Video Article

The FlyBar: Administering Alcohol to Flies

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Abstract

Fruit flies (*Drosophila melanogaster*) are an established model for both alcohol research and circadian biology. Recently, we showed that the circadian clock modulates alcohol sensitivity, but not the formation of tolerance. Here, we describe our protocol in detail. Alcohol is administered to the flies using the FlyBar. In this setup, saturated alcohol vapor is mixed with humidified air in set proportions, and administered to the flies in four tubes simultaneously. Flies are reared under standardized conditions in order to minimize variation between the replicates. Three-day old flies of different genotypes or treatments are used for the experiments, preferably by matching flies of two different time points (*e.g.*, CT 5 and CT 17) making direct comparisons possible. During the experiment, flies are exposed for 1 hr to the pre-determined percentage of alcohol vapor and the number of flies that exhibit the Loss of Righting reflex (LoRR) or sedation are counted every 5 min. The data can be analyzed using three different statistical approaches. The first is to determine the time at which 50% of the flies have lost their righting reflex and use an Analysis of the Variance (ANOVA) to determine whether significant differences exist between time points. The second is to determine the percentage flies that show LoRR after a specified number of minutes, followed by an ANOVA analysis. The last method is to analyze the whole times series using multivariate statistics. The protocol can also be used for non-circadian experiments or comparisons between genotypes.

Video Link

The video component of this article can be found at http://www.jove.com/video/50442/

Introduction

Drosophila melanogaster demonstrate biphasic behavioral responses to alcohol[1] that are analogous to human responses to this drug[2,3]. Upon initial exposure to low concentrations of alcohol, flies exhibit increased locomotor activity, replaced by a lack of motor coordination, the loss of postural control and righting reflexes (Loss of Righting Reflex: LoRR), and sedation (complete lack of motor activity in response to mechanical stimulation) as exposure to alcohol progresses[4-9]. The endogenous circadian clock is a strong modulator of alcohol sensitivity and toxicity as observed in mice[10,11], rats[12], and humans[13]. Recent advances in *Drosophila* research have shown the circadian clock modulates acute alcohol sensitivity but not alcohol tolerance[1]. The powerful genetic approaches available in *Drosophila* through mutant studies and transgenic manipulations of spatial and temporal gene expression provide a system that allows rapid progress in identifying the underlying cellular and molecular mechanisms for complex behaviors. The use of *Drosophila* as an investigative tool has permitted substantive advances in understanding alcohol neurobiology that can be rapidly translated to mammals[14-16]. In order to facilitate understanding of the molecular mechanisms through which the circadian clock modulates alcohol sensitivity and to uniformly measure behavioral responses across circadian time points, an alcohol administration protocol suitable for use in dim red light conditions is required. For *Drosophila*, alcohol may be administered through food supplementation for chronic exposure or reliably through administrating alcohol in the form of vapor for acute exposures. Here, we describe an alcohol administration protocol suitable for the assessment of the circadian modulation of Loss-of-Righting Reflex (LoRR)[1] as well as sedation.

Flies are entrained with 12 hr:12 hr LD cycles at constant temperature and then transferred to a controlled light regime for 2-5 days depending on the experimental question. Flies are exposed to ethanol vapor in a device known as the FlyBar. In this device, controlled amounts of air are bubbled through water and alcohol; the vapors are then mixed and directed into a vial housing the flies. Every 5 min the flies are scored for the number that fails to display righting reflexes or have become sedated. LoRR percentages for each time point are calculated and compared among circadian time points or between strains of flies. The simplicity and reliability of alcohol delivery using the FlyBar alcohol delivery combined with behavioral analysis options provides a significant benefit for circadian experiments conducted under dark conditions.

Protocol

1. Assembly of the FlyBar

Rationale and overview: The system is designed to administer controlled percentages of alcohol vapor to flies. Note: **Figure 1** provides a schematic overview of the FlyBar set-up as described below in three stages (assembly of the air flow, set-up of the alcohol and water bottles, and assembly of the observation vials). In short, a steady airflow is split into two fractions that are bubbled through alcohol and water, respectively, mixed, and administered to 4 observation vials.

