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# Ethanol consumption in mice: relationships with circadian period and entrainment

**Jennifer L. Trujillo**[1], **David T. Do**[1], **Nicholas J. Grahame**[2], **Amanda J. Roberts**[3], and **Michael R. Gorman**[1,4]

[1]Department of Psychology and Center for Chronobiology, University of California, San Diego, CA 92093 USA

[2]Indiana University Purdue University at Indianapolis Department of Psychology, Indianapolis, IN 46202 USA

[3]Molecular & Integrative Neurosciences Department, The Scripps Research Institute, La Jolla, CA 92037 USA

## Abstract

A functional connection between the circadian timing system and alcohol consumption is suggested by multiple lines of converging evidence. Ethanol consumption perturbs physiological rhythms in hormone secretion, sleep and body temperature, and conversely, genetic and environmental perturbations of the circadian system can alter alcohol intake. A fundamental property of the circadian pacemaker, the endogenous period of its cycle under free-running conditions, was previously shown to differ between selectively bred High- (HAP) and Low- (LAP) Alcohol Preferring replicate 1 mice. To test whether there is a causal relationship between circadian period and ethanol intake, we induced experimental, rather than genetic, variations in free-running period. Male inbred C57Bl/6J mice and replicate 2 male and female HAP2 and LAP2 mice were entrained to light:dark cycles of 26 h or 22 h or remained in a standard 24 h cycle. Upon discontinuation of the light:dark cycle, experimental animals exhibited longer and shorter free-running periods, respectively. Despite robust effects on circadian period and clear circadian rhythms in drinking, these manipulations failed to alter the daily ethanol intake of the inbred strain or selected lines. Likewise, driving the circadian system at long and short periods produced no change in alcohol intake. In contrast with replicate 1 HAP and LAP lines, there was no difference in free-running period between ethanol naïve HAP2 and LAP2 mice. HAP2 mice, however, were significantly more active than LAP2 mice as measured by general home-cage movement and wheel running, a motivated behavior implicating a selection effect on reward systems. Despite a marked circadian regulation of drinking behavior, the free-running and entrained period of the circadian clock does not determine daily ethanol intake.

[4]Correspondence Michael Gorman, Department of Psychology, 9500 Gilman Drive, La Jolla, CA 92093-0109, Tel: 858-822-2466, Fax: 858-534-7190, ; Email: mgorman@ucsd.edu

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## Introduction

In all of the mammalian species in which it has been studied, the suprachiasmatic nuclei (SCN) of the anterior hypothalamus function as a daily, or circadian, clock that exerts a marked influence on myriad aspects of physiology and behavior (Liu et al., 2007). At the cellular level, circadian rhythms are generated by interacting transcriptional, translational feedback loops of a few dozen genes including 3 homologs of the period (*per*) gene, so named because point mutations in *Drosophila* altered the circadian cycle length (i.e., period) under constant environmental conditions. While circadian rhythmicity driven by clock gene expression can be seen in both the SCN and in tissues throughout the brain and body, only the rhythms in the SCN are self-sustaining. This master pacemaker thus sits atop a hierarchy where it orchestrates the circadian organization of multiple physiological systems below (Albrecht, 2006; Liu et al., 2007; Yamazaki et al., 2000). The circadian timing system has proven relevant to a wide array of health conditions (Maywood et al., 2006). For example, shift-work that requires people to time their sleep and activity counter to the preferred phase of their circadian clock has been recently classified by the World Health Organization as a probable carcinogen (IARC, 2008). Conversely, incorporation of circadian timing considerations can improve cancer treatment outcomes of chemotherapy by optimizing therapeutic and minimizing toxic actions of drugs (Hrushesky, 1993; Rivard et al., 1985).

The biology of alcohol consumption, likewise, displays a pronounced circadian organization (Rosenwasser, 2001; Spanagel et al., 2005b; Wasielewski and Holloway, 2001). Alcohol consumption in the general human population, for instance, peaks early in the evening, whereas alcohol-dependent subjects report greatest cravings in the morning (Arfken, 1988; Danel et al., 2003). Ethanol acts on numerous physiological systems that are strongly rhythmic (e.g., sleep, body temperature, melatonin) (Danel et al., 2001; Landolt et al., 1996; Rupp et al., 2007) and produces different effects as a function of time of day (Danel et al., 2001). Further, the chronotype of humans, morning “larks” versus evening “owls”, predicts alcohol intake, with greater consumption reported by evening types (Adan, 1994; Wittmann et al., 2006). Shift-workers too have been reported to have increased alcohol consumption or risk for heavy drinking although not consistently across studies (Hermansson et al., 2003; Webb et al., 1990). Among abstinent alcoholics, relapse is predicted by the degree of persistent disruption of the sleep/wake cycle (Drummond et al., 1998). Finally, a single nucleotide polymorphism in the *per2* gene reportedly associates with elevated alcohol intake among a population of human alcoholic subjects (Spanagel et al., 2005a).

Important aspects of the temporal organization of human alcohol consumption are reproduced in rodents, making them ideal subjects for experimental assessment of causal relationships between circadian function and ethanol biology. Mice and rats express pronounced daily rhythms in voluntary alcohol intake and time-dependent responses to ethanol (Baird et al., 1998; Freund, 1970; Trujillo et al., 2009). Repeated shifting of the rat

circadian pacemaker can alter voluntary alcohol consumption (Clark et al., 2007). A null mutation of the *per2* clock gene likewise increases ethanol consumption in mice (Spanagel et al., 2005a). In both rats and mice, artificial selection for high versus low alcohol preference has produced line differences in circadian period measured by wheel running under constant environmental conditions (Hofstetter et al., 2003; Rosenwasser et al., 2005b). Because of its hierarchical nature, however, it is difficult to know at which level of physiological organization that the circadian system is implicated in these effects. The genetic studies raise the possibility of a direct causal relationship between fundamental mechanisms of circadian pacemaker function and an alcohol consuming phenotype. Alternatively, effects on alcohol consumption could occur downstream of the pacemaker on, for example, reward or arousal processes, that have a circadian character (McClung, 2007). Finally, altered entrainment or perturbation of the circadian system may act as a chronic non-specific stressor (i.e., introduce a general allostatic load; Boulos and Rosenwasser, 2004) that could induce changes in drinking behavior.

Circadian biologists have a number of analytical tools with which to assess the nature of circadian influence on physiology and behavior (Daan and Aschoff, 2001; Dunlap et al., 2004). In the absence of temporal cues from the environment, circadian rhythms “free-run” with an endogenous period, τ. The light:dark cycle, however, typically synchronizes (i.e., entrains) the endogenous rhythm to match the 24 hour (h) day by resetting the clock daily to offset any discrepancy between τ and 24 h. Because light can reset the clock earlier or later depending on when it falls in the endogenous cycle, animals can entrain to a range of environmental periods both somewhat longer and shorter than 24 h using so-called T cycles, where T indicates the period of the entraining environmental cycle (e.g., T26 indicates alternating 13 h of light and 13 h of dark). The phase dependence of light’s actions further implies that the phase of the entrained rhythm can be varied systematically: as T lengthens, the endogenous rhythm adopts a progressively earlier alignment with the lighting cycle (i.e., animals become more like “larks”) expressed in circadian terminology as a “phase angle of entrainment” (see methods for precise definition). T cycles may also be used to influence the endogenously expressed free-running period, τ. Transfer to constant conditions from an entraining long T cycle produces a τ that is longer than observed after transfer from an entraining short T cycle. Such period after-effects may persist for at least a month in rodents (Pittendrigh and Daan, 1976).

Using T cycles to induce long-term changes in the functional organization of the circadian system of mice, we tested two hypotheses suggested by epidemiological and correlational studies in humans and rodents: first, that there is a causal relationship between the period of the free-running circadian pacemaker and ethanol intake in C57BL/6J mice; second, that there is a causal relationship between the phase angle of entrainment and alcohol consumption in C57BL/6J mice. Finally, we assessed whether aspects of circadian rhythmicity in addition to the free-running period and phase angle of entrainment distinguished high-alcohol preferring (HAP2) and low-alcohol preferring (LAP2) mice (Grahame et al., 1999; Grahame et al., 2003) not yet studied from a circadian perspective. We provide strong evidence against a direct connection between circadian period or entrainment phase and alcohol intake in mice. Instead, we confirm an association between high alcohol preference and activity levels in these genetically distinct mice.

