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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 one hour 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 minutes. 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.
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Question	**Response**

The FlyBar: Administering alcohol to flies

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Keywords: neuroscience, alcohol sensitivity, *Drosophila*, Loss of Righting Reflex, sedation, circadian

Short Abstract: *Drosophila* has emerged as a significant model system for dissecting the cellular and molecular underpinnings of behavioral responses to alcohol. Here we present a protocol for the collection of alcohol sensitivity data in a circadian context that can be easily applied to other experiments and is well-suited for undergraduate research.

Long 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 one hour 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 minutes. 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.

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 h: 12h LD cycles at constant temperature and then transferred to a controlled light regime for two to five 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 five minutes 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 four observation vials.

1.1. Assembly of the airflow.

1.1.1. 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 1000 mL/min for 4 observation vials).

1.1.2. 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.

1.1.3. Connect tubing to the airflow regulators and then connect two airflow meters.

1.2. Set-up of the alcohol and water bottles

1.2.1. Add flexible tubing to the exit of the airflow meters and insert a thin glass tube (sections of 1 ml glass pipets) with a 90 degree bend into the end of each length of tubing. This will serve as the inlet air flow into the water and alcohol bottles.

1.2.2. Place rubber stoppers with two holes through them into the bottles filled with alcohol and water. Keep both bottles at a constant temperature 2 degrees 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.

1.2.3. Insert the glass pipet for the air inlet through the rubber stopper and extend into the fluid till approximately 1 cm from the bottom of the bottle.

1.2.4. Insert a second straight section of glass pipet 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.

1.2.5. 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 a two-holed rubber stopper. Use another length of silicone tubing for the outlet mixed airstream.

1.3. Assembly of the observation vial

1.3.1. Split the outlet airstream emerging from the mixing flask two or three times to obtain four or eight smaller streams of air so that multiple observation vials can be used in each experiment. Connect the flexible silicone tubing to the observation vials.

1.3.2. 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.

1.3.3. 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.

1.3.4. Insert the second glass tube also with the end covered by netting until it is flush with the inside edge of the rubber stopper.

1.3.5. Place the vials horizontally on a white piece of paper to maximize contrast with the flies under dim red light conditions.

1.3.6. 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 hour 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 hours. 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).

2.1. Rear the flies at 25^{o}C under 12:12 hour light-dark entrainment conditions (LD).

2.2. Collect freshly eclosed flies at the end of the day-light period on day 1 and store them for 24 hours 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.

2.3. 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.

2.4. 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.

2.5. Maintain flies under DD conditions at 25^{o}C for two days.

2.6. 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 one hour prior to the experiment. Acclimation reduces variability due to changes in temperature or humidity.

2.7. 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.

2.8. 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 one hour 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 five minutes during alcohol vapor exposure, and the total number of flies counted at the end of the experiment.

3.1. Before starting the experiment, run the air through the system (air bubbled through water and alcohol bottles) for at least 10 minutes and use that time to calibrate the airflows.

3.2. 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.

3.3. 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-minute intervals.

3.4. Place a piece of white paper under the vials to increase contrast and fly visibility, especially under dim red light conditions.

3.5. 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.

3.6. Count the number of flies that have lost their righting reflex once every five minutes for a one hour 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.

3.7. 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.

3.8. Measure light levels using a light meter to establish standards for all experiments.

3.9. 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 seconds. Flies that display LoRR may still move their legs and wings, but cannot turn themselves upright.

3.10. 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

4.1. Determine the percentage LoRR at each time assessed based upon the total number of flies in each vial.

4.2. 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

4.3. Alternative statistics:

4.3.1. 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 α 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.

4.3.2. 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).

4.3.3. 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 minutes during the whole exposure period. Observations are limited to 60 minutes 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 (α=0.001) were found in this experiment from minute 20 through minute 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 (α=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 minutes (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.

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 one hour during which the number of flies that have lost their righting reflex are scored every five minutes. 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 - 1100 mL/min for 4 observation vials) do not seem to negatively affect the flies.

Observations beyond one hour 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 minutes. 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 12h-12h Light-Dark (LD; Zeitgeber Time) conditions: Maintain flies under 12h-12h LD cycle till the experiment. Transfer the flies to the dark approximately one hour 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 one hour 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 five minutes. Continue the flow of humidified air through the vials during recovery periods.

