Home-Cage Sipper Devices Reveal Age and Sex Differences in Ethanol Consumption Patterns

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Highlights (85 characters max per line)

- Female mice consume more ethanol than males in a continuous access paradigm
- Adolescent male and female mice consume more ethanol than young or mature adult mice
- Automated home-cage sipper devices accurately measure ethanol consumption
- Devices reveal sex- and age-dependent differences in circadian drinking patterns
- Devices reveal distinct individual variation in circadian drinking patterns

Abstract

Free-choice paradigms such as two-bottle choice (2BC) are commonly used to characterize ethanol consumption and preference of rodent models used to study alcohol use disorder (AUD). However, these assays are limited by low temporal resolution that misses finer patterns of drinking behavior, including circadian drinking patterns that are known to vary with age and sex and are affected in AUD pathogenesis. Modern, cost-effective tools are becoming widely available that could elucidate these patterns, including open-source, Arduino-based home-cage sipper devices. We hypothesized that adaptation of these home-cage sipper devices would uncover distinct age- and sex-related differences in temporal drinking patterns. To test this hypothesis, we used the sipper devices in a continuous 2BC paradigm using water and ethanol (10%; v/v) for 14 days to measure drinking patterns of male and female adolescent (3-week), young adult (6-week), and mature adult (18-week) C57BL/6J mice. Daily grams of fluid consumption were manually recorded at the beginning of the dark cycle, while home-cage sipper devices continuously recorded the number of sips. Consistent with prior studies, females consumed more ethanol than males, and adolescent mice consumed the most out of any age group. Correlation analyses of manually recorded fluid consumption versus home-cage sipper activity revealed a statistically significant prediction of fluid consumption across all experimental groups. Sipper activity was able to capture subtle circadian differences between experimental groups, as well as distinct individual variation in drinking behavior among animals. Blood ethanol concentrations were significantly correlated with sipper data, suggesting that home-cage sipper devices can accurately determine individual timing of ethanol consumption. Overall, our studies

show that augmenting the 2BC drinking paradigm with automated home-cage sipper devices can accurately measure ethanol consumption across sexes and age groups, revealing individual differences and temporal patterns of ethanol drinking behavior. Future studies utilizing these home-cage sipper devices will further dissect circadian patterns for age and sex relevant to the pathogenesis of AUD, as well as underlying molecular mechanisms for patterns in ethanol consumption.

Keywords: sex differences, aging, circadian rhythm, home-cage monitoring, consumption

Introduction

Alcohol use disorder (AUD) is a serious neuropsychiatric disorder that impacts millions of individuals worldwide and is influenced by age, biological sex, and circadian rhythms. Almost twice as many adult men are diagnosed with AUD than women, with an alarming increase in the number of adolescents and older adults meeting the criteria for AUD diagnosis in recent decades.[1-5] Age- and sex- differences in ethanol drinking behavior have also been documented in rodent models of AUD, including the C57BL/6J (B6J) inbred strain of mice. Female B6J mice are known to consume significantly more ethanol compared to male B6J mice.[6–8] There are also significant differences in ethanol preference and voluntary self-administration between adolescent and adult B6J mice.[9,10] The underlying factors for such age-and sex-specific differences in ethanol consumption are largely unknown; however, substantial evidence supports the role of circadian rhythms and their dysregulation in the development and maintenance of AUD[13–17]. Individual variation in temporal patterns of behavior is influenced by age, biological sex, and ethanol exposure across species[13–29]. Determining uninterrupted patterns of drinking behavior is important for the analysis and interpretation of ethanol consumption across experimental groups. [30–32]

Lickometers have been previously used to discern patterns of drinking behavior by recording the ability to complete an electrical circuit or through infrared tracking of activity[30,32,34]. Lickometers been used more recently to determine the important role of flavor cues established during adolescent drinking in ethanol craving during adulthood in rats as well as B6J mouse ethanol drinking microstructure during operant ethanol administration following a chronic intermittent ethanol consumption paradigm. Despite

their utility, lickometers can be cost-prohibitive, markedly influence animal behavior, are sensitive to electrical interference, and frequently involve significant changes in animal housing conditions[30,32,34]. In addition, lickometers have not been used to investigate age and biological sex differences in voluntary mouse ethanol drinking behavior in-detail. To overcome several of these limitations, open-source automated home cage sipper devices have been developed for monitoring fluid consumption. In the current study we adapted the original design for these devices to measure drinking patterns from ball-bearing sipper tubes in a continuous-access 2BC paradigm for 10% ethanol, which was originally used to show differences among inbred strains of mice and demonstrate that mice on a C57BL background to readily consume ethanol[33]. We hypothesized that these home cage sipper devices would reveal patterns across 14 days of ethanol consumption related to age and biological sex. Identifying these patterns of ethanol consumption is important for establishing baseline activity and tracking behavioral differences across time in an animal model for alcohol use disorder.

Our studies revealed significant age- and sex- related differences as well as considerable individual variation in total ethanol consumption and temporal patterns of drinking behavior. As expected, B6J female mice drank significantly more ethanol than their male B6J mice across all age groups. Peak ethanol consumption displayed subtle phase advancement with respect to age in B6J female mice, but a delay in B6J male mice. Using the individual patterns of sipper activity across all groups, our results demonstrate that these home-cage sipper devices help predict individual peak ethanol consumption as measured by blood ethanol concentration. Taken together, our findings support the use of these novel automated devices in extended drinking assays for

characterizing mouse models of AUD. Additionally, our studies further highlight the importance of investigating age and biological sex differences in ethanol consumption.

Methods

Animals

Male and female (N=36 per sex) C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Age groups consisted of mice 3-, 6-, and 18 weeks of age (N=12 per age per sex) at the time of receipt. These ages were chosen to represent under-studied age groups with increasing instances of alcohol misuse and were defined in guidelines supported by The Jackson Laboratory as adolescent, young adult, and mature adult mice, respectively[37,38]. Mice were habituated to the University of Pittsburgh animal facility for 48h, then singly-housed on ventilated racks in a temperature- and humidity-controlled room with *ad libitum* access to food and water under 12/12h reverse light-dark cycle (lights on at 22:00, off at 10:00). Mice were habituated to the reverse light-dark cycle for at least 5 days before experiments began. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh and conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Construction of Home-cage sipper devices

Automated home-cage sipper devices were constructed based on Godynyuk et al. with minor modifications. A new device housing was constructed to allow for the use of Amuza ball bearing sipper tubes (Drinko Measurer Sipper Tube, 3.5in) within 10 mL glass bottles (Amuza, San Diego, CA) that allow for easy daily bottle refilling and

switching[35,36]. The housing was designed using Tinkercad and printed on a Sindoh 3DWOX1 printer using PLA plastic. Devices were secured on the wire top of each cage using plastic cable ties and were positioned to ensure mice maintained sufficient access to food, which was distributed across the cage top equally near both bottles to avoid food-related side preference. Two sipper tubes were placed into the sipper apparatus. **Figure 1A** illustrates the completed sipper apparatus in an empty cage. An earlier version of the Python code by Godynyuk et al. (dated October 6, 2018) was used that collected data every ten seconds. Device components, construction directions, and the device Python code can be found at: https://hackaday.io/project/160388-automated-mouse-homecage-two-bottle-choice-test-v2.

