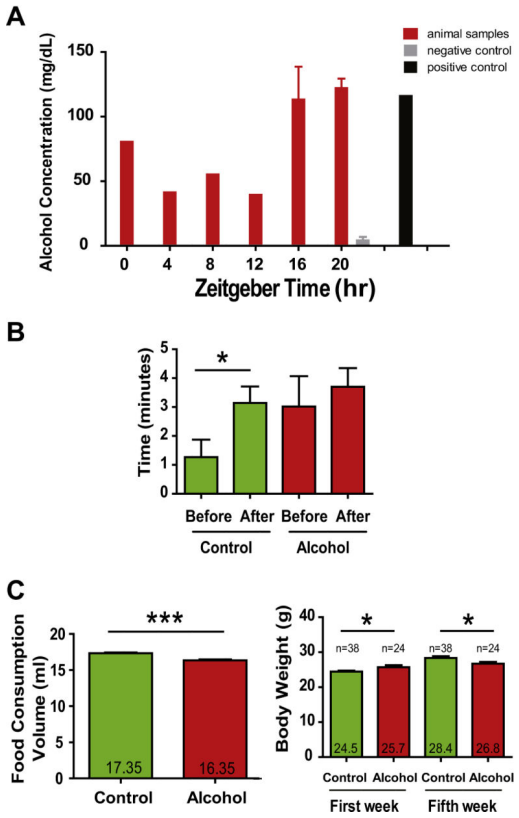

Fig. 3. Biochemical and physiological measurements of mice fed with liquid control or alcohol diet

A) Blood alcohol concentration in ED. n = 1 (ZT0–ZT12); n = 2 at ZT16 and ZT20. Data are presented as mean ± SD. LCD (n = 2) mice are set as negative controls. Alcohol control set (ammonia) as a positive control. **B)** Measurement of feeding time scored during the 1 h before and 1 h after food changing (ZT2–3). **C)** Left: Food consumption volume per day of ED (n = 25) and LCD (n = 38) mice. Right: Body weight measurement of ED (n = 24) and LCD (n = 38) mice before the diet and after 5 weeks of diet entrainment. Data are presented as mean ± SEM. Student's *t* test (*$p < 0.05$, ***$p < 0.001$).