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DISCLOSURES

The authors declare that they have no competing financial interests.

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Figure legends

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 minutes 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)).

*Figure
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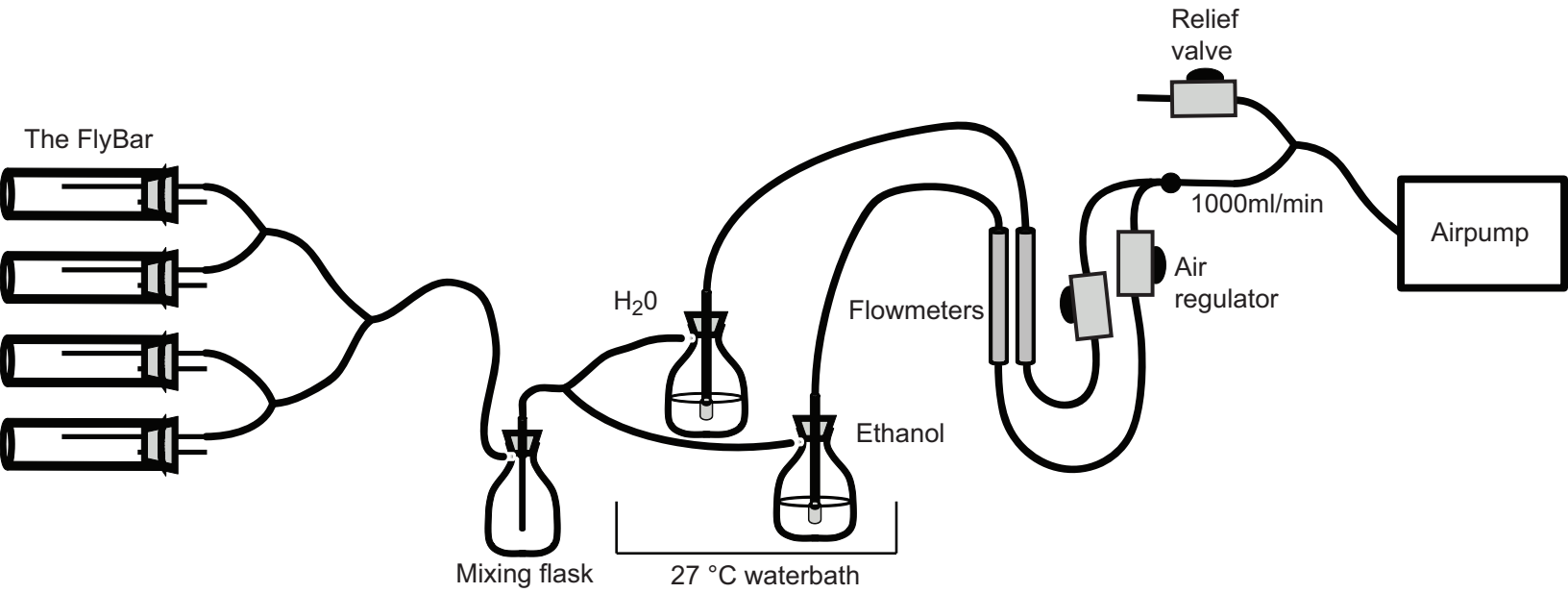