## Materials and Methods

### Subjects and housing

Male C57BL/6J mice (Jackson Laboratories, Sacramento CA) and male and female HAP2 and LAP2 were acquired and housed in standard shoebox cages with food (Mouse diet 5015, Purina Mills) and water available ad libitum. The latter lines were selected for differences in alcohol drinking from the same progenitor population, and using the same phenotype (free-choice consumption of 10% ethanol over a 4 week period) as replicate 1 HAP and LAP mice that showed a difference in free-running period (Hofstetter et al., 2003). Subjects were group housed prior to circadian rhythm or ethanol intake measurement but were moved to individual housing for those measures where they remained for the duration of the experiments. All procedures and animal care were approved by the Institutional Animal Care Use Committee at University of California, San Diego and conducted in accordance with the Guide for the Care and Use of Laboratory Animals.

**Lighting cycles—**Prior to experimentation, animals were maintained on 24 h light:dark cycle with 12 h of light and 12 h of dark (LD12:12). Throughout all experiments, the “light” phases were lit by fluorescent bulbs generating an illuminance of approximately 100 lux, whereas “dark” phases were dimly illuminated with red light (< 1 lux). For all 24 h and non-24 h light cycles (T cycles, where T indicates the cycle period) the lengths of the light and dark cycles were always kept equal (e.g., T22 was 11 h of light and 11 h of dark; T26 was 13 h of light and dark). At transitions between lighting conditions, the timing of lights on was preserved to ensure no sudden phase shifts, and transitions to constant darkness always occurred at the normal time of lights off. For all experiments, exposures to T22 and T26 were preceded by one week of T23 or T25, respectively.

**24 Hour 2 Bottle Choice—**A 10% (w/v) ethanol solution was prepared using 95% ethyl alcohol and water; a separate water bottle was also prepared for the procedure. Fluids were presented to mice in 50 ml conical tubes fitted with sipper tubes. In experimental conditions where alcohol licking was recorded, location of alcohol and water bottles remained constant throughout alcohol exposure; in all other experimental conditions alcohol and water bottle locations were alternated weekly. Bottles stayed on 7 days/week, were checked daily and were changed and weighed 2 times per week (and divided by number of days to obtain a 24 hour average) to determine g/kg intake. During periods of alcohol exposure, animals were weighed at least every 2 weeks. Alcohol intake values were calculated both on a 24 h basis and per circadian cycle when animals were free-running in DD. Because in no cases did adjustment for period alter the results of the statistical tests, all intake measures are reported per 24 h. Ethanol preference was calculated as proportion of ethanol to total liquid consumption. Ethanol intake and preference values were calculated separately for each week of the experimental manipulation. As these measures produced parallel results, only the intake values are presented in repeated measures ANOVAs.

**Activity and licking monitoring—**General activity or ethanol licking activity was recorded continuously and compiled into 6 min bins by Vital View software (Mini Mitter, Bend, OR). When an animal licked at a drinking bottle it closed an electrical contact

between a metal stage (8×13 cm) on the cage floor and an electrode connected to the bottle spout. For recording general activity, the latter electrode was moved from the water bottle to the wire cage lid. Thus, whenever the animals stood on the metal stage and contacted any part of the wire lid, the electrical contact was closed. This occurred during feeding, climbing, rearing and apparently undirected movement within the cage (informal observation), but only when the animal was in approximately half of the cage. Contact with the lid occurred also during drinking but not as a result of licking the bottle, per se. Regardless of recording condition, the cage configuration was identical, but limitations in recording capacity prevented concurrent recording of both measures.

## Procedures

Figure 1A–C schematically represents the manipulations employed in Experiments 1–3.

**Experiment 1—**Male C57BL6/J mice (n = 23), 10 weeks of age, were exposed to light:dark cycles of 22, 24, or 26 h duration (T22, T24 and T26, respectively; Fig. 1A). After three weeks, animals were singly housed and cages were equipped with general locomotor activity sensors and lick monitors. The bright lights were permanently extinguished for assessment of their free-running circadian rhythms in continuous dim red (DD) over the next 28 days. At the onset of DD, animals were also provided ethanol in a 24 h two bottle choice protocol for the next 4 weeks. Licking was recorded except during two intervals (2 days and 5 days) when general activity was recorded instead (see Fig. 2).

**Experiment 2—**Male C57BL6/J mice (n = 40), 10 weeks of age, were entrained to T22, T24 or T26 cycles for 3 weeks (Fig. 1B). Subsequently, mice either continued in T22, T24 or T26, or were transferred from T22 to T24 or from T26 to T24 (n = 8/group) at which point they were allowed ethanol in a 24 h two bottle choice paradigm for 30 days. Ethanol licking activity was continuously recorded throughout the ethanol exposure phase.

**Experiment 3—**Male and female HAP2 (n = 30) and LAP2 (n = 28), 16 weeks old, were retained in T24 or allowed to free run in DD for assessment of the free-running period of general activity in the absence of ethanol (Fig. 1C). Subsequently, mice remained under their respective lighting conditions for an additional 21 days with the addition of 24 h two bottle choice, during which ethanol consumption was measured volumetrically. Ethanol was removed from all animals, and mice that had been in DD were returned to T24 for 2 weeks for re-entrainment. These mice, which had free-run previously, were then randomly assigned to be entrained by long (T26) or short (T22) cycles. After 3 weeks, these mice were again exposed to DD and all mice received 24 h two bottle choice for 21 days. Rhythms of general activity were recorded for all animals throughout the experiment except for controls exposed continuously to T24. For these control mice, general activity was recorded during the first alcohol naïve portion of the experiment only. Thereafter only alcohol licking behavior was assessed.

**Experiment 4—**Male HAP2 (n=14) and LAP2 (n=13) mice were retained from Experiment 3, and re-entrained to T24. At 42 weeks of age, they were transferred to cages equipped with running wheels (13 cm diam) and activity rhythms were recorded over 13

days. Subsequently, these mice were put into DD to freerun for another 13 days for assessment of the free-running rhythm. Two HAP2 males died before the end of data collection.

### Data Analyses

Circadian measures were evaluated using ClockLab software (Actimetrics, Wilmette, IL). Periods of free-running and entrained activity and/or licking rhythms were calculated with chi-square periodogram analysis that determined the best fit of the data at all 0.1 h intervals from 20–30 h. From the periodogram analysis, the statistical power (Q) of the best fitting period was additionally recorded, as this is a quantitative measure of rhythm robustness (Refinetti, 2006; Sokolove and Bushell, 1978). In cases where there was no periodicity detected with $\alpha$ set to 0.001, the animal's period and power data were not included in that analysis. To assess the importance of rhythm phase in Experiment 2, activity profiles were generated by averaging data over 22, 24 or 26 h, respectively, and smoothed with an 18 minute moving average (data from three, 6-min bins). From these smoothed profiles, the onset of elevated drinking was defined as the earliest point that drinking exceeded the daily mean and was sustained for 18 min. These onsets were converted to a phase angle of entrainment, which is defined as the difference expressed in hours between the time of lights off and drinking onset. Negative values indicate that drinking precedes dark onset.

Data were analyzed using univariate and repeated measures ANOVAs run in JMP 7.0 (SAS Institute, Cary, NC). Values for each week of the manipulation were entered as repeated measures. For Experiments 1 and 2, lighting manipulations were the only between subject factors. In Experiment 3, lighting manipulations, sex, and line and all interactions of these factors were assessed in each statistical model. Because after the introduction of alcohol, licking instead of general activity was measured in mice maintained as controls in T24, this lighting condition was not included in circadian analyses after the first phase of the experiment during which activity was recorded from all animals.

