Journal of Visualized Experiments

A simple way to measure alterations in reward-seeking behavior using Drosophila melanogaster

--Manuscript Draft--

Manuscript Number:	JoVE54910R2
Full Title:	A simple way to measure alterations in reward-seeking behavior using Drosophila melanogaster
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	Neuroscience; Drosophila; behavior; Alcohol; addiction; experience; preference; consumption; social; courtship; courtship suppression; mating
Manuscript Classifications:	95.51: Life Sciences (General); 95.53: Behavioral Sciences
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Abstract:	We describe a protocol for measuring ethanol self-administration in fruit flies (Drosophila melanogaster) as a proxy for changes in reward states. We demonstrate a simple way to tap into the fly reward system, modify experiences related to natural reward and use voluntary ethanol consumption as a measure for changes in reward states. The approach serves as a relevant tool to study the neurons and genes that play a role in experience-mediated changes of internal state. The protocol is composed of two discrete parts: exposing the flies to rewarding and non-rewarding experiences and assaying voluntary ethanol consumption as a measure for the motivation to obtain drug reward. The two parts can be used independently to induce modulation of experience as an initial step for further down-stream assays or as a two choice feeding assay on its own. The protocol does not require a complicated setup, and can therefore be applied in any laboratory with basic fly culture tools.
Author Comments:	Dear Editor, Attached please find our revised manuscript and response to the editorial and peer review comments. I would like to extend our appreciations to the reviewers for their important remarks that improved the clarity and accessibility of this visualized protocol to the scientific community. Our response appears in blue below or next to the comments.

Editorial comments:
* JoVE reference format requires that DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information. Done.

* 1.5.1.1: Bloomington #24638 was removed from this step. Please add Bloomington #24638 to the Table of Material. Added to the material excel file.

*Formatting:
-Please spell out author first names and include a comma between Last, First name. Done
-Please include a space between numbers and units. Done

*Grammar:
-Please copyedit the manuscript for numerous grammatical errors. Such editing is required prior to acceptance.
-Line 68 - "using two choice voluntary ethanol consumption assay. We insist that this is not wrong, although this is two choice, it is still an assay.

-Line 103 - "other are needed. Changed to: should take place in a timely manner.

-2.2.1 - "…along the exterior raw of the rack. Changed to: margins of the rack.

-Line 419 - "Other feeding assays that can measure bouts of feeding, their length and temporal regulation like FIIC and EXPRESSO, which are more suitable to achieve high resolution analysis of feeding behavior." - reword to form a complete sentence. We changed the sentence to: Other feeding assays such as FIIC, Flypad and EXPRESSO that measure frequency of feeding events and their bout length, are technologically superior to the CAFE assay and can provide high content temporal data of feeding and ethanol preference.

-Line 423 - "the CAFE approach allow continues monitoring of flies accurate consumption" - several errors here. Changed to: continuous monitoring and accurate consumption of flies over.

-Line 425 - "flies behavior" changed to: interrupting their behavior.

-Line 430 - "as an approximate" - "approximate" not used correctly; "therefor" changed to: consumption assay serves as an estimation for internal reward. Therefor changed to: therefore.

-Line 433 - "the capillaries ends" change to: capillary ends.

*Additional detail is required:

-1.1.1 - Is this a standard fly vial? Yes, details are provided in the Excel material file.

-1.1.2 - Cut in half lengthwise or crosswise? Changed to: Cut across the vial plug.

*Branding: Line 138 "Bloomington", is there a generic term instead? Deleted and added to the "material excel file"
*Results:
-Figure 2: what do error bars represent? SE? SD? Standard Error
-Figure 3: there are no asterisks, were there no P<0.01 results? Fixed, see Fig. 2 and Fig. 3

Reviewers' comments:
Reviewer #1:Manuscript Summary:Details method for reward conditioning and measuring alcohol preference in fliesMajor Concerns:noneMinor Concerns:

Line 56,61 - This manuscript describes - change to "We" changed to: we.

92 - In this publication - "We" changed to: we.

229 - exterior 'row' of the rack Changed to: margins of the rack.

267 - 'Dampen' changed to: Wet the plugs.

Additional Comments to Authors:N/A

Reviewer #2:

Discussion Points-

The sole relationship to measuring fly reward rests on the assumption that ethanol preference is a definitive proxy for changes in reward states following natural reward. The authors should discuss this in the context of their previous findings.