1. Assembly of the Airflow
    1. Connect a short piece of flexible silicone tubing to either building air or an aquarium aerator to generate a consistent air flow and split using a y-connector. Connect the first branch to the air flow regulator that controls the total amount of air through the system (typically 1,000 ml/min for 4 observation vials).
    2. Insert a quick connector in the second tube so that the air stream can be interrupted at the beginning of the experiment without affecting the calibrated airflow. Add a y-connector and connect each branching tube to the airflow regulator.
    3. Connect tubing to the airflow regulators and then connect two airflow meters.

2. Set-up of the Alcohol and Water Bottles
    1. Add flexible tubing to the exit of the airflow meters and insert a thin glass tube (sections of 1 ml glass pipettes) with a 90° bend into the end of each length of tubing. This will serve as the inlet air flow into the water and alcohol bottles.
    2. Place rubber stoppers with 2 holes through them into the bottles filled with alcohol and water. Keep both bottles at a constant temperature 2° C higher than ambient air temperature using a water bath. In our experiments, the environmental room is maintained at 25 °C, while the water bath is at 27 °C.
    3. Insert the straight glass pipette for the air inlet through the rubber stopper and extend into the fluid till approximately 1 cm from the bottom of the bottle.
    4. Insert an elbow section of glass pipette into the remaining hole in the rubber stopper until the end of the glass is flush with the bottom of the stopper inside the bottle. Insert this air outlet in another length of tubing.
    5. Reunite the air streams using a y-connector and use another length of silicone tubing to direct the air flow through an empty mixing flask or bottle with bent glass pipette sections inserted through a two-holed rubber stopper. Use another length of silicone tubing for the outlet mixed airstream.

3. Assembly of the Observation Vial
    1. Split the outlet airstream emerging from the mixing flask 2-3x to obtain 4 or 8 smaller streams of air so that multiple observation vials can be used in each experiment. Connect the flexible silicone tubing to the observation vials.
    2. Set up the observation vials using empty vials sealed with a rubber stopper containing two holes through which glass tubes provide an inlet and an outlet for the alcohol vapor.
    3. Cover the end of the first glass tube with netting and keep the netting in place using a small piece of flexible plastic tubing. Insert this tube through the first hole until it extends to approximately half the length of the vial. If needed, use Teflon tape to obtain a snug fit.
    4. Insert the second glass tube also with the end covered by netting until it is flush with the inside edge of the rubber stopper.
    5. Place the vials horizontally on a white piece of paper to maximize contrast with the flies under dim red light conditions.
    6. Mix appropriate fractions of the airstream bubbled through alcohol and the airstream bubbled through water. Monitor the air pressure continuously and make adjustments as needed to maintain desired mixing of the air streams.

        Note: The continuous running of several Fly Bar assays in parallel or even a single assay in a small room can lead to a noticeable accumulation of alcohol vapor. To avoid continual release of alcohol vapor that potentially can affect the researcher in a closed room, an appropriate system needs to be put in place that adequately removes alcohol vapor generated during the experiment. To remove alcohol vapors, connect a 6-12 inch piece of tubing onto the second glass tube protruding from each vial, bundle them and direct to a funnel-vacuum system. Researchers should also ensure that the experimental testing room is adequately ventilated.

2. Preparation of Experimental Animals

Rationale and overview: Proper culture and housing of the flies will reduce variability in the data. This is achieved through standardization and minimization of stress experienced by the flies. For that reason, no anesthesia (CO_2 or alternatives) is used during any of the following steps of the protocol. Furthermore, flies should be age matched across experiments and time points to minimize variability as is standard for other behavioral analyses including learning and memory experiments[17].

Different light:dark conditions can be used to probe the function of the circadian clock in the behavioral response to ethanol. To determine if a diurnal rhythm exists, experiments can be performed under a defined LD cycle to measure performance at specific Zeitgeber Times (ZT). ZT 0 represents dawn and is defined as the time of lights on under LD cycles, while ZT 12 is the time lights are turned off with a 12:12 hr LD cycle. Under constant conditions, the Circadian time (CT) measures time for the animal in the absence of environmental signals, *i.e.* free-running time, and is related to the previous LD entrainment cycle. In wild-type *Drosophila*, CT reflects the previous ZT for the first several days in constant conditions as the free-running circadian period and rhythms are ~ 24 hr. To measure circadian modulation and eliminate acute light effects on behavior, flies are entrained to light:dark cycles and then transferred to constant dark conditions (DD) prior to experiments. Circadian experiments are performed on the second day of DD to measure performance at specific Circadian Times (CT).