## Results

### Experiment 1

Entrainment to T cycles produced strong after-effects on the period of the free-running general activity and licking rhythms in DD (Fig. 2A–D; $F_{2,20} = 47.8$; $p < 0.001$). All groups differed significantly from one another ($p < 0.05$, Tukey test). Visual inspection of actograms at transitions between licking and general activity confirmed previous findings that these rhythms closely parallel one another. Despite a mean difference in circadian period of over 0.6 h between conditions, there were no significant group differences in ethanol intake (Fig. 2E; $F_{2,20} = 0.2$; $p > 0.7$) or in ethanol preference (Fig. 2F; $F_{2,20} = 0.05$; $p > 0.9$). Repeated measures ANOVA revealed no effect of week ($F_{3,18} = 0.6$; $p > 0.6$) or week by entrainment interaction ($F_{3,19} = 2.6$; $p > 0.08$) on daily average ethanol intake across weeks (data not shown).

## Experiment 2

Indicating successful entrainment, the periods of the licking rhythms determined by periodogram analysis matched the periods, T, of the imposed lighting schedules (22, 24 or 26 h; data not shown). Additionally, as predicted by circadian entrainment theory, the relative phasing of the behavioral and environment rhythms varied with the T cycle (Fig. 3A–C, G; $F_{4,35} = 48.4$, $p < 0.001$): In T22, drinking onset occurred several hours into the dark period whereas drinking onset was earliest in T26. These measures differed significantly from values in T24 ($p < 0.01$, Tukey test). The three groups in T24 did not differ as a function of their prior T cycle history (Fig. 3G). The offset of nocturnal drinking also was advanced in T26 versus T22, whereas the bimodality of the drinking rhythm was lost in T22 (Fig. 3A). Despite these differences in entrainment, there were no differences among the five groups of animals in alcohol intake (Fig. 3H; $F_{4,35} = 0.6$; $p > 0.6$) or preference (Fig. 3I; $F_{4,35} = 1.6$; $p > 0.20$) over the entire period of alcohol exposure. Repeated measures ANOVA showed a main effect of week on daily average ethanol intake ($F_{3,33} = 5.6$; $p < 0.01$; data not shown), driven by higher alcohol intake in the first week of the exposure. There was no significant interaction of entrainment condition by week ($F_{4,35} = 1.5$; $p > 0.22$).

## Experiment 3

**Activity rhythms in T24 versus DD—**Prior to any alcohol exposure, general activity rhythms of mice in DD free-ran with a period less than 24 h as expected whereas mice in T24 had periods near 24 h, producing a main effect of lighting condition (Fig. 4A; $F_{1,49} = 27.2$, $p < 0.001$). There were no main effects of or interactions with either line or sex. In contrast, the amount of activity was significantly greater in HAP2 than versus LAP2 mice (Fig. 4B; $F_{1,50} = 48.8$, $p < 0.001$) and in females versus males ($F_{1,50} = 4.1$, $p < 0.05$). Female mice showed a greater difference in activity counts between LD and DD than did males (sex × lighting condition interaction, $F_{1,50} = 8.4$, $p < 0.01$) and this effect was greater for HAP2 than LAP2 mice (sex × strain × lighting condition interaction, $F_{1,50} = 6.3$, $p < 0.05$). Rhythm power (Q) was higher in HAP2 versus LAP2 mice (Fig. 4C; $F_{1,49} = 16.2$, $p < 0.001$) and was higher in LD compared to DD ($F_{1,49} = 4.6$, $p < 0.05$). Representative actograms of HAP2 and LAP2 females free-running in DD are shown in Figures 4D, E.

**Ethanol consumption in T24 versus DD—**In the first interval of ethanol exposure, HAP2 mice consumed significantly more ethanol daily than did LAP2 mice (Fig. 5A; $F_{1,50} = 152.9$; $p < 0.001$); female mice drank more than male mice ($F_{1,50} = 13.4$, $p < 0.001$); and mice drank more in LD than in DD ($F_{1,50} = 8.0$, $p < 0.01$). The sex difference in intake was greater for HAP2 than in LAP2 mice ($F_{1,50} = 7.7$, $p < 0.01$) and was more pronounced in LD than in DD ($F_{1,50} = 4.6$, $p < 0.05$). Similarly, the line difference was reduced in DD compared to LD ($F_{1,50} = 5.2$, $p < 0.05$). For alcohol preference, HAP2 mice had higher values than did LAP2 mice (Fig. 5B; $F_{1,50} = 126.9$, $p < 0.001$) and females higher than males ($F_{1,50} = 4.9$, $p < 0.05$), but lighting condition exerted no effect. Repeated measures ANOVA indicated no significant differences in average daily ethanol intake across the 4 measurement epochs ($F_{3,48} = 2.0$; $p > 0.13$).

**Free-running rhythms during alcohol consumption—**Analysis of circadian parameters of mice freerunning in DD after the introduction of alcohol yielded several main effects of line: HAP2 mice exhibited longer periods (Fig. 5C; $F_{1,35} = 7.7$; $p < 0.01$), more activity (Fig. 5D; $F_{1,35} = 83.4$; $p < 0.001$) and higher power rhythms (Fig. 5E; $F_{1,35} = 10.0$; $p < 0.01$) compared to LAP2 mice. There were no significant effects of sex.

**Licking patterns of HAP2/LAP2 mice in T24—**Even though the data were collected over different intervals, the alcohol licking rhythms of HAP2 and LAP2 mice maintained in T24 paralleled rhythms of general activity, albeit at lower amplitude (Fig. 6). Sex and strain differences in rhythm amplitudes (see above) are reflected in the differential scaling of their respective ordinates. A scaling ratio of 1:5 (licks to activity counts) produced a close match of the two rhythms for HAP2 female and males, whereas LAP2 animals licked proportionately less per activity count. Qualitatively, the shape of the two rhythms was more similar in LAP2 than in HAP2, with the latter showing divergence between activity and licking late in the night.

**Activity rhythms in DD following T cycles—**As it did with C57 mice, T cycle entrainment produced significant period after-effects in DD (Fig. 7A). Following transfer from T22 to DD, during which animals also had access to alcohol, period was shorter than after transfer from T26 ($F_{1,29} = 96.1$, $p < 0.001$), and period was longer in HAP2 than in LAP2 ($F_{1,29} = 6.6$, $p < 0.05$). Activity levels were also higher for HAP2 animals (Fig. 7B; $F_{1,31} = 27.3$, $p < 0.001$). The prior T cycle had a differential effect on male versus female activity levels ($F_{1,31} = 6.8$; $< 0.05$), more so for HAP2 than for LAP2 ($F_{1,31} = 9.2$; $p < 0.01$). Rhythm power was again greater for HAP2 versus LAP2 mice (Fig. 7C; $F_{1,29} = 11.3$, $p < 0.01$), but no other effects were significant.

**Drinking in DD after T cycles—**Despite large effects on period (see below), the prior T cycle again had no effect on alcohol intake (Fig. 7D; $F_{2,45} = 0.4$, $p > 0.65$) or preference (Fig. 7E; $F_{2,45} = 0.7$; $p > 0.49$) and interacted with no other variable. For both intake and preference, the main effects of line and of sex seen in the first alcohol exposure (Fig. 5) were replicated as was the sex × line interaction on intake. Repeated measures ANOVA revealed that ethanol intake varied with time, peaking in the second week and thereafter declining ($F_{3,43} = 4.0$; $p < 0.05$), but no other variable interacted with time.

## Experiment 4

In T24, male HAP2 mice had significantly greater wheel running activity per day versus LAP2 male mice (Table 1; $F_{1,25} = 21.2$; $p < 0.001$). Furthermore, activity rhythms of HAP2 mice also showed greater periodogram power ($F_{1,25} = 8.0$; $p < 0.01$) than those of LAP2 mice. The phase angle of entrainment to the 24-hour cycle, however, did not differ between strains ($F_{1,24} = 0.1$; $p > 0.7202$). In DD, differences in wheel-running activity characteristics between HAP2 versus LAP2 mice were again observed. HAP2 mice again showed greater wheel running activity per day ($F_{1,23} = 10.7$; $p < 0.01$) and higher power than LAP2 mice ($F_{1,21} = 4.5$; $p < 0.05$). However, there was no significant difference between the HAP2 and LAP2 period ($F_{1,22} = 0.0001$; $p > 0.99$).