Our previous study shows that there is an inverse correlation between naturally rewarding experiences and voluntary consumption of ethanol. We performed a series of experiments to show that the observed changes in the preference to consume ethanol reflect changes in reward states following different types of social experiences. This included experiments showing conditioned preference to a neutral cue associated with mating ethanol exposure, and the identification of a neuronal system (NPF/R) that connects experience input to behavioral output in the form of both ethanol consumption and conditioned odor preference. Altogether, these experiments suggest that voluntary consumption of ethanol is a valid tool to assess motivation to obtain drug rewards in flies.
In this manuscript, we chose to focus primarily on ethanol self administration, as a tool to assess changes in reward state. Still, it is highly recommend supplementing this approach with classical paradigms measuring reward directly such as conditioned odor preference.
Based on the remarks we chose to add a paragraph to the introduction that describes how our previous study with the NPF/R system couples both natural and drug reward.

What are "internal reward levels?" This term is discussed in a few places in the article, but it is not clear what the authors mean. Would motivation be a better term?

The phrase internal reward levels, is used to describe neuronal representation of rewarding experiences within the brain, based on the assumption that the brain encodes the value and intensity of the rewarding experience. As such, depriving animals from natural rewards is hypothesized to lead to reward deficit, which is reflected by the motivation to obtain other drug or natural rewards. Based on the reviewer comments we used reward states to describe changes in reward levels.

I would like to see the authors come up with a better way to define what they are actually testing.

We are measuring changes in the preference to consume ethanol or the motivation to self-administer ethanol. This has previously been shown to model an addiction like behavior, which is affected rather by the hedonic/pharmacological effects of the drug than its caloric value. We changed the text accordingly.

Does the CAFE assay tube have food besides the control (no EtOH) capillary? It is a known fact that flies on the CAFE assay become starved after a few days, and in fact show decreased lifespan in this assay.

The CAFE assay as described in this manuscript does not contain food besides the two types of capillaries. From our experience, as long as the capillaries are replaced on a daily basis, and the number of flies in the vials does not exceed 8 flies, we do not observe flies being starved for the entire duration of the experiment.

This is important because EtOH is caloric, so the EtOH+yeast/sucrose solution is more caloric then the sucrose/yeast solution. Can the no EtOH solution be normalized for equal numbers of calories by using compounds with no-taste but that are caloric (maltodextrin, sorbitol?) This should be mentioned in the discussion in the least, because in my view it is a major flaw in the current assay.

The question of whether flies display preference to consume ethanol for its caloric value or pharmacological properties is still under debate and was examined in the last few years by several independent studies. The approaches to resolve the question included modulation of the food solutions to equalize the caloric value of the non-ethanol solution using some of the suggested compounds or varying the caloric value of the ethanol solution (for review see Devineni at al).
Early studies suggested that flies could survive longer on a diet consisting ethanol as a sole energy source in comparison to complete starvation (Van Herrewege & David 1980). On the other hand, the survival on ethanol as a sole energy source was found to be much less effective than isocaoloric concentration of sucrose, suggesting that the calories present in ethanol are weakly utilized by flies (Xu et al. 2012). Supporting evidence against the hypothesis of the caloric role of ethanol, comes from the observation that although flies modulate their sucrose consumption to maintain equal caloric intake over different concentrations of sucrose, this is not the case for varying concentrations of ethanol (Xu et al. 2012).
In addition, we showed that the preference to consume ethanol can be dissociated from general food consumption; although rejected and mated male flies display different levels of ethanol consumption, the overall food consumption of both cohorts is similar (Shohat-Ophir et al. 2012). To conclude, we cannot rule out the contribution of Ethanol's caloric value to the development of preference, it most likely that this property contributes to the hedonic/pharmacological role and is not the main reason for the development of preference.

The authors prioritized simplicity over accuracy in their choice to use the CAFÉ assay over others such as the FLIC or flypad (or the recently published EXPRESSO); now that the field has switched using high-resolution methods, it would be helpful to add a sentence about how this assay could be adapted to these new platforms, which have superior behavioral and temporal resolution. Added to the discussion.

As flies find initial ethanol exposure aversive (Kaun et al., 2011) within the first 30 minutes following exposure, it is likely that this effect is contributing at least to Day1's variability in Figure 2. Should add to the discussion.