In *Drosophila*, continuous light (LL) conditions result in circadian dysfunction with dampened or abolished molecular oscillations of core circadian genes and disruption of behavioral circadian rhythms as evidenced by arrhythmic locomotor activity[18-21] and arrhythmic short-term memory[17]. The protocol is optimized for circadian studies and can be simplified for other experiments. All circadian experiments are conducted using dim red light (ambient overhead red light < 1 lux on bench top; small red lights used at 12 inches from tubes ~ 1 lux light).

1. Rear the flies at 25 °C under 12:12 hr light:dark entrainment conditions (LD).
2. Collect freshly eclosed flies at the end of the day-light period on day 1 and store them for 24 hr under LD conditions in holding vials containing a small amount of high agar concentration food to minimize food stickiness. Note: To ensure healthy, normally developed flies are collected, only use flies collected within the first days after eclosion starts in a culture bottle.
3. Collect batches of approximately 30 (25-35) flies using an aspirator on day 2 towards the end of the light period, and transfer to fresh holding vials.
4. Use a strong light source to direct flies to the far end of the vial. Within this range, the exact number of flies in each vial is not critical as behavioral observations are reported in percentages with total number of flies counted at the end of each experiment.
5. Maintain flies under DD conditions at 25 °C for two days.
6. On the day of the experiment, place all flies housed in different conditions or incubators other than the experimental behavior room in the room for at least 1 hr prior to the experiment. Acclimation reduces variability due to changes in temperature or humidity.
7. Make observations at six time points a day (CT 1, 5, 9, 13, 17 and 21) in order to test for circadian modulation of behavior.
8. Compare multiple time points within a single set of behavioral experiments to increase the robustness of the experimental design and minimize variability specific to a single experiment. For example, observations of CT 1 and CT 13 can be obtained simultaneously if two incubators with opposite light-dark schedules are used for entrainment.

   Note: The above procedure describes the preparation of experimental animals for assays performed in circadian condition of constant darkness. Different light:dark conditions can be used to probe the function of the clock in the behavioral responses to alcohol. To determine if a diurnal rhythm exists the experiments can be performed under an LD cycle to measure performance at specific Zeitgeber Times (ZT). Additionally, the protocol may be used with flies raised under constant light conditions for experiments testing circadian dysfunction. For alternate protocols that test flies housed in light conditions, behavioral assays should still be performed under dark conditions. Flies should be transferred into the dark for 1 hr prior to the experiment to minimize behavioral variability due to the acute effects of light on behavior.

3. Behavioral Observations

Rationale and overview: The following alcohol administration protocol is optimized for observations under dim red light condition. The two behavioral measures LoRR and sedation represent two distinct points of fly inebriation. LoRR represents a late point of inebriation incorporating the loss of motor and postural control, whereas sedation measures a very late end point of intoxication. Genotype or circadian modulation may affect these two measures differently; hence one may wish to examine both. In short, flies are loaded in the vials, the number of flies displaying LoRR or sedation are scored every 5 min during alcohol vapor exposure, and the total number of flies counted at the end of the experiment.

1. Before starting the experiment, run the air through the system (air bubbled through water and alcohol bottles) for at least 10 min and use that time to calibrate the airflows.
2. Disconnect the quick release to stop the airflow. Load the flies in the vials, and reconnect the airflow and start the timers. Note: If unresponsive flies or dead flies are left in the holding vials, this could be indicative of stress conditions. In general, these conditions may be alleviated by housing fewer flies in the holding vials or decreasing food stickiness using a slightly higher agar concentration during food preparation. For optimal behavioral analyses and minimal variability between experiments, flies should be healthy prior to experiments.
3. For accurate time keeping, use one timer to keep track of the total time of alcohol exposure and use a second count-back timer to mark the 5-min intervals.
4. Place a piece of white paper under the vials to increase contrast and fly visibility, especially under dim red light conditions.
5. Check the airflow regularly during an experiment to maintain constant levels. Generally, once airflows have stabilized, they remain stable during the length of the experiment.
6. Count the number of flies that have lost their righting reflex once every 5 min for a 1 hr period. As the alcohol sensitivity varies between genotypes and genetic backgrounds, it may be desirable to perform more frequent assessments or to conduct the experiment for a longer period of time.
7. Lift the vial slightly from the surface, and direct the light from a red-light flashlight toward the paper behind the vial. Keep the hand-held red flashlights at a distance of at least 12 inches to the experimental vial to maintain light levels no greater than 1 lux for all experiments under dim red light conditions.
8. Measure light levels using a light meter to establish standards for all experiments.
9. Determine the number of flies that have lost their righting reflex by applying a firm tap to the vial and count how many flies fail to right themselves within approximately 4 sec. Flies that display LoRR may still move their legs and wings, but cannot turn themselves upright.
10. At the end of the session, count the total number of flies in each vial.