## Discussion

Evidence suggesting a deep mechanistic connection between the circadian clockwork and alcohol consumption includes circadian differences between high and low-preferring lines of rodents (Hofstetter et al., 2003; Rosenwasser et al., 2005b), *per2* SNP associations with human alcoholism (Spanagel et al., 2005a), and contributions of clock genes to physiological processes (e.g., sensitization, reward) that have been implicated in addiction (Abarca et al., 2002; McClung, 2007). The present study, however, unambiguously establishes that gross experimental manipulation of the most fundamental clock property -- its endogenously expressed period -- produces no change in alcohol intake or preference in three mouse genotypes: under constant darkness, prior T cycles produced robust group differences in circadian period of 0.6 h of C57 and of 1.0 h in both HAP2 and LAP2 mice. In no case, however, was there any evidence of either increases or decreases in ethanol intake or preference. Driving the pacemaker at even more extreme periods – 22 and 26 h – with the consequent changes in phase angle of entrainment, also had no effect on alcohol consumption. This lack of effect on alcohol drinking was seen across a variety of baseline alcohol consumption levels, including low (LAP), moderate (C57), and high (HAP). The absence of effects is not attributable to our manipulations being too subtle because our induced period after-effects were markedly greater than period differences between selected strains. Nor were null effects on drinking due to low statistical power, because we were able to discern effects of sex, other dimensions of the lighting condition and their interaction. In short, alcohol intake is very stable and well conserved across these circadian manipulations. Thus, we can conclude that prior associations between circadian period and phase on the one hand, and ethanol intake or preference on the other, despite repeated co-occurrence, are not likely to be causal. Instead, these associations may reflect selection artifacts and/or pleiotropic actions of genes.

Aside from prior empirical associations, there are several reasons why a causal relationship between circadian period and alcohol intake could be reasonably expected. The first relates specifically to lighting. Gross experimental manipulation of the lighting cycle (e.g., constant light, constant dark) reportedly alters the drinking behavior of rats (Burke and Kramer, 1974; Geller, 1971; Geller and Purdy, 1979), although a lack of adequate control groups (Sinclair and Geller, 1972) leaves these studies open to alternative interpretations. In general support of this proposition, Experiment 3 showed increased drinking of HAP2 and LAP2 mice in T24 compared to DD (Fig. 4), an effect of light that is opposite in direction to that reported in rats. This effect could be either a direct action of light or a secondary consequence of pacemaker entrainment. Arguing against a direct effect of light during the daytime, we previously reported equivalent drinking behavior in male C57 mice kept under a normal 12 h light phase and those under “skeleton” photoperiods (Trujillo et al., 2009). In the latter case, entrainment is maintained by 1 h light pulses at dawn and dusk that replace the entire 12 h light phase, which is otherwise dark. Conversely, breaking up daily presentation of 12 h of light into two 6 h epochs decreased consumption in this same strain (Millard and Dole, 1983). Because this manipulation alters the manner in which the circadian system is entrained, at least some actions of light depend on their interaction with the circadian clock.

Actively driving the pacemaker at periods of 22 or 26 hours as in Experiment 2 induces differential engagement of the circadian pacemaker by light. In the former case, entrainment requires the endogenous period (~24 h) to be shortened, and this is achieved by light falling late in the subjective night to produce daily phase advances of ~ 2 h. For entrainment to T26, light must fall in the early subjective night to produce daily ~ 2 h delays. The mechanisms and consequences of phase advances and delays, moreover, differ markedly (Illnerova, 1991; Yan and Silver, 2004): for example, the circadian pacemaker requires longer to return to its steady state after advances than after delays, and repeated exposure to advances but not delays accelerates mortality in aged mice (Davidson et al., 2006). T22 mimics the “owl” chronotype associated with higher alcohol intake in humans and exposes mice to health compromising phase advances, whileT26 mimics the “lark” chronotype through less stressful phase delays. Thus, although this and prior studies establish entrainment-mediated effects of light on alcohol intake (Fig. 5; Millard and Dole, 1983), the relative phasing of light and the pacemaker is of no measurable significance to male C57 mice. A subsidiary hypothesis – that an abrupt transition between entrainment periods would alter alcohol intake – was also not supported.

The effects of the lighting cycle (versus DD) in Experiment 3 differed by sex and line, with HAP2 females drinking nearly twice as much in T24, and males little affected. Sex affects myriad aspects of ethanol response in rodents (Devaud et al., 2003), and sex by lighting interactions specifically have been reported in HAD1 rats under a repeating jetlag protocol (Clark et al., 2007). In HAP2 mice, as well, sex can determine the influence of external factors (e.g., stress) on ad libitum alcohol consumption (Chester et al., 2006).

A second reason for expecting a connection between circadian period and alcohol intake relates to the temporal organization of multiple physiological systems under SCN control. Just as the light/dark cycle entrains the master pacemaker, the SCN entrains the oscillatory function of a vast array of tissues through diverse signals via both humoral and neural mechanisms (Guo et al., 2006; Liu et al., 2007). Thus, cells in the heart, liver, spleen, thymus, esophagus, kidney, etc. are themselves rhythmic and the phase relation between these various tissues is determined by the SCN (Guo et al., 2006; Yamazaki et al., 2000). Both entrainment theory and empirical results establish that period after-effects would produce changes in phase relationships among peripheral oscillators (Molyneux et al., 2008). As temporal ordering in the internal milieu is of profound significance for physiological organization (Hrushesky, 1993), rearrangements of temporal order would be considered by many chronobiologists to be a form of circadian disruption and thus a general physiological stressor. Because of the formal properties of entrainment mechanisms, even 0.5 to 1.0 h changes in circadian period could be expected to produce substantial changes in phase angle.

Whereas substantial functional reorganization of the circadian system did not alter total alcohol intake, we nonetheless confirm an important role of the circadian system in drinking behavior. Regardless of whether they are in DD or on T cycles, C57 mice are shown here to express very distinct circadian rhythms in alcohol licking. In HAP2 and LAP2 mice, drinking rhythms free-running in DD were not directly assessed, but drinking and activity

rhythms were very similar in T24 (Fig. 6) just as they were as previously reported in C57 mice (Trujillo et al., 2009).

An unexpected finding was the association between high activity levels and high ethanol intake (e.g., female HAP2 mice were most active and drank the most). In this study, HAP mice ran three times as much LAP mice. Alcohol was not present during this testing, and therefore these differences cannot be caused by its acute pharmacological effects. Wheel-running behavior has been linked to motivation and reward systems (Meeusen, 2005), and wheel running and ethanol consumption can substitute for one another in C57 mice (Ozburn et al., 2008). As a motivated and rewarding behavior, wheel-running might be expected to serve as an index of the tone or sensitivity of reward systems relevant also to drugs of abuse (de Visser et al., 2007; Ozburn et al., 2008). Future work might also consider general home cage activity as this also strongly correlates with drinking behavior, but which, to our knowledge, has not yet been shown to have the same reinforcing properties as wheel-running. Post-hoc analysis showed that wheel-running and general activity levels are themselves highly correlated ($r=0.53$, $n=26$, $p < 0.01$) even though these measures were taken 5 months apart. In the present case, activity was detected when a mouse simultaneously touched the wire cage lid and a stainless steel plate on the cage floor and thus may have captured potentially rewarding behaviors such as play, exploration or climbing.

It is unclear why artificial selection for alcohol intake commonly brings along a circadian activity phenotype. Prior studies document shorter free-running periods in three independent selections for high- versus low-preferring rodents (Hofstetter et al., 2003; Rosenwasser et al., 2005b). This convergence is impressive given that the free-running period is often considered to be the most fundamental clock property and a close reflection of the activity of the master pacemaker. The current data from HAP2 and LAP2, however, do not conform to the pattern. Period differences between lines appeared only in the presence of alcohol, and here the line difference was opposite that reported in other line pairs (Hofstetter et al., 2003; Rosenwasser et al., 2005b). Because alcohol may alter circadian period (Rosenwasser et al., 2005a; Seggio et al., 2009), the more relevant measures to compare with the prior literature are those collected from alcohol naïve or abstinent mice. Neither general locomotion nor wheel-running periods collected under these conditions evince any suggestion of a period difference. While the lack of difference between replicate 2 HAP and LAP mice is puzzling, genetic correlations do not always replicate. Drawing from suggestions provided by Crabbe et al. (1990), the overall pattern of differences in *tau* between HAP and LAP mice (a significant difference in one set of replicates but not the other) may be considered to be moderately supportive of a genetic correlation between *tau* and ethanol consumption. The convergence of period effects is qualified also by their occurrence only in constant light and not constant dark in ethanol-preferring (P) versus nonpreferring (NP) rats (Rosenwasser et al., 2005b), suggesting in that case that the strain difference might derive from altered light sensitivity rather than to pacemaker period, per se.