Devineni et al analyzed the role of different sensory modalities in the development of preference to consume ethanol and found that the taste of ethanol aversive (Devineni et al). Both this and the short-term aversive response to ethanol intoxication can contribute to low preference and variability observed in the first day. Additional factors affecting the development of preference are: learning process associated with two choice assays, and the need to reach a consumption threshold that will facilitate the pharmacological effects of ethanol.

The timeline at the interface of the end of the courtship training and the beginning of the ethanol preference assay was confusing. In Figure 3, if one assumes the "Day" labels refer to the age of the fly as in part A, why in part B do we see ethanol preference for 4-day-old flies, which should not have begun to consume ethanol as they are in the first day of courtship suppression training? It seems that flies in B should be tested during days 8-11 on EtOH, as showed in the A schematic. Also, in Fig. 2, why are flies tested during the "aging" phase, 1-4 days? Changed accordingly.

The figures are very confusing and poorly constructed, the X axis needs to be clear. When are days compared to Fig. 3A? The authors should also add ul consumed, not just preference index, since they are doing CAFÉ assays.
Figures were changed and the requested data was added.

Line 162, what is meant with naïve?
Naïve means virgin males without prior social experience. We added an explanation in

	the text. Line 167, what is meant with individually housed? Can't flies be housed in groups? One can control the social experience of flies and house them either singly or together with other flies in a group (referred as grouped housed). The conditioning protocol is based on single males that interact with either virgin or mated female flies. Prior to the conditioning session, flies can be collected and kept in groups or maintained singly from eclosion on. Line 182, define "virginator stock" by genotype added to the "material excel file Line 219, what is a behavior box? A behavior box is a chamber that minimizes environmental variations and is routinely used in behavioral experiments. One can use even a carton box for this purpose, or build a behavior chamber with temperature, lighting and humidity control. We changes behavior box into behavior chamber. -Major Concerns:CAFE assay is a very primitive tool given the FLIC and FLYpad assays published in 2014Figures need to be better explained We agree with the comment that the CAFE is very basic compared to the technologically advanced FLIC, FLYpad and Expresso systems. As such it requires manual recording and offers only basic consumption readouts compared to the high content data and high throughput features of the above-mentioned tools. Still, the simplicity of the system, being constructed from basic components that are available in every lab, and the simple but continuous readout, offer robust and reliable consumption data. We do not try to claim by any mean that this method is superior, but just to demonstrate visually for the first time the one of the behavioral paradigms we use in lab, that can be useful for others. -Minor Concerns: Some editing and rephrasing necessary -Additional Comments to Authors: N/A Reviewer #3:Manuscript Summary:A simple way to measure alterations in reward-seeking behavior in Drosophila melanogaster" by Zer and others describes a simple set up to measure ethanol consumption preferences after previous mating or non-mating experience of male flies. The assay is thought to measure experience dependent changes in internal state, e.g. reward. It requires a two step procedure. Does the assay measure reward seeking?I am missing one proof of principle. For example dopamine is known as a "reward molecule" in Drosophila. What happens when dopamine is altered? So far the assay measures only ethanol consumption preference and we do not know whether there is a relationship to reward. The CAFE assay is analogous to the two bottle choice assays used in rodent studies for drug self-administration, and has been shown to reflect certain properties of addiction like behavior. When given the choice to consume food or food supplemented with 5-20% ethanol, flies display strong preference to consume ethanol that increases over time and can reach pharmacologically relevant concentrations. Flies would exhibit preference to ethanol even if it contains aversive compounds and show relapse like behavior by rapidly returning to high levels of ethanol preference after a period of two days deprivation Devineni and Heberlein 2009). The link to reward comes from experiments showing that activation of NPF neurons inhibits ethanol preference, and perceived as rewarding in the well-established conditioned odor preference assay (measuring conditioned responses to a neutral cue associated with the activation). This inverse correlation suggest that rewarding experiences, reduce the motivation to self administrate ethanol. From the presentation within the draft we do not know whether reward related behaviors are indeed altered. The increased "drinking" in non-mated males could be also due to training effects and not related to reward. Mating males have other things to do than drinking and have less drinking experience. Alternatively the assay could be renamed and the title of the manuscript changed.