    Note: Occasionally, a single fly may be caught between the stopper and the side when loading flies into the experimental vials. As this is done in the dark, it may not be readily noticed so it is necessary to count the total number of flies at the end of the experiment to correctly calculate percentages.

Additionally, this procedure also may be used to measure sedation of flies, which represents a different behavioral endpoint. While sedated flies have lost their right reflex, sedation requires greater alcohol exposure. Behaviorally, sedation may be characterized by the complete lack of apparent motor activity with flies remaining motionless in the vial following a a firm tap to the vial. For sedation, count the number of flies that remain motionless with no leg waving following delivery of a firm tap of the vial. Additionally, the vial can be rolled side to side to determine whether individual flies still retain their grabbing reflex.

4. Data Analysis

1. Determine the percentage LoRR at each time assessed based upon the total number of flies in each vial.
2. Estimate differences between circadian time points or strains by calculating the 50% LoRR for each sample, which falls within the linear portion of the sigmoid curve (See **Figure 2** below).
3. Alternative statistics:
    1. If comparisons between genotypes are planned, it is desirable to analyze the whole time course using repeated measures ANOVAand to determine the range of time points that the differences are significant with post-hoc tests (**Figure 3**). For these tests, we prefer to use an a value of 0.001. This allows differences at individual exposure times to be assessed as well as differences between genotypes in the slope of the curve.
    2. Differences in sensitivity for can be determined for specific times of alcohol exposure for a particular response such as sedation from the linear portion of the graph (**Figure 4**).
    3. Differences between strains or circadian time points can be estimated using standard F-statistics and post-hoc tests.

Representative Results

Circadian Modulation of Alcohol Sensitivity using the 50% LoRR as a Marker.

A representative example showing circadian modulation in alcohol sensitivity during the day is presented in **Figure 2**. LoRR was measured at six time points during the 2nd day of DD in Canton-S and the 50% LoRR was determined for each time point. Analysis showed a significant effect of time of day (ANOVA: $F_{5,45}$=7.39, p<0.001, N=6-10 per time point). The Fisher LSD test showed significant differences between CT1 vs. CT5, CT5 vs. CT13, CT5 vs. CT17, CT5 vs. CT 21, CT9 vs. CT13, CT9 vs. CT17, and CT9 vs. CT 21. This result is consistent with our previously published results[1].

Differences Between Wild-type and Mutant Flies.

The second example uses time series to show differences in the LoRR and sedation between wild-type Canton-S flies and flies carrying a loss-of-function *white* mutation (w^{1118}) in the same genetic background (**Figure 3**). The w^{1118} mutation is of particular interest to *Drosophila* researchers as transgenic lines are often created using these flies and many mutant lines for circadian clock genes also have the w^{1118} mutation. The results are presented as a time series (**Figure 3**) with data shown for every 5 min during the whole exposure period. Observations are limited to 60 min to avoid the effects of rapid tolerance buildup[22-24]. The w^{1118} mutants display significantly reduced LoRR sensitivity in response to ethanol vapor than does Canton-S (ANOVA between subjects $F_{1,10}$ = 57.12, p<0.001, N = 6). Significance differences (a = 0.001) were found in this experiment from min 20 through min 60 (**Figure 3A**). Differences between w^{1118} and Canton-S were also found in the rate of sedation (ANOVA between subjects $F_{1,10}$ = 137.301, p<0.001, N = 6). In the sedation assay, significant differences (a = 0.001) were found in minutes 50, 55, and 60 (**Figure 3B**). In addition to the lack of screening pigments in their eyes, the w^{1118} mutants also have reduced levels of serotonin, dopamine and histamine[25,26]. These changes in biogenic amine levels may account for the altered sensitivity to ethanol in the w^{1118} mutants[27,28]. Hence, controlling the level of *white* expression in the assayed genotypes may be essential for accurate assessment of ethanol sensitivity.

Circadian Modulation of Sedation.