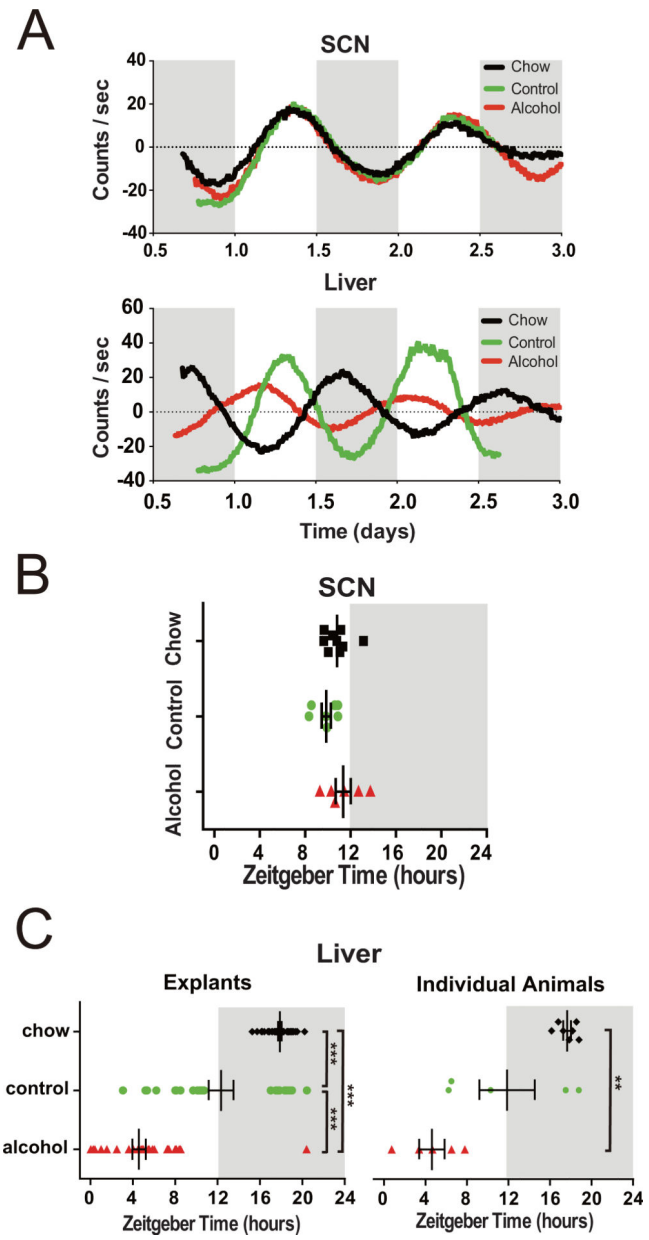

Fig. 4. Bioluminescence analysis of suprachiasmatic nucleus (SCN) and hepatic PERIOD2 protein expression

A) Circadian expression of PER2::LUC protein from a representative culture of SCN or liver explants from chow-, liquid control-, and alcohol-fed mice. Data plotted as baseline subtracted. **B)** Peak phase distribution of SCN explants from chow- (RD), liquid control- (LCD), and alcohol-fed (ED) mice (one-factor ANOVAs, n.s.). **C)** Left: peak phase distribution of liver explants PER2 expression from RD, LCD, and ED mice. Right: peak phase distribution of individual animal hepatic PER2 expression. Values are means ± S.E.M. One-factor ANOVAs were performed followed by Tukey's *post hoc* tests, $^{**}p < 0.01$, $^{***}p < 0.001$.

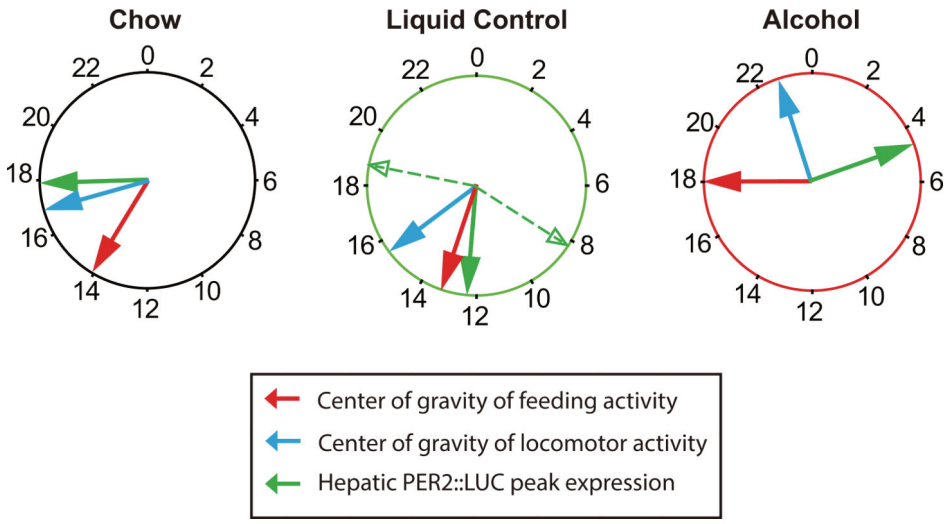

Fig. 5. Summary of the effect of Lieber-DeCarli diet (LCD and ED) on animal behavior and hepatic circadian system compared to normal chow diet

The mean feeding and locomotor CG or PER2 peak phase of each animal group are indicated by the arrows within the 24–h circle. The PER2 peak expression of the LCD mice fell into two distinct groups. The means of these groups are shown by the two dash-lined/ open arrows. Numbers on outside of circle are Zeitgeber time (h).