	Previously it has been shown that mating experience and ethanol intoxication is rewarding to flies using conditioned responses to a neutral cue associated with mating or ethanol intoxication (Kaun, Shohat-Ophir). In addition, as mentioned above we identified a molecular signature of both mating experiences and ethanol intoxication in the form of changes in NPF levels and shown that there is a causal link between the NPF system, ethanol consumption and perception of reward. When I have decided that I would like to use this assay, I would have questions related to the experimental strategy. I am not sure how alterations of the behavior would look like and how to plan my experiments in respect to the number of animals required to perform an experiment successfully. I need to know how many animals I have to take and how high the variance of the assay is.In general it would be nice to be briefly introduced into the assay as a whole. This could be done by modifying Figure 1, before mentioning all the details for the used vials. In addition it would be easier to understand when fig2 and fig 3 are pooled together. I would have liked to know the infos earlier, the infos in the REPRESENTATIVE RESULTS appear quite late. Do we need a JOVE for it?Yes, it is important to describe such a tricky assay in detail! What I like about it is that it is a two-step assay that could be separated. However the second part is still highly variable. It would benefit from a second JOVE that complements this approach. We feel that both parts can still go together. How robust is the assay? 1.1.5 "Maintain the positions of the two types of the capillaries consistently…."Why? This should not matter for the choice of the flies, otherwise this raises questions regarding positional effects of the capillary and its influence on feeding. When analyzing the consumption data along the days of the experiment, flies display constant consumption of non-ethanol containing food, and increase their consumption of ethanol containing food, resulting in overall increased preference over time. Altering the position of the ethanol-containing capillary on a daily basis resulted in a constant, rather than increasing, level of preference (Xu et al. 2012). This may reflect learning and memory processes that allow flies to reliably discriminate which capillaries contain the ethanol food and reinforce their preference, possibly by leaving chemical cuses next to the preferred capillaries. Statistic is missing (Fig. 2) and a discussion of possible pitfalls. Done The variance of the preference appears quite high, number of experiments and tested animals needs to be mentioned and discussed. We made changes to explain this better. Are the data significant different from random choice? Done Some details: *Page4: The preparation for the CAFE set up refers to Fig.1a and Fig.1b. There is only one Figure 1. Figure 1: Experimental set up contains one picture. Changed to Fig.1 *Step two: cut micro-pipets…what kind of pipets? This is difficult to understand. Explained in the "material excel file" *Preparing small glass food vials: Bloomington cornmeal molasses recipe: I would like to have the recipe without looking somewhere else. A link to the recipe was added. *2.1.1: Here it would be good to mention that the 12h/12h light cycle could be changed to a convenient starting time. Added accordingly. *2.3.1 At the end of the last conditioning session (fourth day), aspirate the male flies into the capillary feeder vials and pool single males into groups of 6-8 fliesit would be better to mentioned that single males are grouped to perform the second part of the assay. Done

*2.3.4 It is not clear what a capillary adapter is (maybe a picture?). This is indicated in the figure.

*2.3.7. In addition to vials that contain experimental flies, prepare a mock control vial without flies to assess natural evaporation…."Here it would be interesting to know how high the evaporation rate for the experimentar normally is…to assess the variance of the assay. Evaporation is usually up to 1-1.5 mm.

*Figure 2. Preference of EtOH intake: here we need statistics. How many N's and is the PI different from random? In addition it would be nice to see the total consumption of non EtOH versus EtOH solutions. Done

*Line 372: The present experiment supports our previous findings, suggesting that sexual experience and not housing conditions modulate ethanol consumption.I believe that this is true. However to support the experimental set up and robustness of the behavior a statistical analysis of the data is important for the procedure. Exist

*Fig3. In the pict there is no statistics. Done.

*For the controls: the evaporation control for the CAFÉ assay is rather vague, since it is not known how variable the assay in general is. When relative humidity is maintained around 60%, the evaporation reaches no more than 1mm in the mock vials.

*The pitfalls need to be discussed and the pro and cons in comparison to other techniques. Discussed

*It would be nice to know how the flies are set up that are used for the experiments: The following statement is weak:"start with healthy and large male flies eclosed from a well-populated fly bottle". We changed this sentence.

More general concerns:I am wondering whether it is technically possible to see within 200 vials every 15 min whether males mate. Is "mating" = copulation? There is a lot of literature out there that described that mating is more complicated than that.
Thank you for this remark; we meant copulation. Courtship ritual in flies is indeed more complicated, and the analysis of required recording the behavior and manually going over the movies and scoring the behavior (e.g. wing vibration, attempted copulation, licking and so on). Once copulation is achieved, it lasts for about 20min and is very obvious and easy to see by naked eye, therefor it is very easy to quickly watch all the vials and identify copulation events.