In the third example, we measured the percentage of Canton-S flies sedated after a set amount of time to determine whether there is a circadian effect of alcohol on sedation (**Figure 4**). We compared the percentage of flies sedated at 40 min (30% alcohol vapor) at CT 5 and 17 and the results show that there are significantly fewer sedated flies during the day compared to alcohol exposure during the night (ANOVA: $F_{1,20}$ = 6.21, p = 0.022, N =10 (CT5) & 12 (CT17)). The flies did not reach the 50% sedation mark within the hour as the observations were made under our standard LoRR conditions in order to make a direct comparison possible. Observations beyond the hour are problematic due to the buildup of rapid tolerance[22-24]. In this experiment, less than 25% of the flies at either circadian time point were sedated at 40 min, indicating that there is a difference in leading edge sedation sensitivity between these groups. Collecting data at this early point in sedation is a useful indication that differences exist, however the ability to determine the effect of treatment on the shape of the distribution in sedation responses is limited. To determine if there is difference in the entire sedation distribution, a higher ethanol concentration should be used to ensure a faster rate of sedation.

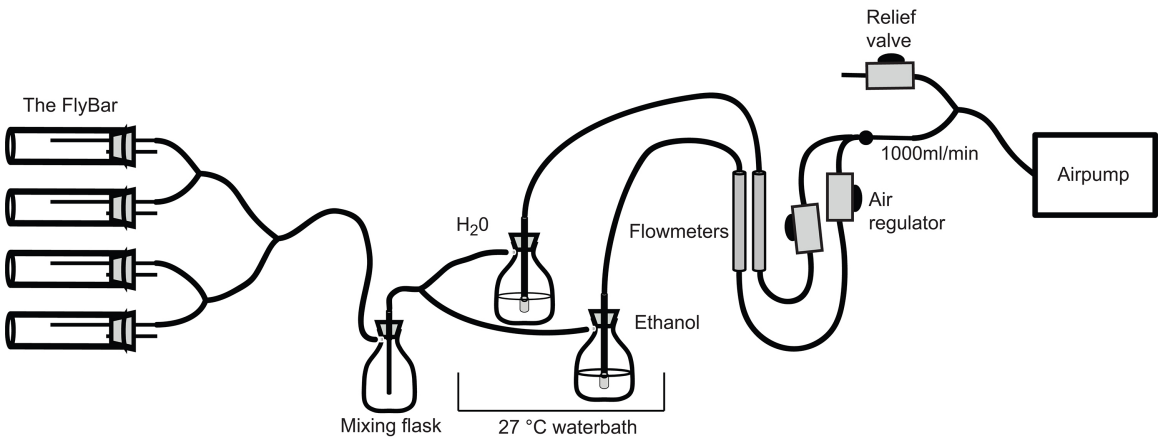

Figure 1. The FlyBar to measure alcohol sensitivity and sedation in fruit flies.