Continuous access two-bottle choice

Mice were acclimated to the sipper devices for 3-5 days with water before continuous 2BC began. 2BC was performed for 14 days. Half of each cohort (N = 6 per age per sex) was given a choice between ethanol (10%; v/v) and water, while the other half comprised water-only controls. All bottles were weighed every 24h and switched in position and data from the devices were collected by experimenters. Bottles were placed into the device apparatus so that each sipper was flush with the plastic housing, meaning mice could equally access each sipper and the sippers did not interfere with the device photobeams. An empty control cage with the device housing and sipper tubes was maintained to account for spillage. During these experiments, left and right side were defined as experimenter left/right facing the cage front (**Figure 1A**).

Blood ethanol concentration analysis

Peak ethanol consumption period was determined via R statistical analysis by averaging sipper counts from the continuous access period. For two days following 2BC, the ethanol bottle was maintained on the left side and tail vein blood was collected from ethanol mice at predicted at individual peak blood ethanol concentration (BEC) times. Blood was collected and centrifuged for two minutes at 12,000 rpm in heparin-coated glass capillary hematocrit tubes (Fisher Scientific, Cat #22362566) in a Unico Microhematocrit centrifuge (Model C-MH30, Dayton, NJ). Serum was collected and analyzed for ethanol concentration on an Analox Alcohol Analyzer (AM1, Analox Instruments, London, UK). BEC measurements were corrected using a standard curve constructed via a serial dilution of Analox 100mg/dL standards in distilled water. Overall experimental design is summarized in **Figure 1B**.

Statistical Analyses

Ethanol consumption data were analyzed by two-way ANOVA. Linear regression analysis was performed on average consumption per day in week 1 compared to week 2 to examine similarities in drinking between the two weeks. Linear regression and Pearson correlation analyses were performed on total daily fluid consumption from experimenter measurements versus device measurements as well as on BECs versus average daily ethanol sipper counts. Individual variation of ethanol drinking behavior was assessed using principal component analysis (PCA) and pairwise correlation analysis of daily sipper activity. 3-way ANOVA analysis including post-hoc Tukey tests and power analysis was performed on daily sipper data to determine statistically

significant differences for age, biological sex, and time. All analyses were conducted in the R statistical environment (R 4.1.0)[39].

Results

Ethanol consumption in a continuous access two-bottle choice paradigm

Male and female animals, aged 3-, 6-, and 18-weeks, were allowed free access to one bottle containing ethanol and one containing drinking water for 14 days. Male animals consumed between 1.5 and 10 g/kg of ethanol per day, while female animals consumed between 5 and 15 g/kg of ethanol per day. Two-way ANOVA did not reveal a statistically significant main effect of time or age, or a time x age interaction, for either males **(Figure 2A)** or females **(Figure 2B)**. Ethanol consumption over the 14-day period was summed to determine the total amount of ethanol consumed over the course of the experiment. Two-way ANOVA of total ethanol consumption revealed a statistically significant main effect of age ($F_{2, 28} = 5.672$, $P = 0.0085$) and sex ($F_{1, 28} = 9.726$, $P = 0.0042$), although no age x sex interaction **(Figure 2C)**. To demonstrate that ethanol consumption remained stable between the two weeks of testing, linear regression analyses were performed on average consumption per day for week 1 and week 2 of the 2BC paradigm. Statistically significant correlations were observed in both male ($r = 0.8691$, $P < 0.0001$, **Figure 2D**) and female ($r = 0.9301$, $P < 0.0001$, **Figure 2E**) mice.

Correlation analysis of experimenter-measured fluid consumption and sipper counts

Linear regressions were performed to determine the accuracy of the sipper devices in measuring the amount of ethanol consumed by B6J mice. We sought to establish whether the daily total recorded sip counts logged by the device correlated

with the consumed grams/grams per kilogram of ethanol measured by the experimenter. Here we use terms such as “strong” or “weak” to denote correlation strength according to previously-discussed guidelines for medical/biomedical research to discuss correlation coefficients [46,47]. Scatter plots were constructed and Pearson correlation coefficients and best fit linear regression equations were calculated for all age and biological sex (collapsed across age) subgroups. Measurements with null sipper values were omitted from analysis. Pearson correlation coefficients and linear regression equations are summarized in **Figure 3**. All correlations performed in these analyses were statistically significant ($p < 1*10^{-4}$ for all analyses), with very strong correlation coefficients > 0.8 for all age and biological sex subgroups (**Figure 3**). Subsequent analyses were split by fluid type and done in grams per kilogram of mouse bodyweight. Ethanol correlations were very strong ($r > 0.8$) for all mouse subgroups: $r = 0.81$ for females and 3-week mice, $r = 0.82$ for males, $r = 0.84$ for 6-week mice, and $r = 0.89$ for 18-week mice **(Figure 3)**.

Circadian and individual differences in ethanol consumption by age and sex

Sipper count data were analyzed in R to construct circadian histograms averaging 14-day continuous 2BC data within age-sex subgroups for both water and ethanol consumption. Ethanol consumption for 3-week-old female mice peaked approximately 6 hours into the dark phase **(Figure 4A)**, while 3-week-old male animals peaked much earlier, about 2-3 hours into the dark phase **(Figure 4B)**. Adolescent females also displayed higher peak ethanol counts than adolescent males. When comparing young adult animals, ethanol consumption in 6-week-old females **(Figure**

4C) peaked about 5 hours into the dark phase, compared to 3 hours in 6-week-old males **(Figure 4D)**. As with adolescent animals, females displayed higher peak ethanol counts than males. Finally, peak ethanol consumption occurred in 18-week-old females about 5 hours into the dark phase **(Figure 4E).** Male 18-week-old animals demonstrated a large shift in consumption from other age groups, with peak ethanol consumption occurring 6 hours into the dark phase **(Figure 4F).** When comparing within sex, a subtle phase advancement can be observed in female mature adult animals compared to adolescents and young adults **(Figure 4G)**. The opposite appears to be true in males, where peak consumption shifts later with increasing age **(Figure 4H)**. All animals consumed significantly more fluid (both water and ethanol) in the dark photoperiod **(Figure 4, Figure S3)**. Interestingly, distinct sex differences in fluid consumption during the light and dark photoperiods were observed. Females drank a significantly greater percentage of their daily fluid intake during the light cycle than males, while the opposite was true during the dark photoperiod, where males drank a significantly greater percentage of their daily fluid intake than females (**Figure S3)**.

Principal component analysis was performed for averaged individual ethanol sipper counts for the ethanol group mice in 60-minute bins from the 14 days of continuous 2BC. High individual variation was observed, as the first two principal components accounted for only 20% and 6.6% of variance, respectively, though samples clustered according to biological sex (**Figure 5A**). To further dissect individual variation recorded by the devices, pairwise correlations were performed on 60-minute binned data, where ethanol sipper counts were correlated at matching timepoints across consecutive days within each mouse to generate a set of Pearson correlation

coefficients. Boxplots were constructed for these within-subjects analyses (**Figure 5B**), as well as averaged values between subjects in each subgroup (**Figure 5C**). Subjects within each group displayed variation in the consistency of ethanol drinking behavior across days, as seen in the spread of within-subjects r values near 0.5 **(Figure 5B).** While these correlations were fair strength, correlation coefficients were stronger within subjects than between subjects (**Figure 5B/C**). Average median r values were comparable between age groups, though slightly higher for 3-week mice, followed by 6-week mice (**Figure 5C)**. Averaged group data used to plot **Figure 5C** can be found in **Table S3**. Representative visualizations of individual drinking behavior separated by day, as well as averaged across the 2BC paradigm are provided in **Figures S2 and S3**.