As an analytical tool, T cycles have been successfully used to define the contribution of the circadian pacemaker to diverse aspects physiological and behavior (Carmichael et al., 1981). Here we used T cycles to define better the nature of the circadian contribution to alcohol

consumption. Although mice exhibit marked circadian rhythms in alcohol intake, the amount of alcohol consumed is broadly independent of how the circadian clock is entrained or free-runs. The reorganization of the cellular and network properties of the circadian pacemaker induced to run at different periods, thus, is insufficient to affect alcohol intake or preference. Alteration of the phasing of the pacemaker relative to the entraining light/cycle likewise does not imply any change in alcohol consumption nor does rearrangement of the phase relationships among multiple organ systems downstream of the pacemaker. In totality these results establish a great resilience of alcohol intake mechanisms to major variations in the organization of the circadian system. Instead, many of the myriad processes that influence alcohol intake (e.g., taste preference, arousal, reactivity, reward, etc.) are certainly targets of circadian regulation. Systems operating at this level of physiological organization, between the master pacemaker on one hand and global internal milieu on the other, are the most likely conduits for circadian influence. Finally, these data do not exclude the possibility that more dynamic disruption of circadian organization could influence alcohol consumption such as may occur, for example, outside the limits of entrainment or with repeated jetlag or shift-work (Clark et al., 2007; Martino et al., 2008; Woelfle et al., 2004).

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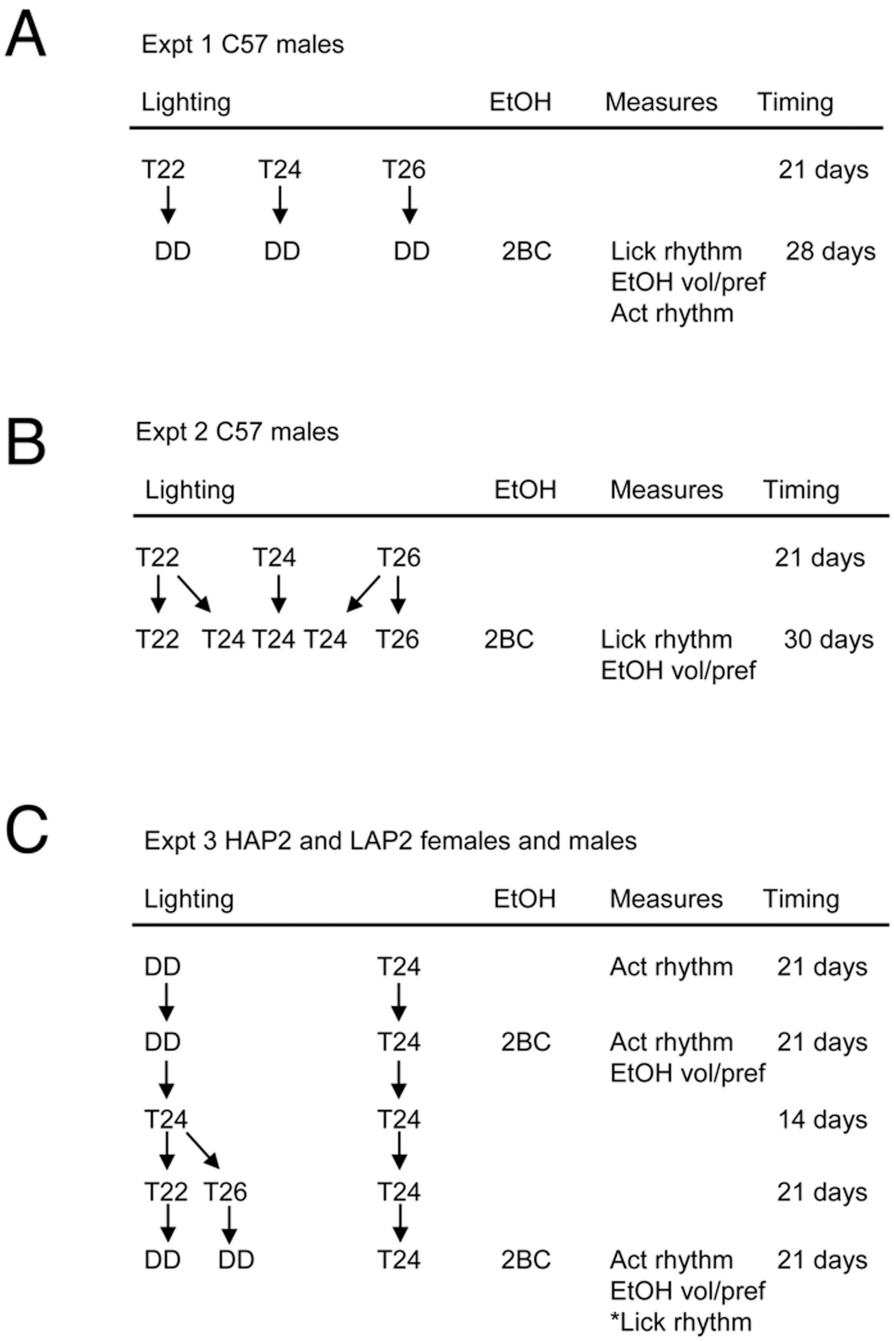


**Figure 1.**

A–C. Schematic representation of the experimental protocols used in Experiments 1–3. Lighting conditions for each phase are indicated with arrows showing the trajectory of exposure for different groups. When ethanol was present, it was always delivered in the form of 2 bottle choice (2BC) during which EtOH intake and preference were always determined volumetrically. The measures collected and reported are indicated for each experimental interval. In Experiment 3, asterisk denotes that licking rhythms were recorded in lieu of activity rhythms for the control subjects in T24.