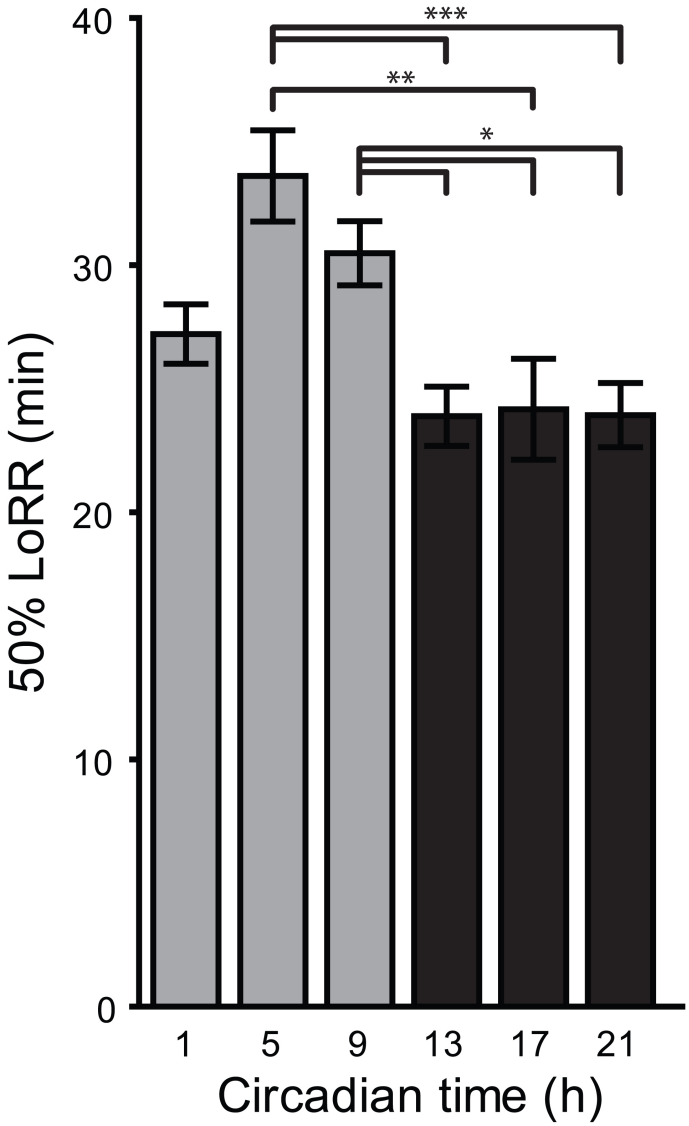

Figure 2. Representative example showing significant circadian modulation in alcohol sensitivity during the day. (ANOVA: $F_{5,45}$=7.39, *p*<0.001, N=6-10 per time point; significant differences between CT5 vs. CT1, CT13, CT17, & CT21 and CT9 vs. CT13, CT17, & CT21).

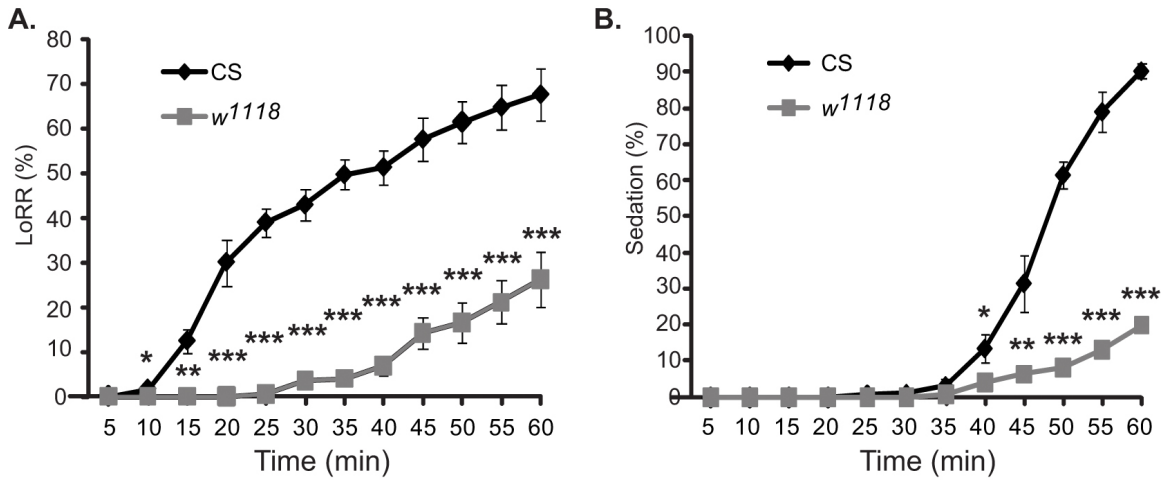

Figure 3. Effects of alcohol on behavioral responses are significantly different between wild-type Canton-S flies and the loss-of-function *white*^{1118} mutant flies. A) Canton-S flies show significantly increased sensitivity to alcohol as measured by LoRR compared to flies with the same genetic background carrying the *white* mutation (ANOVA between subjects $F_{1,10}$ = 57.12, p<0.001, N = 6). **B)** Canton-S flies are more susceptible to alcohol sedation than *w*^{1118} mutants (ANOVA between subjects $F_{1,10=}$137.301, p<0.001, N=6). * p<0.05, ** p<0.01, *** p<0.001.