Correlation analysis of blood ethanol concentration at predicted peak from sipper data

Linear regression was performed on BECs measured at peak consumption time from individual sipper bout data analyzed in R (**Figure S3**). BECs were correlated to sipper counts at the time of sampling. This correlation was statistically significant ($p < 0.05$), and overall strength was fair ($r = 0.36$, **Figure 7**).

Discussion

Open-access, automated home-cage sipper devices were validated in an extended, continuous access 2BC ethanol drinking paradigm to investigate age- and sex differences in ethanol consumption and temporal drinking patterns. Experimenter-collected ethanol consumption data revealed increased consumption in female compared to male animals, as well as in adolescent compared to young and mature adult mice. Statistically significant correlation of ethanol consumed and sips counted by

the devices validated their accuracy for use in this paradigm. Device sipper data revealed subtle circadian differences between sexes and ages, as well as distinct day-to-day variation in individual drinking behavior.

Pairwise correlations yielded lower-strength r values for 18-week-old mice. This could be explained by older mice drinking the least ethanol out of all the age groups. BEC correlations yielded overall only fair strength, though with a low sample size. Further studies can more definitively dissect temporal drinking patterns and their consistency and could use more quantitative tools for predicting individual peak ethanol consumption period. Machine learning algorithms will be useful in future applications of these devices as their use is scaled up with this low-cost equipment. Indeed, a recent study with these devices used a feedforward artificial neural network to analyze sipper microstructure in a DID paradigm that successfully predictively classified fluid (ethanol versus water) and groups of mice injected with different viral vectors [Haggerty et al].

Our data more concretely validate these automated home-cage sipper devices by expanding their use to more extended periods as well as to measure ethanol consumption. In their initial validation of these devices, Godynyuk et al. performed correlations of sipper counts to experimenter-measured volume of water or chocolate milk consumed overnight via time lapse video recording [35]. Resulting R^2 values ranged from 0.26 to 0.85 (average 0.46) between individual devices[35]. With their modified version of these sipper devices using hydrostatic depth sensors and tested in rats, Frie and Khokhar also performed a separate correlation assay resulting in an R^2 value of 1, though through incremental addition of water during a brief testing period [36]. In our study, linear regression analysis of g/kg of ethanol consumed versus sipper counts from

all 14 days of 2BC yielded statistically significant correlations for all age/sex subgroups. These data demonstrate the overall relative accuracy of these automated home cage sipper devices in measuring ethanol consumption by B6J mice during extended testing periods with Amuza ball bearing sipper tubes.

Analysis of ethanol consumption data in our experiments revealed that female C57BL/6J mice, across all age groups, drank significantly more ethanol than their male counterparts. This finding is supported by numerous studies that have shown adult, female mice of this strain and multiple other strains drink significantly more ethanol than males[6–8]. While we and others employed a continuous access 2BC paradigm [7], similar results have been demonstrated in the Drinking in the Dark (DID) paradigm [6]. There are multiple hypotheses as to why female mice drink more than males: females could be more sensitive to ethanol's rewarding effects and/or could metabolize ethanol more quickly, meaning ethanol and its toxic metabolites (such as acetaldehyde) would remain in the body less long to induce negative side effects such as oxidative stress[41-44]. Multiple studies have found no sex differences in ethanol conditioned place preference [11,40], though, and while difference in metabolism may underly sexually dimorphic differences in ethanol consumption, future studies are necessary to fully understand this phenomenon.

In addition to metabolism changes, it is possible that variations in circadian rhythm could underly the sex and age differences we observed in ethanol consumption, as well as the differences in ethanol drinking patterns seen among individuals within the same age/sex subgroups. Interestingly, our data suggests that females drink significantly more fluid during the light cycle than males, while the reverse is true during

the dark cycle. Another study measuring consumption in a continuous-access 2BC paradigm found that the female-specific increase in consumption occurred only during the dark cycle [8]. While circadian rhythms can vary up to an hour among individuals of similar age and sex [22], distinct sex differences have also been noted[18]. Male mice display more accurate onset of activity when lights go off in a light/dark setting [18], which is consistent with our findings, as peak ethanol consumption occurred earlier in males than females. Sex differences in the strong relationship between ethanol and sleep/activity patterns have also been documented in humans, though details remain unclear due to male sex bias and omission. While insomnia as a whole is more common among women, men may be more likely to self-medicate their insomnia with ethanol in both adolescents and adults [Baker, Carskadon, and Hasler 2020]. Less is known regarding sex differences in the effects of chronic alcohol consumption/AUD on sleep disturbances and vice versa [Inkelis, Hasler, and Baker 2020]. Further research can validate sex differences in circadian ethanol consumption and apply these findings to clinical studies in humans.

Few studies have observed differences in drinking behavior across multiple age groups, with most published findings focusing on the differences between adolescent and adult mice. Studies comparing ethanol consumption in young adult and mature adult mice are severely lacking. Findings on ethanol consumption differences across age groups have been inconsistent, with some studies noting no change in drinking between ages and others finding increased consumption among adolescent animals compared to adults [9,26,45]. As we hypothesized with female animals, it is possible that differences in consumption observed in our study are due to quicker metabolism of

ethanol in adolescents, as adolescent mice are significantly more sensitive to ethanol-induced anxiolysis, locomotor activity, and ataxia [9,12]. Additionally, circadian rhythms phase advance as organisms age, and the activity period of older mice becomes shorter [19,20]. Our oldest mice, 18-weeks of age, displayed peak ethanol consumption earlier than the 3- and 6-week animals regardless of biological sex. A shorter activity period in older mice may underly the reduction in consumption observed in our cohort. As we know there is a strong reciprocal relationship between circadian dysregulation and AUD [14,16,17,24,25,27–29], future studies examining the relationship between age, sex, and circadian rhythms in the context of ethanol consumption is imperative. Determining how consumption and ethanol metabolism changes across the lifespan will aid our understanding of the changing demographics of AUD subjects.

In summary, the automated home-cage sipper devices employed in this study accurately measure drinking behavior in a continuous access 2BC ethanol consumption paradigm. In addition to collected consumption data, these devices are able to detect subtle age- and sex- differences in circadian drinking behavior, as well as capture individual variation among animals. Widespread use of these devices provides a low-cost option for in-depth studies of animal models of AUD and related circadian dysfunction as well as optimization of extended ethanol-related assays *in vivo*. Better understanding of the relationship between AUD and circadian rhythms may lead to better treatments for this disorder.