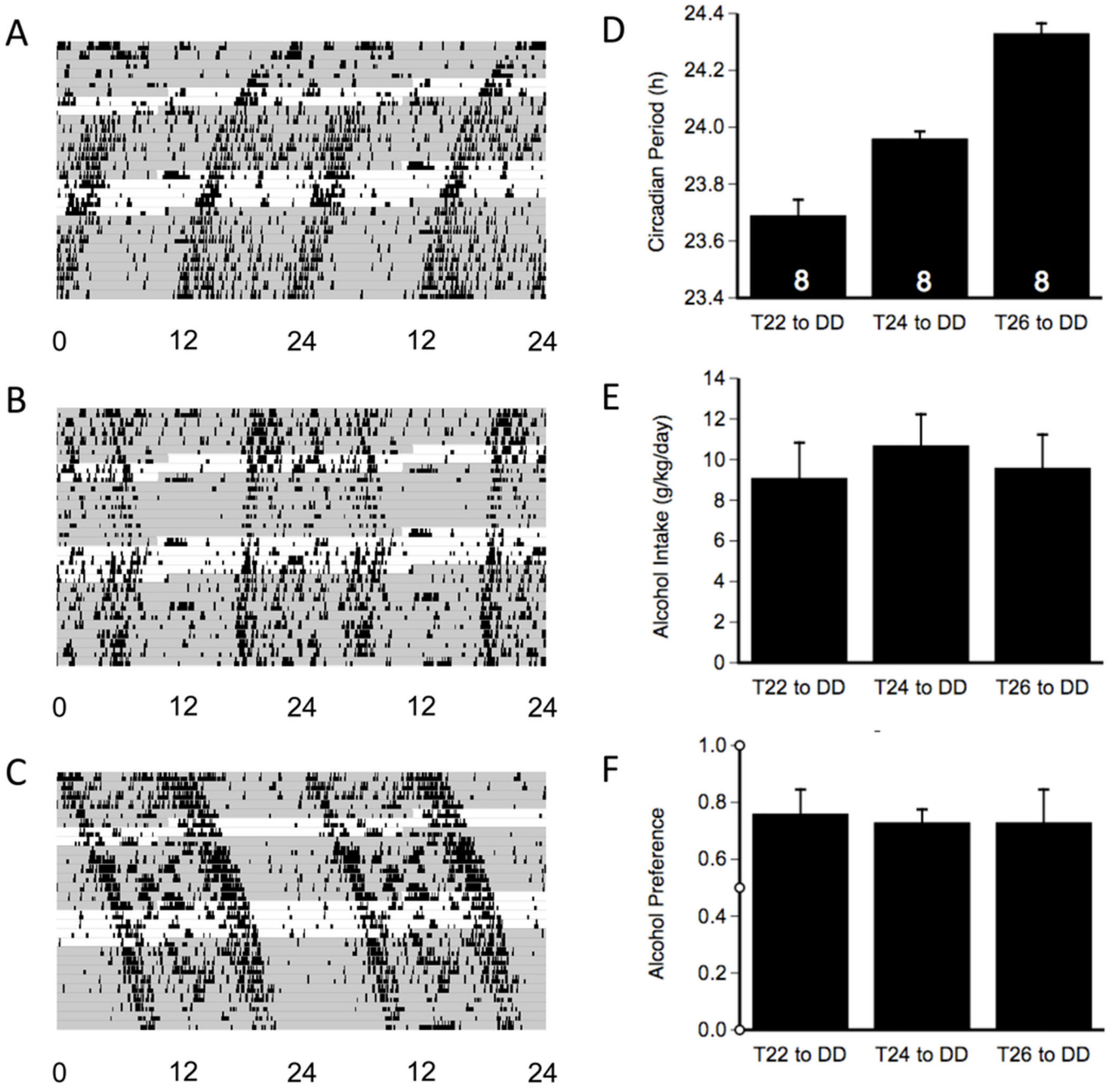


**Figure 2.**

A–C. Representative double-plotted actograms of locomotor and licking activity by C57BL/6J mice during 28 days of 24 hour ethanol exposure in constant dark (DD) following exposure to T22 (A), T24 (B) and T26 (C). Recording was alternated between licking and general locomotion, and intervals of licking data are indicated with background shading. D. Mean (± SEM) τ over the same interval (n = 7–8/group). E–F. Mean (± SEM) ethanol intake per 24 h (g/kg) and alcohol preference by group.

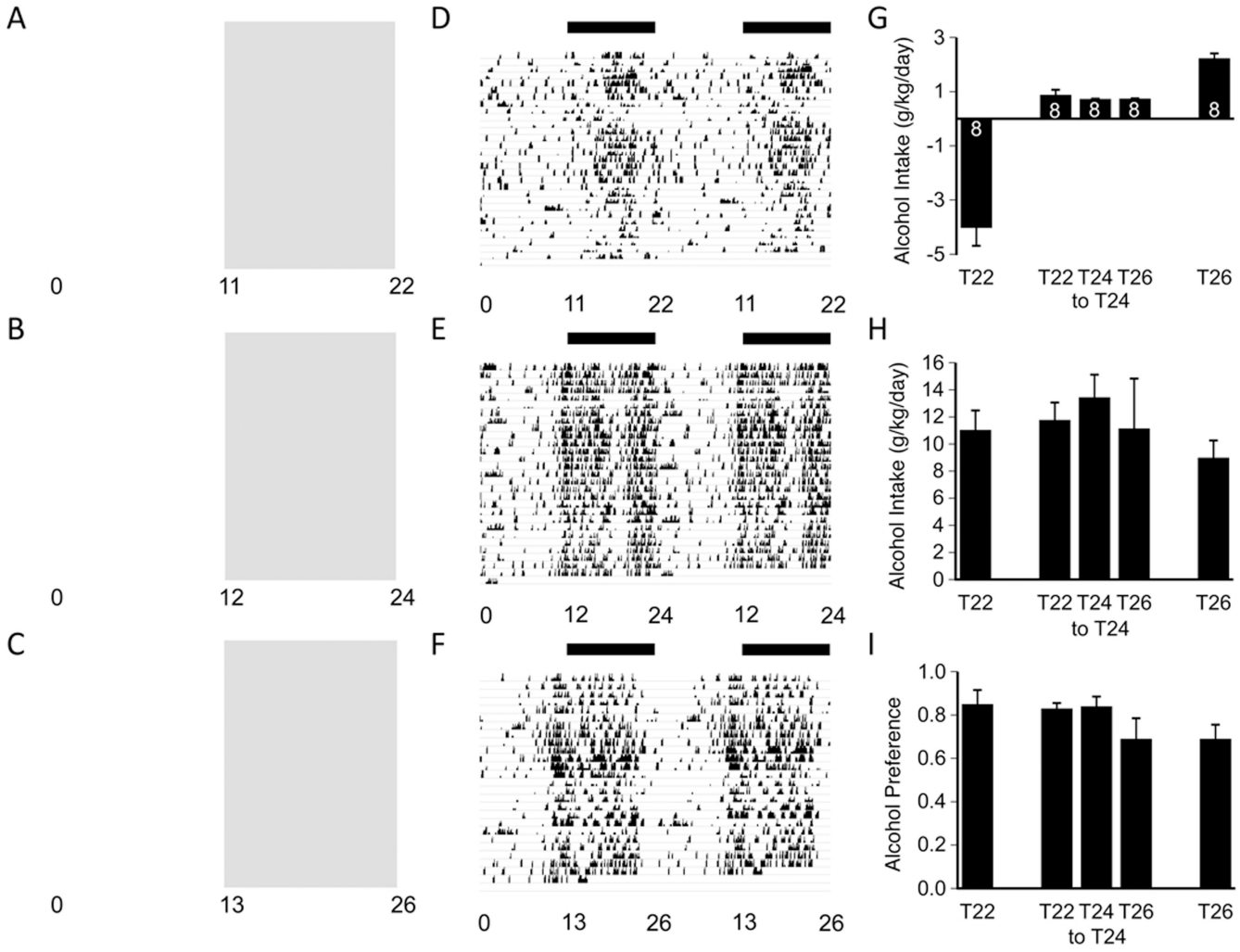


**Figure 3.**

A–C. Average licking rhythms of mice during entrainment to T22 (A), T24 (B) and T26 (C). Shown are activity profiles over 24 to 30 cycles at their respective environmental period, T, and averaged across the 7–8 animals in each group. Profiles are scaled from zero to the averaged group maximum. Shading indicates the half of the cycle in darkness. D-F. Representative double-plotted licking rhythms of individual animals maintained continuously in T22, T24, and T26, respectively. Examples of animals transferred from T22 or T26 to T24 are not shown. G. Mean (± SEM) phase angle of entrainment for drinking rhythm relative to lights off (negative number indicates drinking begins ahead of lights out). H–I. Mean (± SEM) ethanol intake per 24 h (g/kg) and alcohol preference by group.