One more general comment: the introduction gives a reference of 3 - 35 papers for one sentence and the next 10 citations are in the discussion. This is an interesting format and might not reflect an in depths discussion of the pros and cons of the assay and the possible mistakes/ technical problems.
We moved most of the references to the discussion and shorten the list.

Major Concerns:N/AMinor Concerns:N/AAdditional Comments to Authors:N/A

[Editorial recommendation: Please keep JoVE's protocol requirements in mind as you address the above comments - the protocol must contain sufficient detail in order to enable users to accurately replicate your technique. We recommend NOT removing any details from the protocol text.]

Bets wishes,

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Question	**Response**
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TITLE:
A simple way to measure alterations in reward-seeking behavior using *Drosophila melanogaster*

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KEYWORDS:
Neuroscience, *Drosophila*, behavior, alcohol, addiction, experience, preference, consumption, social, courtship, courtship suppression, mating.

SHORT ABSTRACT:
We describe a protocol for inducing rewarding and non-rewarding experiences in fruit flies (*Drosophila melanogaster*) using voluntary ethanol consumption as a measure for changes in reward states.

LONG ABSTRACT:
We describe a protocol for measuring ethanol self-administration in fruit flies (*Drosophila melanogaster*) as a proxy for changes in reward states. We demonstrate a simple way to tap into the fly reward system, modify experiences related to natural reward, and use voluntary ethanol consumption as a measure for changes in reward states. The approach serves as a relevant tool to study the neurons and genes that play a role in experience-mediated changes of internal state. The protocol is composed of two discrete parts: exposing the flies to rewarding and non-rewarding experiences and assaying voluntary ethanol consumption as a measure of the motivation to obtain a drug reward. The two parts can be used independently to induce the modulation of experience as an initial step for further down-stream assays or as an independent two-choice feeding assay, respectively. The protocol does not require a complicated setup and can therefore be applied in any laboratory with basic fly culture tools.

INTRODUCTION:
Modification of behavior in response to experience allows animals to adjust their behavior to changes in their environment[1]. During this process, animals integrate their internal physiological state with the changing conditions of the external environment and subsequently choose one action over another to increase their chances of survival and reproduction. Reward systems evolved to motivate behaviors that are required for the survival of individuals and species by reinforcing behaviors that enhance immediate survival, such as eating or drinking, or those that ensure long-term survival, such as sexual behavior or caring for offspring[2]. Artificial compounds such as drugs of abuse also affect reward systems by co-opting neural pathways that mediate natural rewards[2].

During the last two decades, the fruit fly *Drosophila melanogaster* has been established as a promising model for studying the molecular and neuronal mechanisms that are shaping the effects of ethanol on behavior[3,4].

Previously, we have identified a subset of peptidergic neurons in flies (NPF/NPF receptor neurons) that couple natural rewards, such as sexual experience, to the motivation of obtaining drug rewards[5]. NPF expression is sensitive to both sexual experiences and to drug rewards, such as ethanol intoxication. Changes in NPF expression levels are converted to alterations in ethanol self-administration[5], where high NPF reduces and low NPF increases the preference to consume ethanol. Activating NPF neurons is rewarding for flies, as they display strong preference for an odor paired with the activation, which is also reflected by reduced ethanol consumption. More importantly, activation of NPF neurons interferes with the ability of flies to form a positive association between ethanol intoxication and an odor cue. The causal link between the NPF/R system, reward memory, and ethanol consumption suggests that one can use ethanol self-administration as a measure for changes in reward states[5].

In this publication we demonstrate an integrated approach for tapping into the fly natural reward system and assaying changes in reward states. The approach consists of two separate parts, a training protocol for manipulating natural reward-related experiences, followed by a two-choice capillary feeder assay (CAFE) to assess ethanol self-administration as an estimate for changes in reward states. The CAFE assay is analogous to the two-bottle choice assays used in rodent studies for drug self-administration and has been shown to reflect certain properties of addiction-like behavior in flies[6].

PROTOCOL:

Note: General overview of the experimental design: The experimental design includes an adapted protocol for courtship suppression[7-9] in which male flies are exposed to rewarding and non-rewarding experiences in 3 consecutive training sessions over the course of 4 days. At the end of the experience phase, the flies are tested in a two-choice voluntary ethanol consumption assay for 3-4 days. The protocol herein includes several preparatory steps, some of which can be done in advance to be used in more than one experiment, while others should take place in a timely manner before the beginning of the experiment (Table 1).