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Figures

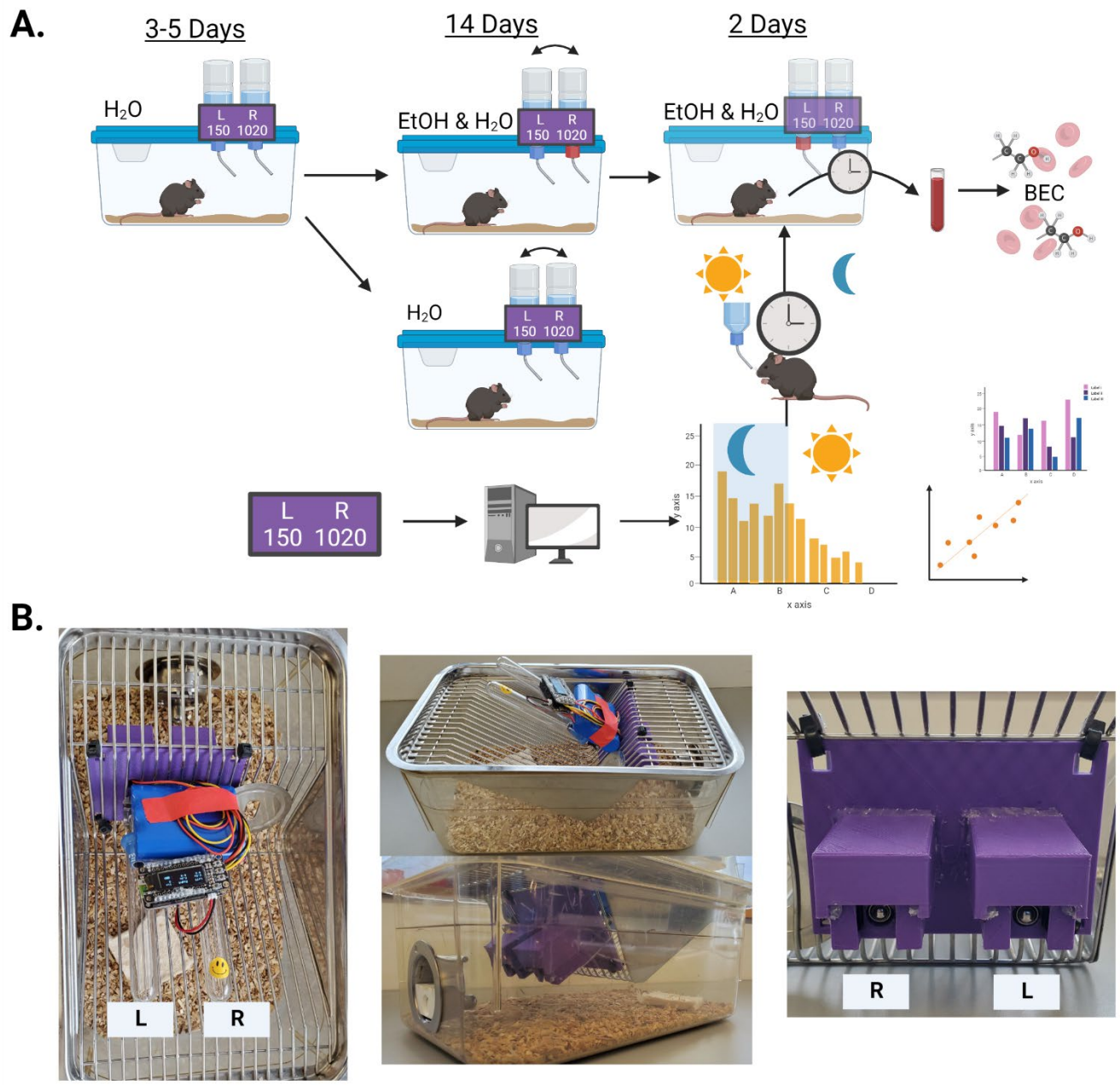


Figure 1. (A) Overall experimental design. Mice were first acclimated to the devices with water in either sipper bottle, after which they were split into water-only control and ethanol experimental groups. Continuous access 2BC was performed for 14 days, after which two days of BEC sampling were performed on ethanol drinking mice using predicted peak BEC periods from 2BC sipper data. **(B)** Representative images of home-cage sipper device on an empty cage and cage top. “Left” and “Right” were defined from experimenter view facing the front of the cage. Wires were taped out of reach of mice, and screens were taped during experiments to avoid excess light. Food was distributed evenly below the bottles, and sipper tubes were placed in the apparatus to ensure they were flush with the plastic housing and equally accessible.

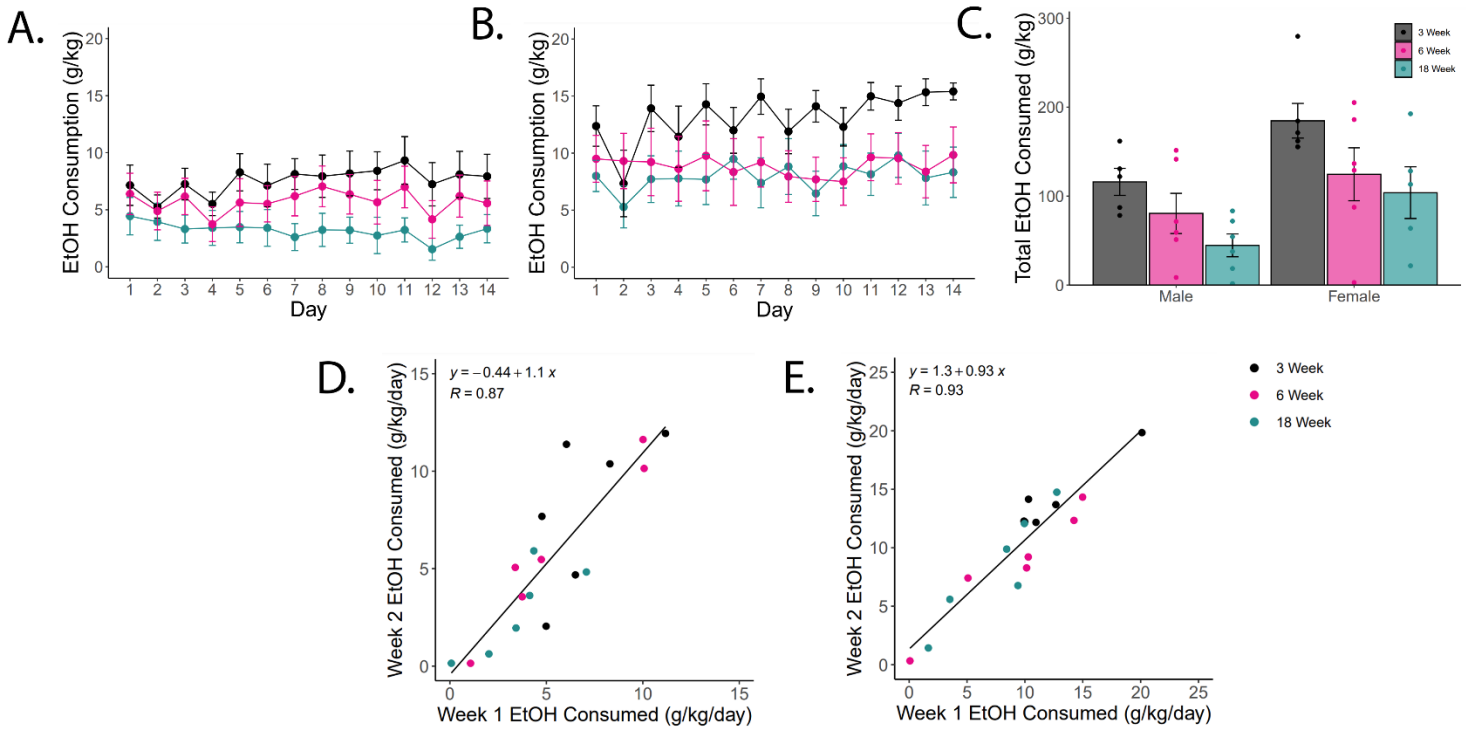


Figure 2. Ethanol consumption during the two-bottle choice (2BC) paradigm. Daily ethanol consumption during the two-week 2BC paradigm for **(A)** male and **(B)** female animals. **(C)** Total ethanol consumption over the entire 2BC paradigm. Linear regression analysis of average daily ethanol consumption for week 1 versus week 2 of the 2BC paradigm for **(D)** male and **(E)** female animals. All data are mean ± SEM. N = 6 per group.