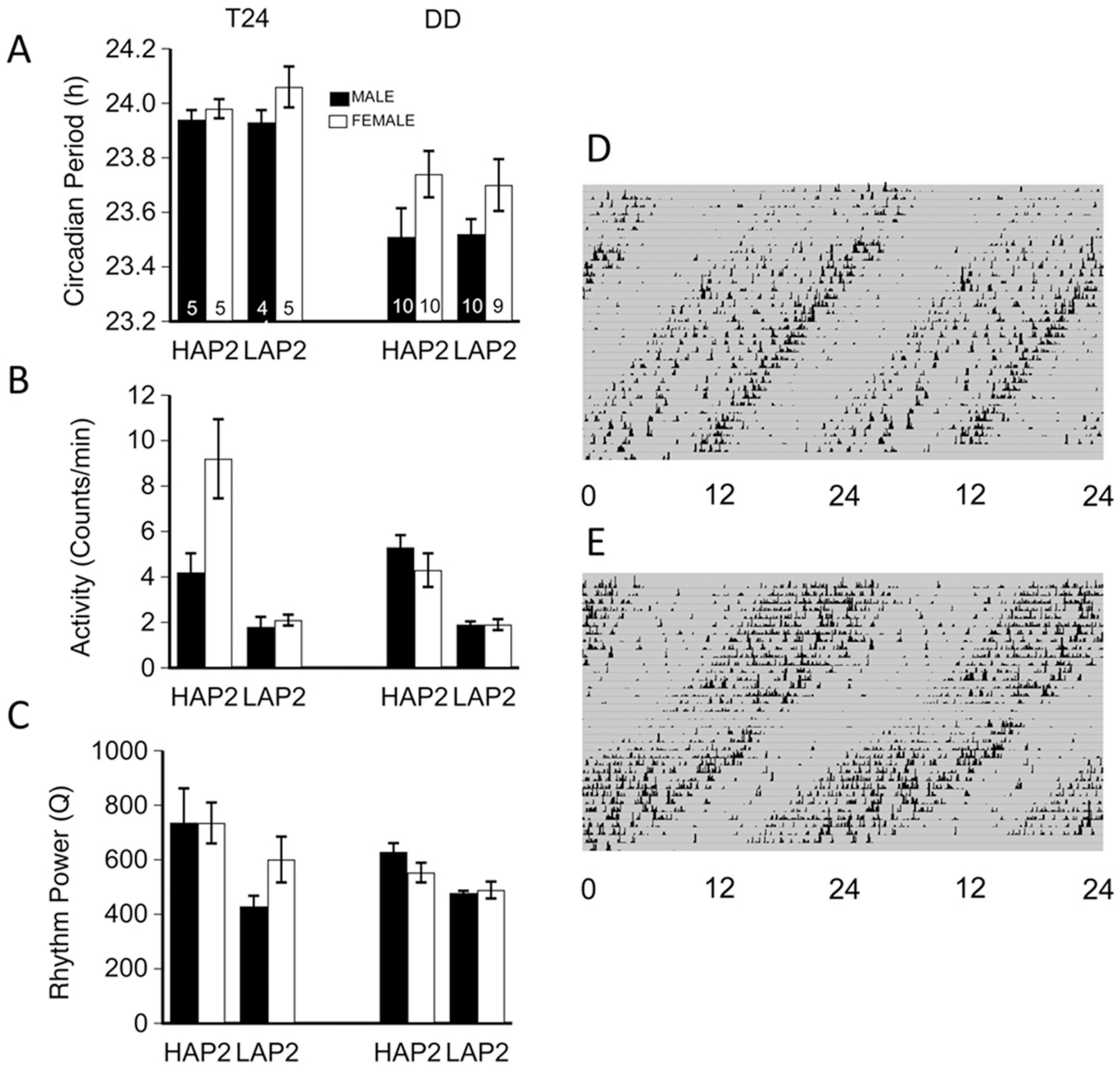


**Figure 4.**

A–C. Mean (± SEM) circadian period, activity counts, and rhythm power of general locomotor activity of HAP2 and LAP2 mice maintained under T24 or exposed to DD. Sample size for respective conditions is indicated in A. D–E. Representative double-plotted actograms of female HAP2 and LAP2 mice in DD.

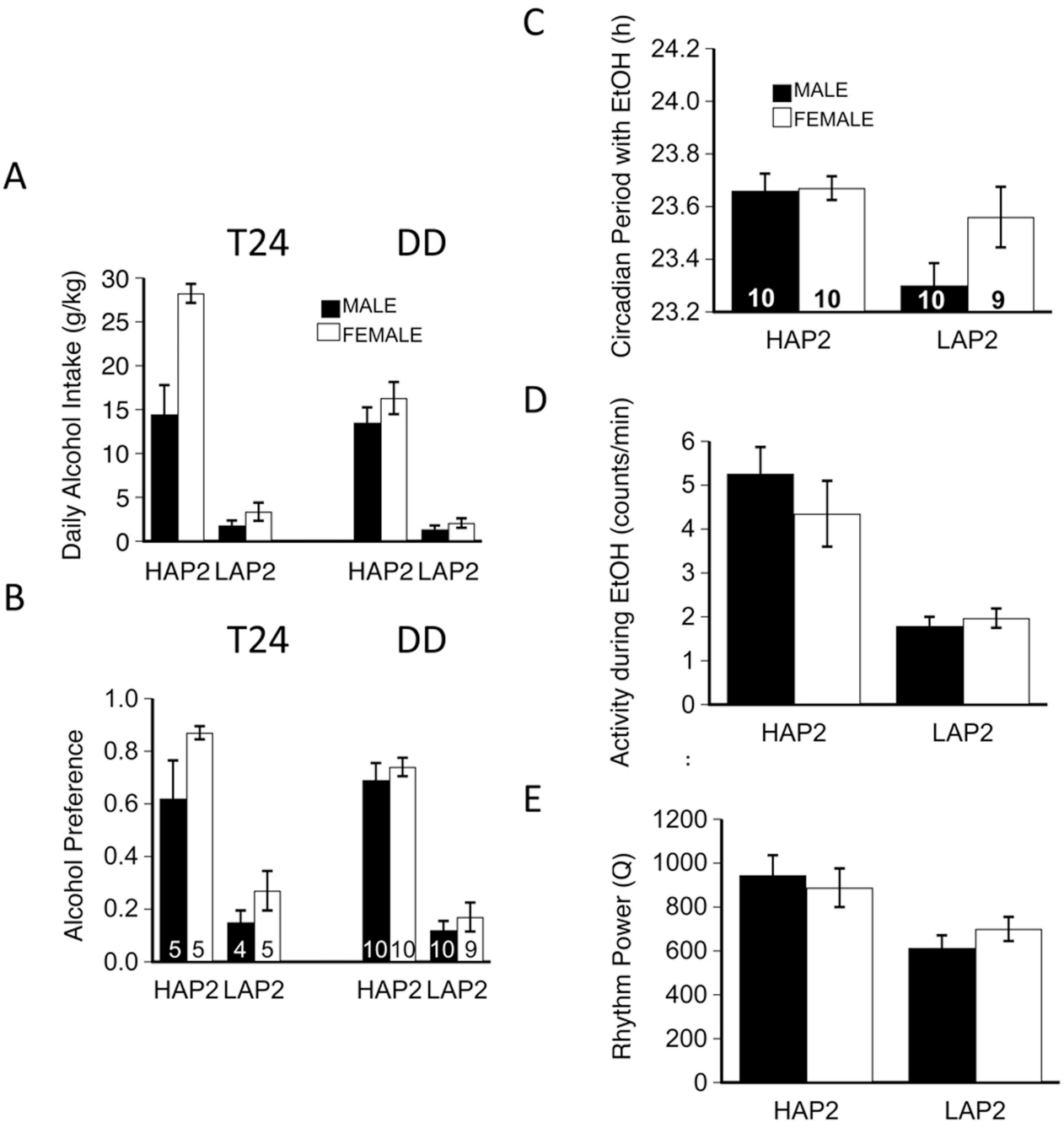


**Figure 5.**

A–B. Mean (± SEM) ethanol intake per 24 h (g/kg) and alcohol preference of male and female HAP2 and LAP2 mice maintained on T24 or in DD. C–E. Mean (± SEM) measures of circadian rhythmicity in DD.