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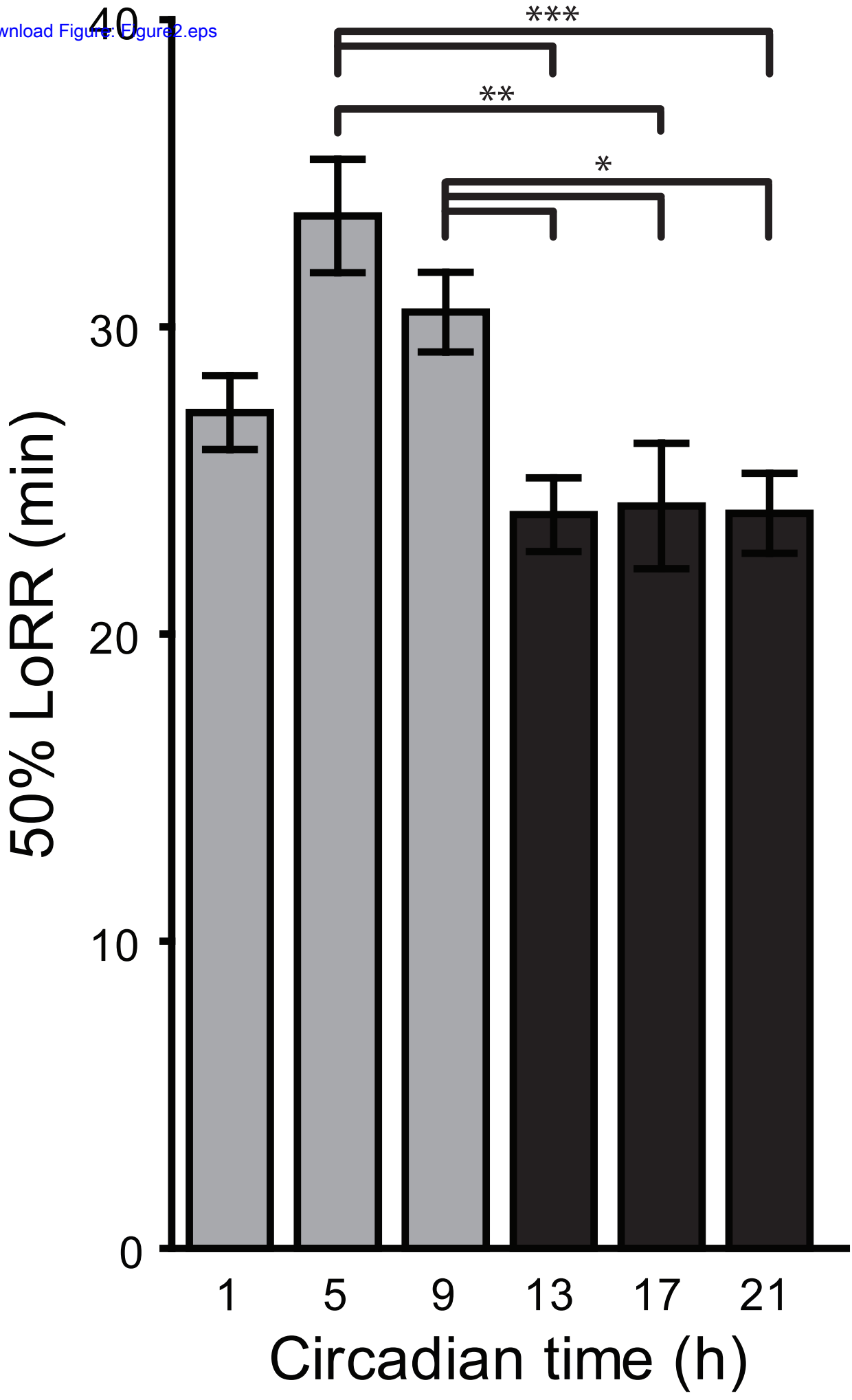

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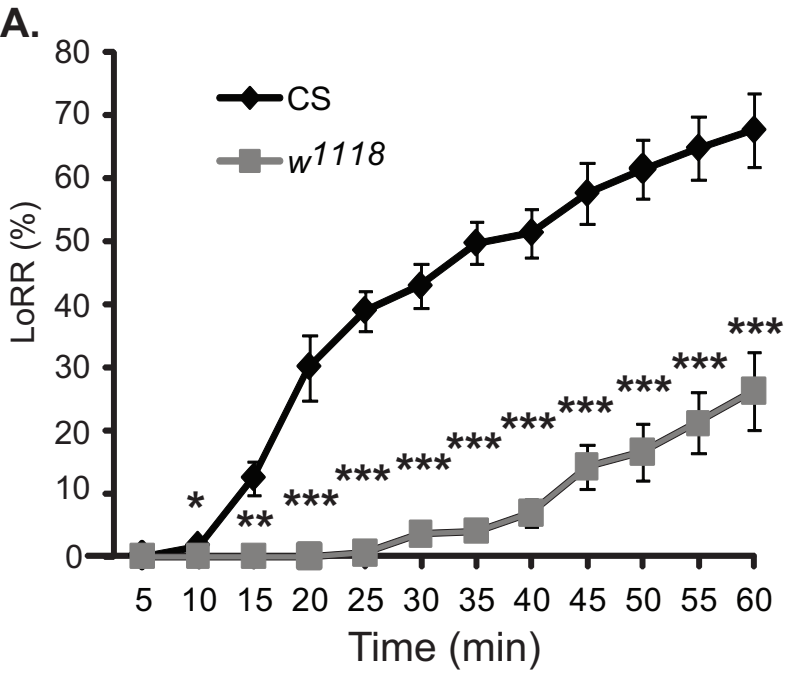