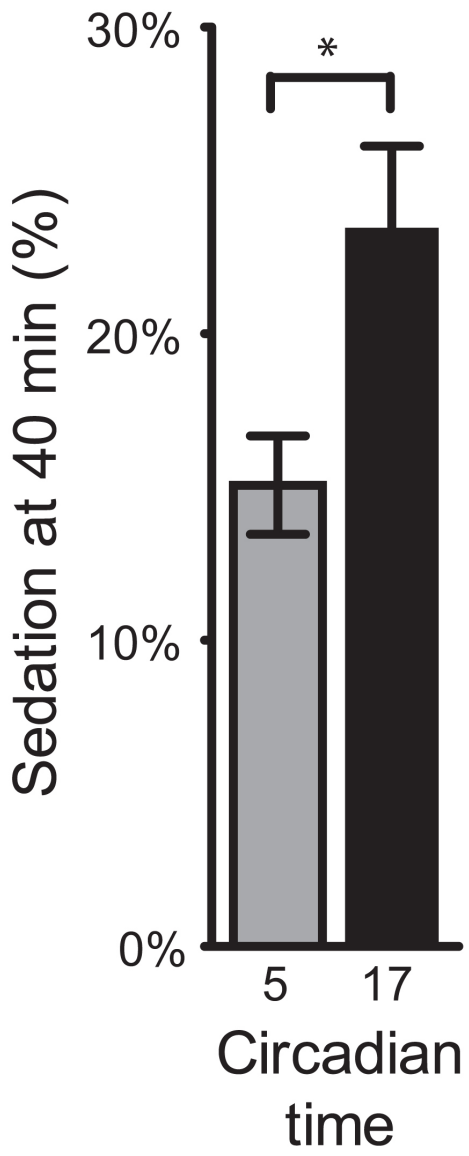

Figure 4. Representative data comparing the percentage of Canton-S flies sedated at 40 min between CT 5 and CT 17. Wild-type flies demonstrate significantly greater increases in initial sedation at CT 17 compared to CT 5 (ANOVA: $F_{1,20}$=6.21, p=0.022, N =10 (CT5) & 12 (CT17)).

Discussion

The costs of alcohol abuse and alcoholism for society is tremendous, both in terms of human[29] and economic costs[30,31]. *Drosophila* as a model offers a fast and versatile system to quickly examine the behavioral responses of a large number of individuals and as such has been extensively used for both alcohol[5,7,32-34] and circadian research[35-37].

Here, we described a straightforward protocol for the controlled administration of alcohol vapor to adult flies under circadian conditions.

Flies cultivated under standardized conditions are exposed to alcohol vapor for 1 hr during which the number of flies that have lost their righting reflex are scored every 5 min. The protocol described herein is optimized for circadian experiments because of the additional requirements for entrainment and housing under constant conditions. Various steps can be simplified for generic studies by removing those steps that are essential for the circadian experiments such as storage in the dark for at least one day or doing the experiments under dim red light conditions. This protocol also can be used to expose large number of flies in a controlled manner to varying alcohol concentrations for subsequent biochemical or molecular analyses. For consistency with other *Drosophila* behavioral experiments such as learning and memory observations, measurement of behavioral responses in dim red light conditions may be desired even for non-circadian experiments.

Variation between independent replicates of the experiment can obscure small differences between mutants or transgenic strains or between circadian time points. It is therefore recommended to test samples of multiple strains or circadian time points simultaneously (see our examples) so that 'replica' can be added as a random variable in order to eliminate the effect of the variation between replicas.

Sensitivity to alcohol varies between strains. Alcohol percentages (the percentage of airflow bubbling through alcohol) need to be adjusted accordingly. As seen in **Figure 3**, flies carrying the white mutation are less susceptible to the effects of alcohol exposure than wild-type Canton-S flies. For analysis of lines carrying the *white* mutation, it may be desirable to increase the percentage of alcohol to which the flies are exposed so as to perform the behavioral assay in the same time frame of other experiments as longer exposure to alcohol can result in rapid tolerance development. For extremely sensitive mutants and for experiments in which it is necessary to decrease the percentage of alcohol used, such as observed for flies containing a mutation in the *yellow* gene, the airflow may have to be increased to calibrate the alcohol saturated airflow accurately. Small increases or decreases (±10%) in total airflow (workable airflow range from 900-1,100 ml/min for 4 observation vials) do not seem to negatively affect the flies.

Observations beyond 1 hr should be avoided when possible because of the potential of rapid tolerance buildup in the flies[22-24] which affects alcohol sensitivity. Instead, determine the alcohol percentage for each strain that results in an approximate 50% LoRR by 30-40 min. If comparison of multiple independent strains is required, choose a single percentage of alcohol that works for all strains.