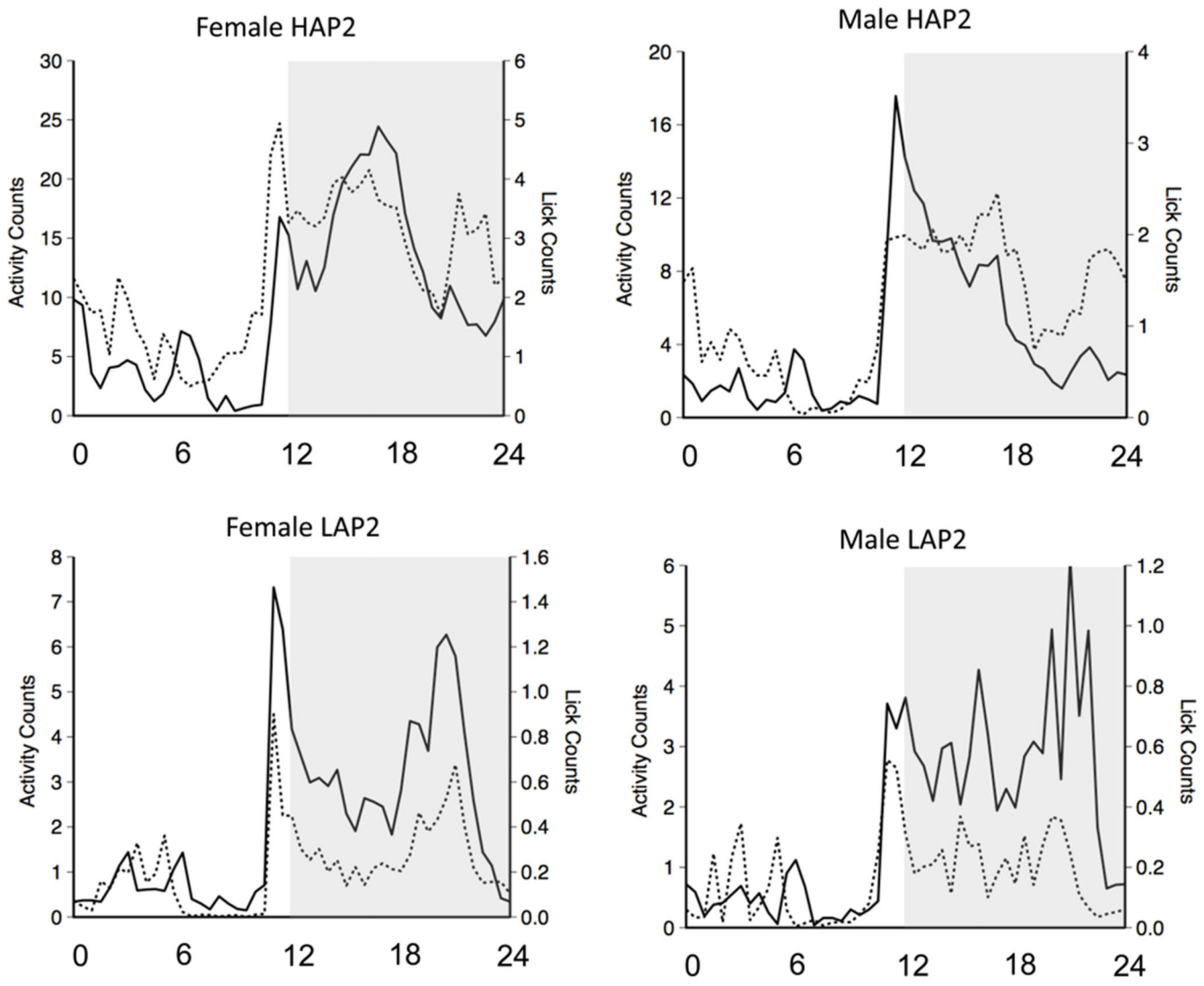


**Figure 6.**

Average daily rhythms in general locomotor activity (solid lines) and in ethanol licking (dashed lines) of female and male HAP2 and LAP2 mice maintained under T24. General locomotor activity was averaged between animals (n = 4–5) in 30 min bins over 15 days. Licking data were processed similarly over 15 days that followed the activity data. Note that groups are scaled differently, but in all cases, licking rhythms are scaled at one fifth that for activity.

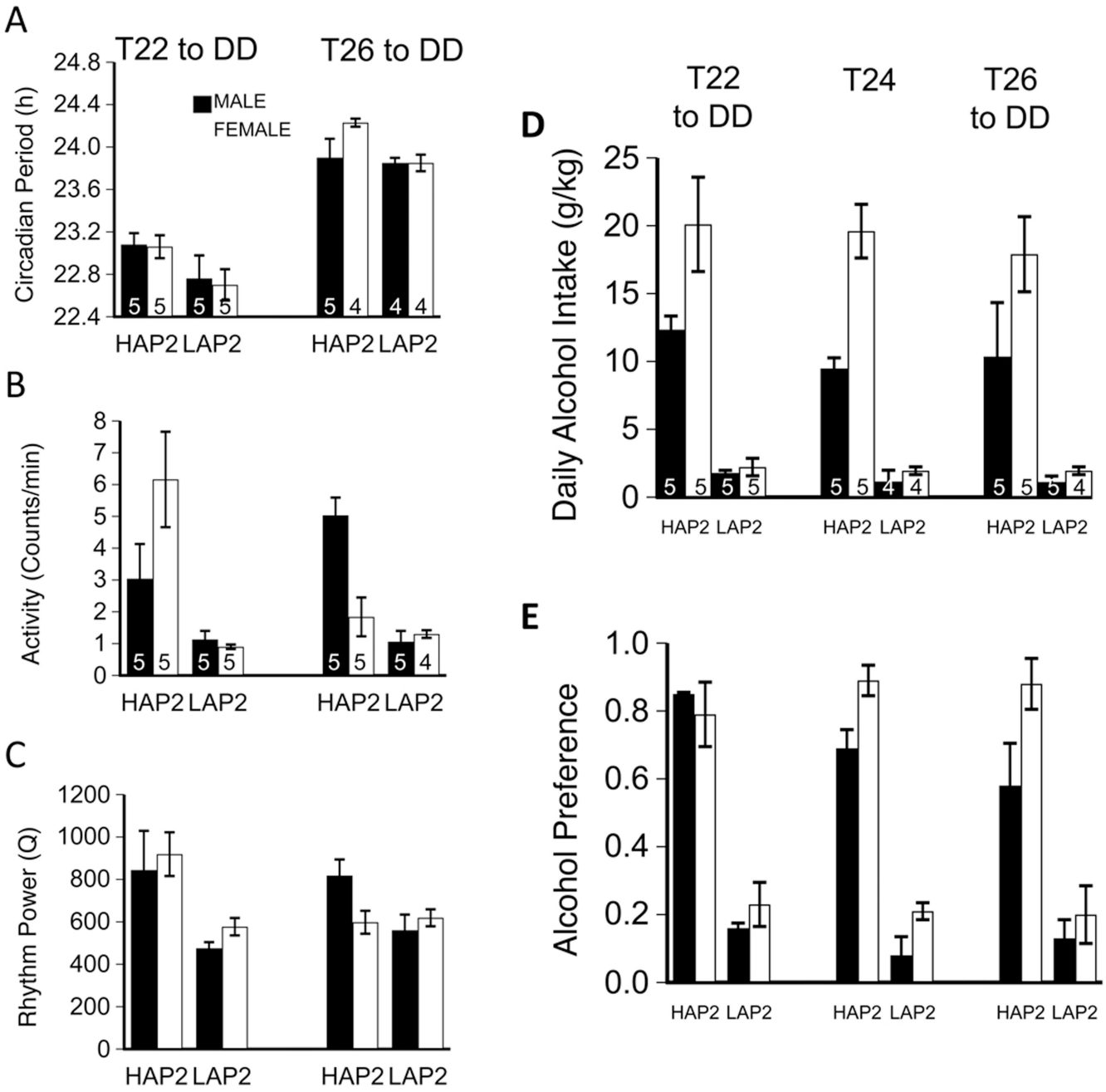


**Figure 7.**

A–C. Mean (± SEM) circadian period, activity counts, and rhythm power of general locomotor activity of HAP2 and LAP2 mice maintained in DD following T22 or T26. D–E. Mean (± SEM) ethanol intake per 24 h (g/kg) and alcohol preference of male and female HAP2 and LAP2 mice under the same conditions and in T24. Sample size in C and E as in A and D, respectively.

## Table 1

Wheel-running measures (mean ± sem) of male HAP2 and LAP2 mice under entrained and free-running conditions.

| **LD 12:12** | | |
|---|---|---|
| | **HAP2** | **LAP2** |
| Phase angle of entrainment (h) | −0.05 ± 0.04 | −0.07 ± 0.05 |
| Statistical power (Q) | 1,652 ± 113 | 1,074 ± 174 |
| Running intensity (rev/day) | 25,294 ± 1,936 | 10,127 ± 2,715 |
| **DD** | | |
| | **HAP2** | **LAP2** |
| Phase angle of entrainment (h) | 23.23 ± 0.12 | 23.24 ± 0.08 |
| Statistical power (Q) | 1,164 ± 159 | 734 ± 123 |
| Running intensity (rev/day) | 19,459 ± 3,438 | 6,319 ± 2,190 |