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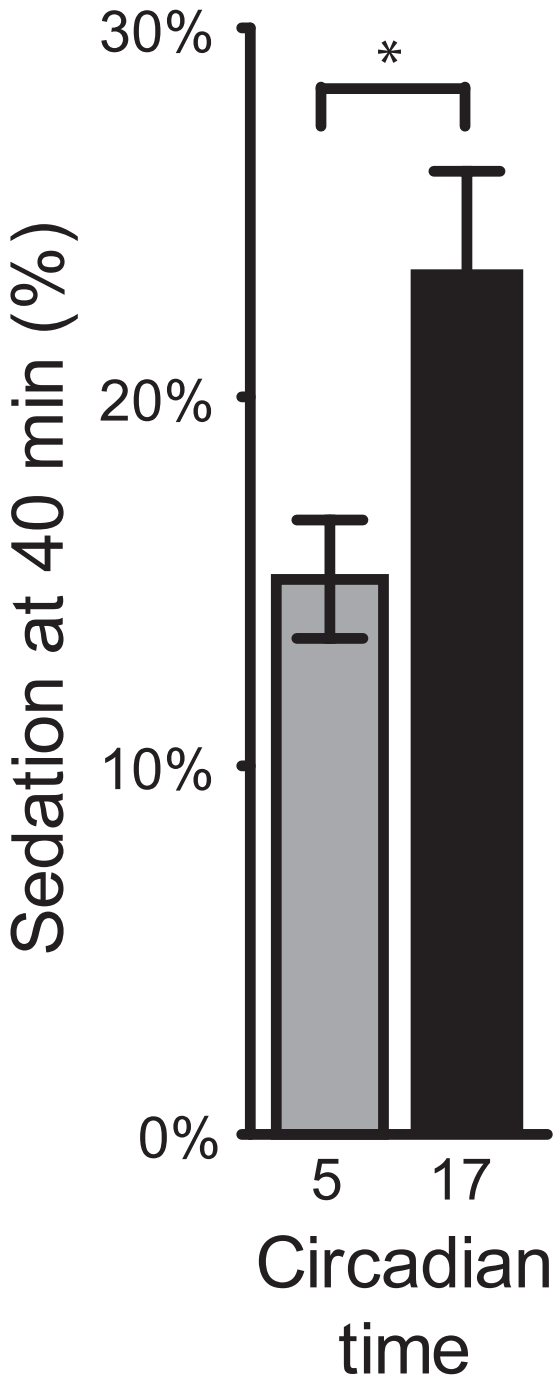

*Table of Reagents/ Materials Used

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Name of Reagent	Company	Catalog Number	Comments
Alcohol 190 proof	Various		

Name of Material	Company	Catalog Number	Comments
Aerator	Local pet store		We use Whisper 60
Silicone tubing 1/8”	VWR	408060-0030	
120° Y Connector	VWR	82017-256	
Quick disconnects	VWR	46600-048	
Plastic tube clamps	Bell-art products	132250000	Either this or next
Miniature Air Regulator	McMaster-Carr	8727K11	Either this or previous
Miniature Air Regulator Mounting Bracket	McMaster-Carr	9891K66	
Gilmont size 12 flow meter	VWR	29895-242	
Tool clips	McMaster-Carr	1722A43	To hold flow meters
Vial	VWR	89092-722	
Rubber stopper with two holes	VWR	59585-186	Fits in vials
5 mm Pyrex Glass tubes	Trikinetics	PGT5x65	Fits best in previous stopper.
Teflon tape	Hardware store		To achieve snug fit in stoppers if necessary
Rubber stopper with two holes	VWR	59582-122	Fits our bottles
Disposable glass pipets	VWR	53283-768	Cut to length and bend by heating
Very fine nylon netting	VWR	Various	
15 watt bulbs	Hardware store		Overhead red light
Photographic red safe light filters			Overhead red light
Mini Flashlights with red filters	Mag-light		