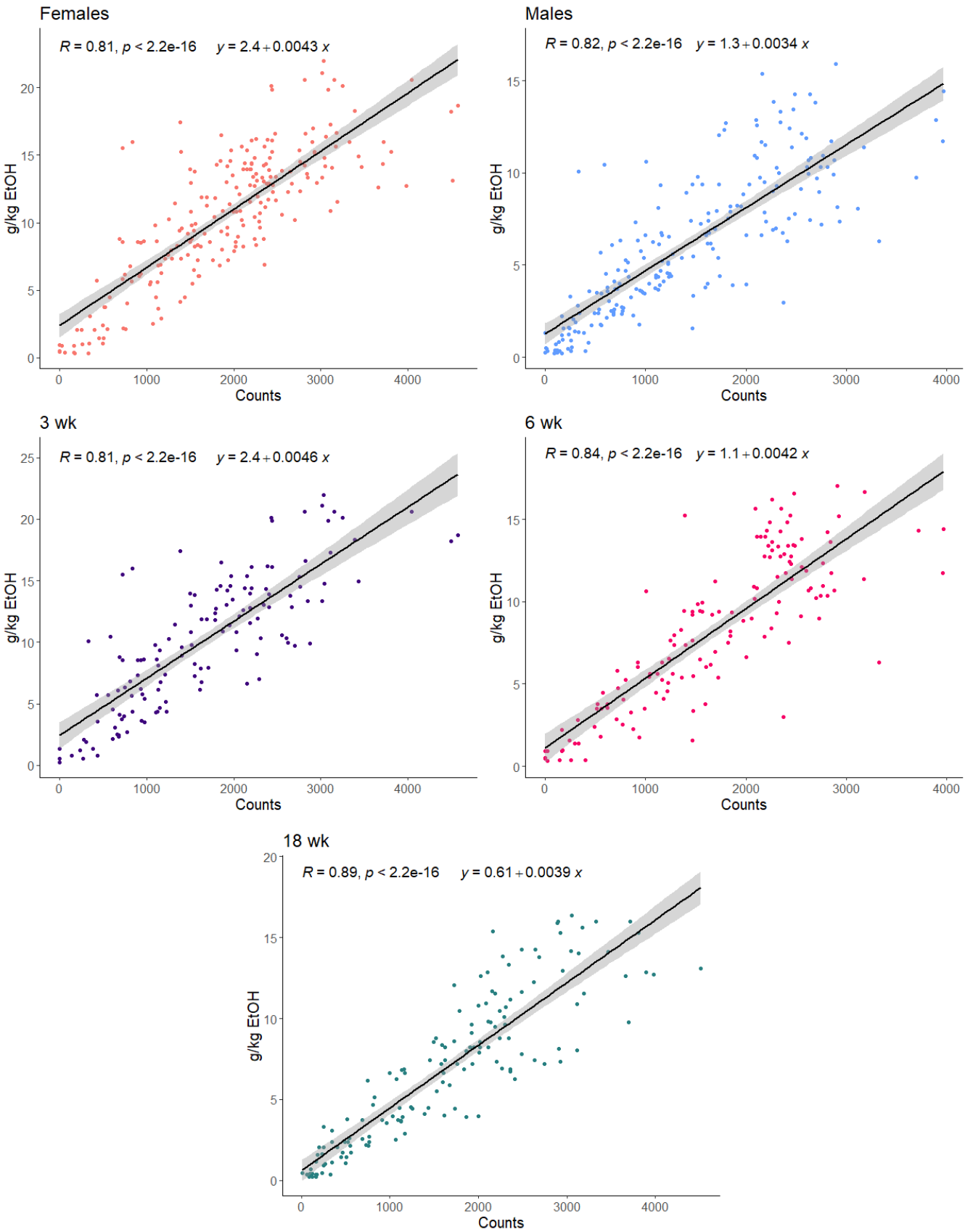

Females
R = 0.81, p < 2.2e-16 y = 2.4 + 0.0043 x
Males
R = 0.82, p < 2.2e-16 y = 1.3 + 0.0034 x
3 wk
R = 0.81, p < 2.2e-16 y = 2.4 + 0.0046 x
6 wk
R = 0.84, p < 2.2e-16 y = 1.1 + 0.0042 x
18 wk
R = 0.89, p < 2.2e-16 y = 0.61 + 0.0039 x
g/kg EtOH
Counts
0
1000
2000
3000
4000

Figure 3. Scatterplots and correlation analyses for total daily ethanol consumed measured by experimenter versus total daily sipper counts logged by the devices for ethanol group mice, split by age and biological sex (collapsed across age groups).