This protocol depends heavily on behavioral observations, so strict adherence to a standardized protocol is essential to avoid drift in behavioral observations over time. If possible, behavioral observations should be performed so that the observer is blind to the genotype or time point being tested. In order to detect potential bias based on these and unknown other factors, it is advisable to examine the data on a time course and to verify that observations remain within the same range throughout the experimental series.

The FlyBar set-up provides certain advantages over other methods of alcohol administration for flies, particularly for undergraduate researchers or circadian studies on the negative effects of ethanol. An alternate device to measure the effect of alcohol on motor control in flies is the inebriometer, a vertical column in which ethanol vapor is circulated through rising baffles and the loss of postural control or sensitivity of the fly can be measured by determining the time it takes to fall to the bottom of the column[38,39]. The inebriometer provides an automated readout of loss-of-postural control and has proven valuable for alcohol research in *Drosophila*[9,22,39,40], but this behavioral paradigm requires relatively expensive equipment, space for the apparatus, and time to calibrate and optimize conditions. Thus, the inebriometer may not be well suited for many undergraduate teaching laboratories with limited budgets or space, or for researchers performing circadian assays. Another method of delivering alcohol to flies and measuring sedation involves placing a small amount of liquid alcohol on an absorbent material either at the top or at the bottom of a vial and then allowing the alcohol to vaporize with time[41,42]. As the concentration of alcohol vapor increases with time, behavioral responses can be assessed. While this delivery method is easy to set-up, the amount of alcohol vapor to which the flies are exposed varies with time and conditions. For experimental questions in which differences are assessed in initial rates of sensitivity or sedation, such as circadian modulation, it is desirable to have a constant level of alcohol vapor delivered to the flies. Additionally, the exposure of a large number of flies to a constant amount of alcohol exposure, as performed with the FlyBar, is desirable for the accurate performance of downstream cellular or biochemical assays. Another method of delivering alcohol to flies common in early *Drosophila* alcohol research involved mixing the alcohol into the food as it was prepared. While this method is easy and requires little set-up, it is best–suited for chronic alcohol exposure over days as the concentration of alcohol changes with time.

More sophisticated, automated methods are also available for assessing locomotor responses of flies to alcohol exposure including video activity recording and image analysis software[7,43]. These are particularly powerful for assessing the positive, hyper-activating effects of ethanol. However, these automated methods may be prohibitively expensive for undergraduate research projects or teaching laboratories and may not be optimally designed for analysis of a large number of flies under circadian conditions (*e.g.*, video capture under dark conditions requires balanced and diffuse infrared lighting and infrared sensitive cameras). We believe that the FlyBar provides an easy to set-up, cost-efficient method for alcohol delivery system and the assessment of behavioral responses to alcohol that is well-suited to a variety of conditions and laboratory designs.

PROTOCOL MODIFICATIONS:

The protocol described above is aimed at examining the effect of alcohol exposure on the Loss-of-Righting-Reflex in a circadian context. However, the protocol can be easily modified to accommodate other types of alcohol experiments.

Examining the response to alcohol under 12 hr-12 hr Light-Dark (LD; Zeitgeber Time) conditions: Maintain flies under 12 hr:12 hr LD cycle till the experiment. Transfer the flies to the dark approximately 1 hr before the experiment conducted during the light phase (ZT 1, 5, and 9) of the day. This will ensure that the acute effect of light is not confounding the results.

Examining the response to alcohol under constant light conditions: Culturing flies under constant light conditions results in the disruption of their circadian clock[18-21] and arrhythmic responses to alcohol exposure[1]. Flies may be transferred to the dark approximately 1 hr before the experiment so that the flies are tested under the same conditions as flies maintained in LD or DD conditions.

Sedation: Flies that are sedated can be separated from LoRR flies because flies remain motionless on the bottom of the vial, while LoRR flies will still move their wings, head and legs. Flies that exhibit LoRR still respond with subtle movements when the vial is disturbed. Sedation of flies is determined by the counting the number of flies remaining motionless after a firm tap to the vial. Additionally, rolling of the vial can be used to determine whether individual flies still retain their grabbing reflex.

Recovery: The behavioral assay can be extended by measuring recovery as an additional parameter of alcohol response. Discontinue alcohol exposure and continue making observations regarding the LoRR every 5 min. Continue the flow of humidified air through the vials during recovery periods.

Disclosures

The authors declare that they have no competing financial interests.

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