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MS # (internal use):

Dear Dr. Jarzylo,

We are submitting our revised manuscript "The FlyBar: Administering alcohol to flies" for consideration for publication in JOVE. We were pleased that the reviewers felt that the technique would be useful and broadly applicable to undergraduate courses and undergraduate researchers. We greatly appreciated the thoughtful comments and suggestions by the reviewers and have revised the manuscript accordingly. Most of the reviewers' concerns were addressed through manuscript revisions to increase clarity, understanding and applicability of the techniques. However, as Reviewer 2 and Reviewer 4 were concerned with the data presented in the original Figure 3, we have eliminated this figure as we did not want to detract from the instructional value of the manuscript. To better exemplify the points and techniques in the manuscript, we have replaced the original figure with a new figure (new Figure 3) that shows the impact of genotype on behavioral responses. Consequently, the revised manuscript has included Dr. Emiliano Fumagalli as an author on the manuscript. With the manuscript revisions and the addition of new experimental data to illustrate the usefulness of the techniques presented in this paper and the methods through which the data can be analyzed, we think that we have addressed all of the reviewers' concerns. We believe that the revisions we have made in the manuscript highlight the usefulness of the presented technique and illustrate its broad applicability for researchers ranging from undergraduates to experienced scientists.

As detailed below, we believe that we have answered all of the reviewers' concerns. Revisions are shown in BLUE in the marked copy. We have also highlighted 2.75 pages of text to identify the most important portions of the protocol for filming.

Specific Responses to Comments from the Editor and the Reviewers:

Response to the Editor's Comments: As requested, we have highlighted 2.75 pages of the protocol that are most important for filming. We have checked to ensure that we have highlighted complete statements, rather than portions of sentences.

Reviewer #1 Comments:

Manuscript Summary:

This manuscript provides a concise summary of an ethanol administration technique to Drosophila melanogaster. It should be applicable to undergraduate laboratory courses where it can be further adapted and customized.

Major Concerns:

N/A

Minor Concerns:

1.3.7: preventing ETOH exposure to the 'researcher'. The authors might want to emphasize this point a bit more strongly if this technique is to be widely adopted to undergraduate teaching laboratories.

Response: We agree with the reviewer that the remover of alcohol vapor is essential and have strengthened the language in the section on the removal of alcohol to stress this point.

Revised Test for 1.3.7.

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.

Reviewer #2 Comments:

van der Linde and colleagues demonstrate how to administer alcohol to a number of flies, how to measure their response, and how to use circadian entrainment to measure the impact of the internal/external clock on said behavior.

The article is clearly written, and demonstrates a useful behavioral paradigm that will be of considerable interest to the readership of JoVE.

The biggest issue with the paper is that it suffers from unexplained choices offered (discussed in detail below). Given that a JoVE article is a tutorial on how to do things, if choices are offered, they should be explained: you can do A or B, which gives this, or that result/interpretation. Or "we do A, because it allows us to?, which is useful for?"

Once these choices are explained (or removed), the MS is ready for production.

Response: We apologize for the lack of clarity and explanation regarding the possible alternative uses of the protocol in the original submission. As we think it is important to delineate variations and alternative uses of the procedure to make this protocol more broadly applicable to undergraduate as well as graduate research, we have significantly revised the manuscript to clearly explain protocol variations and the possible uses and results available from each alternative. We also included an additional section at the end of the protocol detailing protocol modifications.

Revised Text:

2.7. 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 one hour prior to the experiment to minimize behavioral variability due to the acute effects of light on behavior.

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 12h-12h Light-Dark (LD; Zeitgeber Time) conditions: Maintain flies under 12h-12h LD cycle till the experiment. Transfer the flies to the dark approximately one hour 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 (Hamblen-Coyle et al., 1992; Konopka et al., 1989; Power et al., 1995; Yoshii et al., 2005) and arrhythmic responses to alcohol exposure (van der Linde and Lyons, 2011). Flies may be transferred to the dark approximately one hour 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 five minutes. Continue the flow of humidified air through the vials during recovery periods.