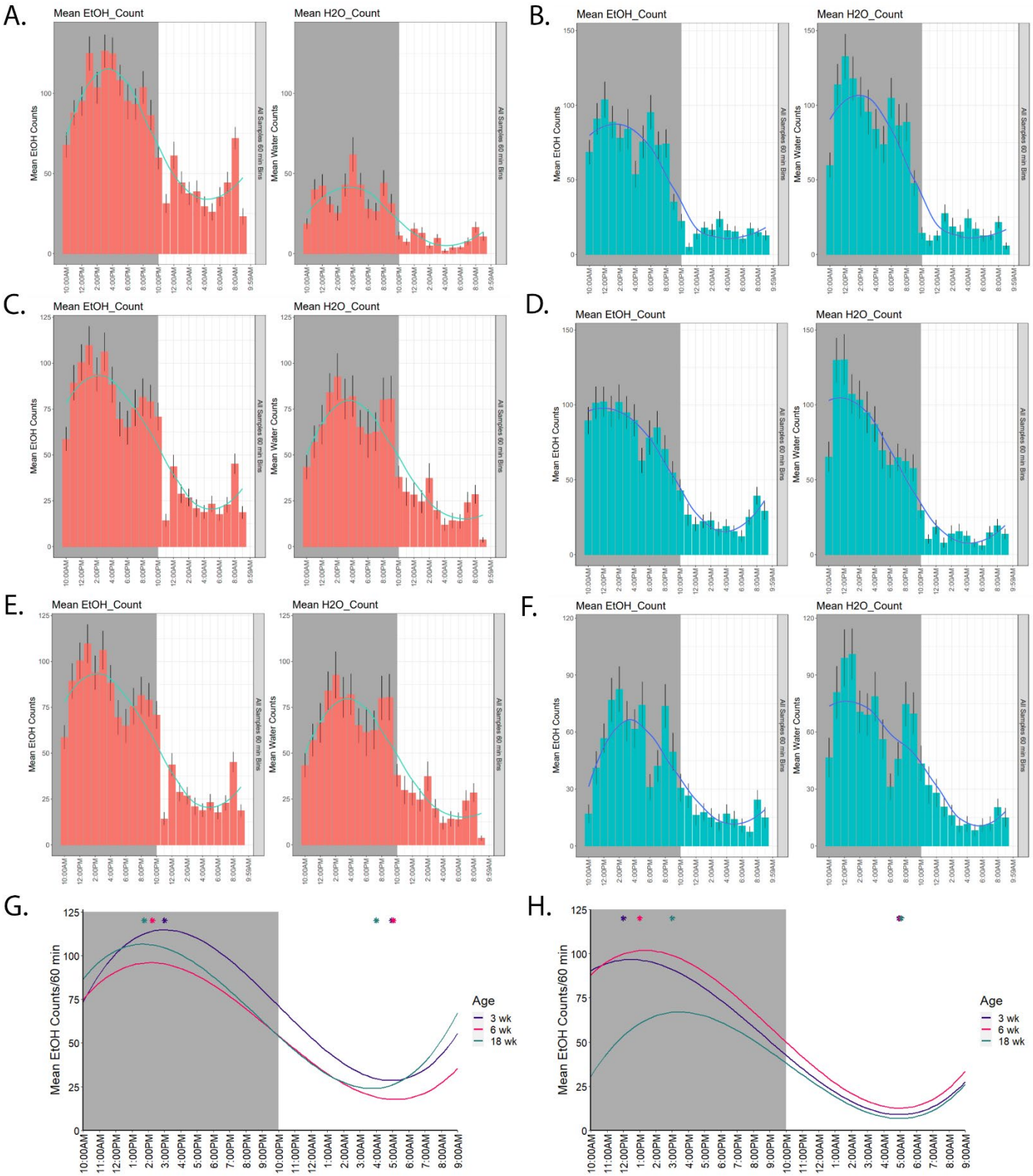
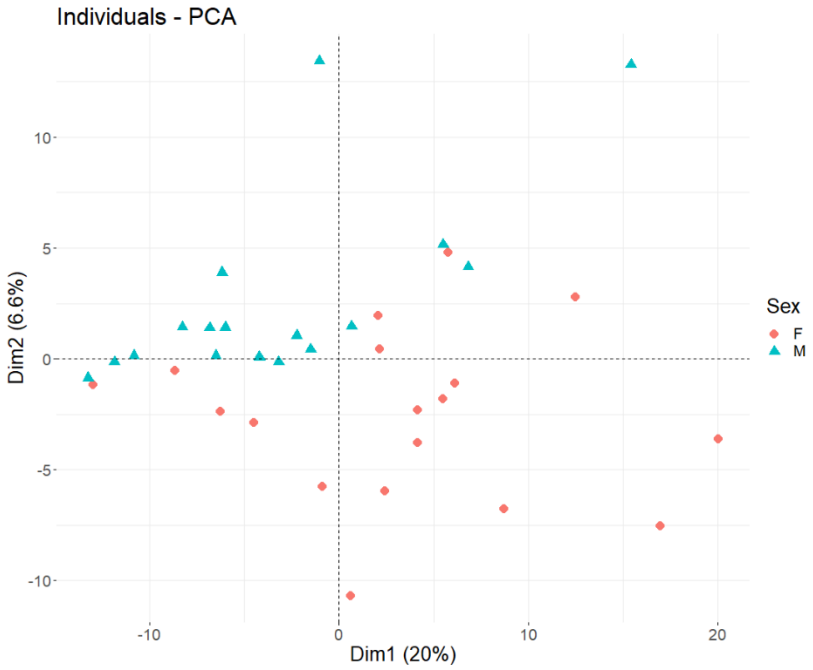


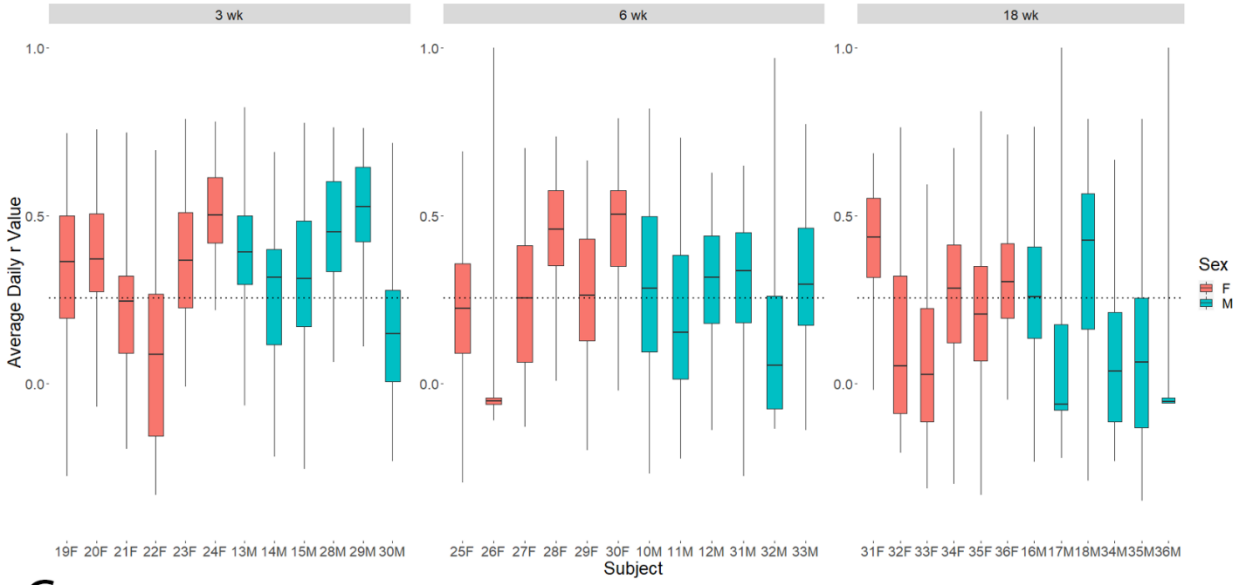
Figure 4. Circadian histogram plots for daily sipper counts across age-sex subgroups for the 14 days of continuous 2BC in 60-min bins. **(A, C, E)** Females, **(B, D, F)** males, **(A-B)** 3-week, **(C-D)** 6-week,

and **(E-F)** 18-week. Nonlinear regression plots summarizing ethanol consumption by age group for **(G)** females and **(H)** males. Data are presented as mean ± SEM. N = 6 per group.

A.


Individuals - PCA
Dim2 (6.6%)
Dim1 (20%)
10
5
0
-5
-10
-10
0
10
20
Sex
F
M

B.


3 wk
6 wk
18 wk
Average Daily r Value
1.0
0.5
0.0
19F 20F 21F 22F 23F 24F 13M 14M 15M 28M 29M 30M
25F 26F 27F 28F 29F 30F 10M 11M 12M 31M 32M 33M
31F 32F 33F 34F 35F 36F 16M 17M 18M 34M 35M 36M
Subject
Sex
F
M

C.

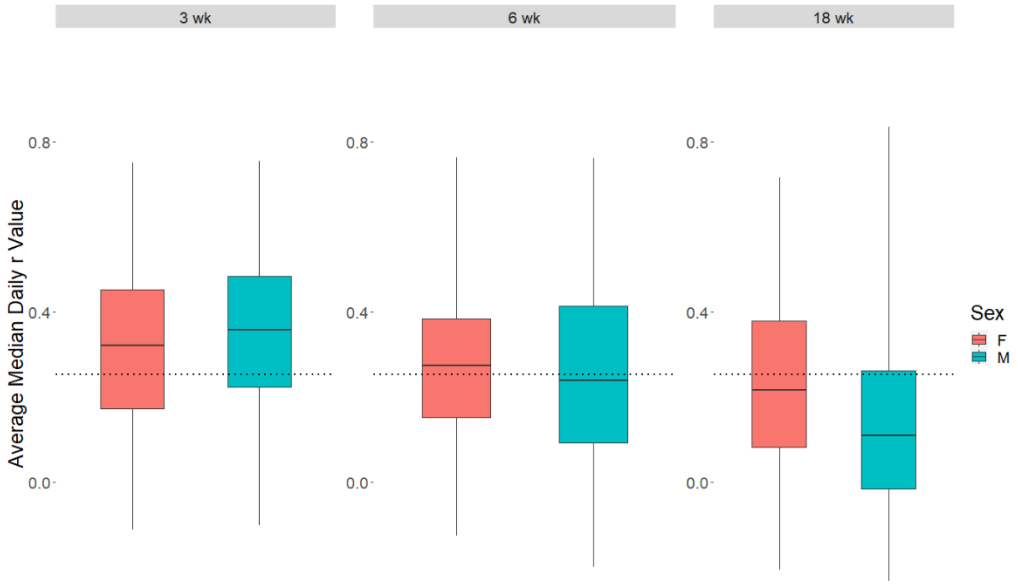

3 wk
6 wk
18 wk
Average Median Daily r Value
0.8
0.4
0.0
Sex
F
M

Figure 5. Pairwise and principal component analyses comparing 60-minute binned ethanol sipper data for ethanol group mice. **(A)** Principal component analysis of average 60-minute binned ethanol sips across the 14 days of continuous 2BC, with biological sex highlighted. The first two principal components are shown, accounting for 20% and 6.6% of variance. **(B-C)** Pairwise comparisons of **(B)** correlation coefficients for individual animals (within-subjects) and **(C)** average correlation coefficients by age-sex group. Average median r-value for all mice (r = 0.253) is plotted as a dotted line across all box plots.

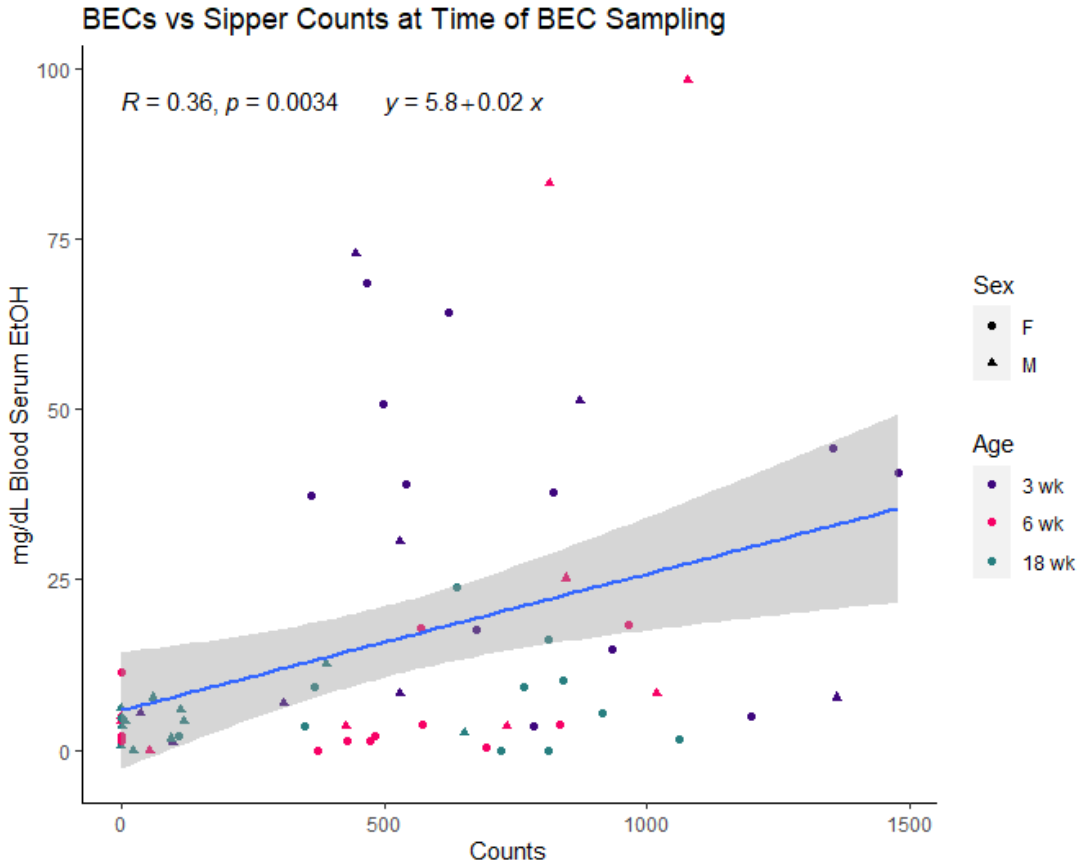


Figure 6. Scatterplot and correlation analysis for measured BECs calculated from a standard curve (in mg/dL) versus total daily sipper counts for ethanol for ethanol group mice for the two days of BEC measurement.

References

1. Center for Behavioral Health Statistics and Quality. 2019 National Survey on Drug Use and Health: Methodological summary and definitions. *Substance Abuse and Mental Health Services Administration* (2020).

2. Martin, C. S. & Winters, K. C. Diagnosis and assessment of alcohol use disorders among adolescents. *Alcohol Health Res. World* **22**, 95–105 (1998).

3. Weitzman, E. R. & Ong, M.-S. Rising prevalence of comorbid alcohol and opioid use disorders in adolescents and young adults in the united states. *J. Gen. Intern. Med.* **34**, 1987–1989 (2019).

4. DiBartolo, M. C. & Jarosinski, J. M. Alcohol use disorder in older adults: challenges in assessment and treatment. *Issues Ment. Health Nurs.* **38**, 25–32 (2017).

5. Han, B. H., Moore, A. A., Sherman, S., Keyes, K. M. & Palamar, J. J. Demographic trends of binge alcohol use and alcohol use disorders among older adults in the United States, 2005-2014. *Drug Alcohol Depend.* **170**, 198–207 (2017).

6. Rhodes, J. S. *et al.* Mouse inbred strain differences in ethanol drinking to intoxication. *Genes Brain Behav.* **6**, 1–18 (2007).

7. Yoneyama, N., Crabbe, J. C., Ford, M. M., Murillo, A. & Finn, D. A. Voluntary ethanol consumption in 22 inbred mouse strains. *Alcohol* **42**, 149–160 (2008).

8. Middaugh, L. D., Kelley, B. M., Bandy, A.-L. E. & McGroarty, K. K. Ethanol consumption by C57BL/6 mice. *Alcohol* **17**, 175–183 (1999).

9. Hefner, K. & Holmes, A. An investigation of the behavioral actions of ethanol across adolescence in mice. *Psychopharmacology (Berl)* **191**, 311–322 (2007).

10. Ho, A., Chin, A. J. & Dole, V. P. Early experience and the consumption of alcohol by adult C57BL/6J mice. *Alcohol* **6**, 511–515 (1989).

11. Cunningham, C. L. & Shields, C. N. Effects of sex on ethanol conditioned place preference, activity and variability in C57BL/6J and DBA/2J mice. *Pharmacol. Biochem. Behav.* **173**, 84–89 (2018).

12. Linsenbardt, D. N. *et al.* Sensitivity and tolerance to the hypnotic and ataxic effects of ethanol in adolescent and adult C57BL/6J and DBA/2J mice. *Alcohol. Clin. Exp. Res.* **33**, 464–476 (2009).