Comment 2. ? "Circadian experiments are performed on the second day of DD. ", yet in

2.4 "flies are transferred to the dark approximately one hour before the experiment.", which means it is not a DD experiment. Then in:

2.5 "observations are made at six time points a day (CT or ZT 1, 5, 9, 13, 17 and 21). " which is either CT or ZT.

This needs rewording along the lines of: if you want to know LD modulation of ethanol sensitivity of your genotype/experimental, you have to entrain in ZT. If you want to know whether the rhythm persists in DD, or if there is a period change in your genotype, then do it in DD. But which day in DD depends on the period change (day2 is ok for a 27hr mutant, but not a 25hr mutant). This needs some fleshing out, given that the FlyBar setup is sold as a circadian assay.

Response: We appreciate the reviewer's suggestions to make circadian protocols more accessible to a broader audience, particularly for undergraduate research. We have revised the wording in the manuscript to more precisely explain the protocol as well as providing additional background information.

Revised Text:

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 hour 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 hours. 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).

A few times "LoRR or sedation" is mentioned. Specifically in 3.5 and 3.6.

Which one should the ignorant reader use, and why? It seems LoRR would precede sedation by the authors' definition, so they are not the same. Can one easily determine motionlessness under dim red light? The authors might want to pick one, or discuss the differences/advantages of the two.

Response: Although it may be surprising, once an individual is used to working under dim red light conditions, it is relatively easy to determine the LoRR or leg movements or other small movements under dim red light conditions. One of the reasons holding the vials in front of white paper we listed as a protocol step is because the increased contrast with the dark fly makes it easier for students to reliably detect movement. We agree with the reviewer that clarification is needed between LoRR and sedation for a novice in the area of alcohol research and we have revised the manuscript to include additional explanation in the Rationale and Overview section.

Revised Text in Section 3. Behavioral observations:

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.

4.2. data analysis three ways.

Again, which one to use, and why? Do they give different insights?

Response: We agree with the reviewer that additional clarification needs to be provided as to the appropriate analysis for particular experimental designs as this technique is well-suited for investigators new to the field of alcohol research and undergraduate researchers. As the appropriate method for data analysis depends upon the questions asked in the experiment, we

have included additional explanation to provide more guidance in choosing a data analysis method.

"4.2.2." claiming to be 4.2.2./Figure 4, does not seem the right choice. This is barely in the linear range of the experiment, and the authors are essentially only counting the 20% of the flies that are most sensitive, the other 80% are excluded. Therefore this is really a measure of the tail end of the distribution of individuals in the two genotypes, not a measure of the "average" fly in the genotype. Is this really a desired measure? (I cannot recall a single Drosophila ethanol paper talking about the tail ends as opposed to the mean/median). While not wrong, the (severe) drawback of this measure needs to be highlighted. (Also, Fig.4 legend does not mention any genotypes, though one assumes they are from Figure 3, albeit with a different color scheme).

Response: Figure 4 assesses sedation in wild-type Canton-S flies at two time points under constant conditions. We have now clarified the genotype within the figure legend. We have also added the following text to the section "Circadian Modulation of Sedation": 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 3 is a rather unfortunate example to put into a methods paper. First, the major point it makes is that (at least) one of the two controls does not behave like wild type. If that's the message the authors wish to convey, they might want to at least turn it into a teachable moment. Second, white and grey are no different around 60 min, where the median is, again highlighting the front end of the distribution of flies, but not the average fly. In this experiment (disregarding elav-Gal4 alone), the 50%LoRR is no different, but the ANOVA/post-hoc is. While this may be true for the given genotypes, can a weak phenotype like this really tell us much? And is it a good case study? If the authors really like this data, they have to address 4.2.1 vs 4.2.2 vs 4.2.3 with this specific example, and what it means.

Response: While we disagree with the reviewer on the interpretation of the data presented in the original Figure 3, we agree with the reviewer that highlighting differences in responses due to mutations or genetic backgrounds provides an important teachable moment and is one of the functions of a technique manuscript. To better address this point, we have eliminated the original figure to maintain the focus on the technique and the important consideration in *Drosophila* research of including genotypic background controls when designing experiments. Instead, we have added a new figure (new Figure 3) that shows the difference between Canton-S (wild-type) and flies carrying the *white* mutation. It is important to look at baseline responses of lines with the *white* mutation as this is frequently the parental line used for making transgenic flies.