13. Damaggio, A. S. & Gorman, M. R. The circadian timing system in ethanol consumption and dependence. *Behav. Neurosci.* **128**, 371–386 (2014).

14. Danel, T. & Touitou, Y. Chronobiology of alcohol: from chronokinetics to alcohol-related alterations of the circadian system. *Chronobiol. Int.* **21**, 923–935 (2004).

15. Davis, B. T., Voigt, R. M., Shaikh, M., Forsyth, C. B. & Keshavarzian, A. Circadian mechanisms in alcohol use disorder and tissue injury. *Alcohol. Clin. Exp. Res.* **42**, 668–677 (2018).

16. Meyrel, M., Rolland, B. & Geoffroy, P. A. Alterations in circadian rhythms following alcohol use: A systematic review. *Prog Neuropsychopharmacol Biol Psychiatry* **99**, 109831 (2020).

17. Spanagel, R., Rosenwasser, A. M., Schumann, G. & Sarkar, D. K. Alcohol consumption and the body's biological clock. *Alcohol. Clin. Exp. Res.* **29**, 1550–1557 (2005).

18. Kuljis, D. A. *et al.* Gonadal- and sex-chromosome-dependent sex differences in the circadian system. *Endocrinology* **154**, 1501–1512 (2013).

19. Weinert, D. Age-dependent changes of the circadian system. *Chronobiol. Int.* **17**, 261–283 (2000).

20. Valentinuzzi, V. S., Scarbrough, K., Takahashi, J. S. & Turek, F. W. Effects of aging on the circadian rhythm of wheel-running activity in C57BL/6 mice. *Am. J. Physiol.* **273**, R1957-64 (1997).

21. Eckel-Mahan, K. & Sassone-Corsi, P. Phenotyping circadian rhythms in mice. *Curr. Protoc. Mouse Biol.* **5**, 271–281 (2015).

22. Horne, J. A. & Östberg, O. Individual differences in human circadian rhythms. *Biol. Psychol.* **5**, 179–190 (1977).

23. Gentry, N. W., Ashbrook, L. H., Fu, Y.-H. & Ptáček, L. J. Human circadian variations. *The Journal of Clinical Investigation* (2021).

24. Clark, J. W., Fixaris, M. C., Belanger, G. V. & Rosenwasser, A. M. Repeated light-dark phase shifts modulate voluntary ethanol intake in male and female high alcohol-drinking (HAD1) rats. *Alcohol. Clin. Exp. Res.* **31**, 1699–1706 (2007).

25. Farnell, Y. Z., West, J. R., Chen, W.-J. A., Allen, G. C. & Earnest, D. J. Developmental alcohol exposure alters light-induced phase shifts of the circadian activity rhythm in rats. *Alcohol. Clin. Exp. Res.* **28**, 1020–1027 (2004).

26. García-Burgos, D., González, F., Manrique, T. & Gallo, M. Patterns of ethanol intake in preadolescent, adolescent, and adult Wistar rats under acquisition, maintenance, and relapse-like conditions. *Alcohol. Clin. Exp. Res.* **33**, 722–728 (2009).

27. Hasler, B. P., Soehner, A. M. & Clark, D. B. Sleep and circadian contributions to adolescent alcohol use disorder. *Alcohol* **49**, 377–387 (2015).

28. Jia, Y.-F. *et al.* Type 1 equilibrative nucleoside transporter (ENT1) regulates sex-specific ethanol drinking during disruption of circadian rhythms. *Addict. Biol.* **25**, e12801 (2020).

29. Ruby, C. L. *et al.* Differential Sensitivity to Ethanol-Induced Circadian Rhythm Disruption in Adolescent and Adult Mice. *Alcohol. Clin. Exp. Res.* **41**, 187–196 (2017).

30. Dole, V. P., Ho, A. & Gentry, R. T. An improved technique for monitoring the drinking behavior of mice. *Physiol. Behav.* **30**, 971–974 (1983).

31. Dole, V. P. & Gentry, R. T. Toward an analogue of alcoholism in mice: scale factors in the model. *Proc Natl Acad Sci USA* **81**, 3543–3546 (1984).

32. Eisenhardt, M., Leixner, S., Spanagel, R. & Bilbao, A. Quantification of alcohol drinking patterns in mice. *Addict. Biol.* **20**, 1001–1011 (2015).

33. McClearn, G. E. & Rodgers, D. A. Differences in Alcohol Preference among Inbred Strains of Mice. *Q. J. Stud. Alcohol* **20**, 691–695 (1959).

34. Weijnen, J. A. Lick sensors as tools in behavioral and neuroscience research. *Physiol. Behav.* **46**, 923–928 (1989).

35. Godynyuk, E., Bluitt, M. N., Tooley, J. R., Kravitz, A. V. & Creed, M. C. An Open-Source, Automated Home-Cage Sipper Device for Monitoring Liquid Ingestive Behavior in Rodents. *eNeuro* **6**, (2019).

36. Frie, J. A. & Khokhar, J. Y. An Open Source Automated Two-Bottle Choice Test Apparatus for Rats. *HardwareX* **5**, (2019).

37. Flurkey, K., Currer, J. & Harrison, D. Mouse Models in Aging Research. in *The Mouse in Biomedical Research* 637–672 (Elsevier, 2007). doi:10.1016/B978-012369454-6/50074-1.

38. Dutta, S. & Sengupta, P. Men and mice: Relating their ages. *Life Sci.* **152**, 244–248 (2016).

39. R Core Team. R: A language and environmentfor statistical computing. *R Foundation for Statistical Computing* (2019).

40. Nocjar, C., Middaugh, L. D. & Tavernetti, M. Ethanol consumption and place-preference conditioning in the alcohol-preferring C57BL/6 mouse: relationship with motor activity patterns. *Alcohol. Clin. Exp. Res.* **23**, 683–692 (1999).

41. Kishimoto, R. *et al.* Gender-related differences in mouse hepatic ethanol metabolism. *J. Nutr. Sci. Vitaminol.* **48**, 216–224 (2002).

42. Baliño, P. *et al.* Effects of acute ethanol administration on brain oxidative status: the role of acetaldehyde. *Alcohol. Clin. Exp. Res.* **43**, 1672–1681 (2019).

43. Hoyt, L. R. *et al.* Mitochondrial ROS induced by chronic ethanol exposure promote hyper-activation of the NLRP3 inflammasome. *Redox Biol.* **12**, 883–896 (2017).

44. Cui, J. *et al.* Acetaldehyde Induces Neurotoxicity In Vitro via Oxidative Stress- and Ca2+ Imbalance-Mediated Endoplasmic Reticulum Stress. *Oxid. Med. Cell. Longev.* **2019**, 2593742 (2019).

45. Moore, E. M., Mariani, J. N., Linsenbardt, D. N., Melón, L. C. & Boehm, S. L. Adolescent C57BL/6J (but not DBA/2J) mice consume greater amounts of limited-access ethanol compared to adults and display continued elevated ethanol intake into adulthood. *Alcohol. Clin. Exp. Res.* **34**, 734–742 (2010).

46. Akoglu, H. User's guide to correlation coefficients. *Turk. J. Emerg. Med.* **18**, 91–93 (2018).

47. Schober, P., Boer, C. & Schwarte, L. A. Correlation coefficients: Appropriate use and interpretation. *Anesth. Analg.* **126**, 1763–1768 (2018).