Minor points:

Substitute Rationale for Rational.

Response: We have made the corrections throughout the manuscript. .

2.2 what is a sub-optimal individual? Maybe rephrase "to ensure healthy, normally developed flies are collected, only?"

Response: We agree with the reviewer's suggestion and have made the change accordingly.

Result, Figure2: Not sure Fisher LSD adjusts for multiple comparisons, thus not the right statistical test.

Response: We have updated the statistics and described appropriately.

Reviewer #3:

Manuscript Summary:

van der Linde et al. present a brief and clear account of how to set up and use a convenient system to quantify behavioral responses to alcohol in Drosophila with regard to circadian cycles. They also show example data that may be recorded from this affordable system and suggest ways to simplify the system for less elaborate experiments. This description should be useful for new investigators setting up systems to record the responses of flies to alcohol, especially in a primary-undergraduate setting.

Major Concerns:

Pg9. I think it would be very useful to add a brief paragraph to note the pluses and minuses of the proposed system with other systems by other fly labs.

Response: We agree with the reviewer that it is important to present comparisons of available systems so we have added a paragraph in the Discussion comparing our method with other methods to administer alcohol in *Drosophila*.

Additional Text in the Discussion:

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[34,35]. The inebriometer provides an automated readout of loss-of-postural control and has proven valuable for alcohol research in *Drosophila*[9,22,35,36], 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[37,38]. 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[7,39]. 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.

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Minor Concerns:

Minor edits:

Page 4, "manifold" is vague. Is it simply the large Erlenmeyer flask downstream of the water and etoh flasks? Reservoir may be a better description. Please describe what this is, and label it on the diagram.

Response: Manifold was the incorrect term as we really meant a manostat used to regulate the air pressure. To avoid any confusion, we have used the term air regulator in the manuscript and the figure as this more correctly describes the equipment.

Fig 1. Label relief valve in figure.

Response: We have revised the figure as requested.

Pg 9 clarify "Alcohol percentages" in "Alcohol percentages need to be adjusted"

Response: As requested, we have provided additional explanation for clarification.

Revised Text:

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 - 1100 mL/min for 4 observation vials) do not seem to negatively affect the flies.

Pg 9. Would be helpful to note useful range of airflows after "Small increases in airflow do not seem to negatively affect the flies."

Response: As requested, we have included an airflow range that we have found workable.

Pg 13. "Representative example of showing significant" delete "of"

Response: We have corrected the figure legend.

Pg 13. "and the resulting cross, showing show that" delete "showing"

Response: The point is no longer applicable as the figure and legend has been replaced.

Figs 2,3 and 4. Note data with statistical differences with standard symbols and brackets.

Response: We have revised the figures to illustrate significant differences with brackets and asterisks.

Reviewer #4:

Minor Concerns:

What is difference between LORR and sedation? My understanding is that LORR is a measure of sedation.

Response: As detailed above, we have revised the manuscript to clearly explain the difference between LoRR and sedation.

Not sure what the point of 3.7 is.

Response: As flies are not anesthetized when dispersing them between vials to avoid any lingering consequences of anesthesia, it is necessary to verify the total number of flies in each vial at the end of the experiment. Occasionally, a single fly may also 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. We have added clarification in the revised manuscript with this point now listed as 3.9.

It would be great if the authors could show an example of a genetic experiment that actually worked (fig 3).

Response: We disagree with the reviewer's comment that the original Figure 3 represents an experiment that did not work. Figure 3 demonstrates the variation that can occur in the response to alcohol due to genetic background and clearly illustrates the difference in responses between the two parental lines used in the experiment. In our original manuscript, we presented the most conservative statistics by comparing the reduced sensitivity of the offspring only with the least sensitive parent line. One would predict that when the two parental lines were crossed the offspring would exhibit alcohol sensitivity intermediate to the parental responses if there was no impact of suppression of the circadian clock gene cycle. However, as the point of the figure was to illustrate the importance of genotype in behavioral responses to alcohol, we have eliminated this figure and replaced it with a new figure comparing the alcohol sensitivity responses of wild-type flies and the white mutation as this mutation is frequently present in lines used to create transgenics.

Thank you for your consideration of the revised manuscript.

Sincerely,

Lisa C. Lyons