1. Preparatory steps

1.1 Preparing the capillary feeder system

1.1.1 Heat a 27-G needle and use it to puncture small holes along a vial (Figure 1).

1.1.2 Cut the vial plug in half (crosswise) using a razor blade. Mark four points on the surface of the plug, creating a square shape to position the capillary holders into. Use an 18-G needle to make four holes through the plug (Figure 1b).

1.1.3 Use a razor blade to cut micro-pipets so that they can firmly hold 5 µL glass capillaries, which serve as adaptors.

1.1.4 Insert four capillary adaptors into the prepared plugs.

1.1.5 Mark the position of the two ethanol- and the two non-ethanol-containing capillaries on the plug.

Note: It is recommended to keep the positions of the two types of capillaries consistent across all vials. Pay attention to the size of the holes, as large holes allow the escape of flies out of the vials. In addition, it is recommended to use the smoother side of the plug (usually opposite the cut side) as the side that faces down when inserted into the vial, as cutting the plug creates some small, loose fibers that the flies can get tangled in.

1.1.6 Prepare a large number of vials and adaptors to be used for the capillary feeder system (CAFE) experiments described in step 2.3.

1.2 Preparing small glass food vials

1.2.1 A day before collecting the flies (step 1.4), arrange 200 glass vials on microcentrifuge tube racks. Melt fly food (according to the cornmeal molasses recipe, see the table of

materials) using a microwave. Pour 2 mL of fly food into the glass vials (try not to smear the food on the vial walls). Let the food harden, and cover the vials with plastic wrap.

Note: The glass vials containing food can be kept at 4 °C for several weeks.

1.2.2 On the day of fly collection, take the vials out of the refrigerator and let them reach room temperature before using them to house flies.

Note: If food vials are several weeks old, make sure that the food is still intact and not detached from the vial, as flies can be trapped in the cracks between the food and the glass.

1.3 Making stock food solution for the capillary feeder assay

1.3.1 To prepare the stock food solution (5% sucrose and 5% yeast extract), dissolve 5.88 g of yeast extract and 5.88 g of sucrose in 100 mL of distilled water.

Note: The stock solution is highly prone to contamination. Therefore, it is recommended to autoclave it (121 °C, 30 min, and without a drying cycle) and aliquot it into 850-µL working aliquots to be kept at 4 °C until further use. The concentration of the ingredients in the stock solution is 5.88% and will reach the final concentration of 5% after the addition of 15% ethanol or water (150 µL into 850 µL of premade food solution) just before its use in step 2.3.

1.4 Collecting experimental flies

1.4.1 Collect 200 naïve male flies using a CO_2 pad.

Note: Use any lab strain relevant for the experimental question. 250 flies can be easily collected from five bottles of flies at their eclosion pick phase. When collecting the flies, pay careful attention to collect flies that have recently eclosed (virgin-looking flies: bright flies with a meconium spot on their abdomen). Experimental flies should be kept singly in small glass vials containing food and aged to 3-4 days old before being used in the experiment.

1.4.2 Use soft plugs to close the vials.

Note: The same soft plugs will be very useful for the training phase when using mouth aspiration to insert females into and out of the vials during the experience phase.

1.5 Collecting trainer flies

Note: All collected flies should be kept in a 12-h/12-h light/dark incubator at 25 °C and 70% relative humidity (RH). The 12-h/12-h light/dark cycle can be changed to a convenient starting time.

1.5.1 Virgin female trainer flies

1.5.1.1 Collect 500 virgin female trainer flies from a wild type stock or a virginator stock for use as virgin receptive females. Keep the female flies in groups of 25 per regular-size food vial, and allow them to age to 3-4 days old before using them in the experiment.

1.5.2 Mated female trainer flies

1.5.2.1 Collect 300 virgin female flies from a wild type stock or a virginator stock to undergo mating before the training phase (step 1.6.1).

Note: Female flies are kept in groups of 10 females per regular-size food vial and aged to 3-4 days old before being used in the experiment.

1.5.3 Male flies used to generate mated female trainer flies

1.5.3.1 Collect 150 male flies (not necessarily virgin).

Note: Flies are kept in groups of 15 males per regular-size food vial and aged to 3-4 days old before being paired with virgin female flies to create the mated trainer females.

1.6 Generating mated female trainer flies

1.6.1 To generate mated female flies to be used as trainers, pair groups of 10 virgin female flies with 15 male flies by flipping one male-containing vial into a female-containing vial 16-18 h before the training phase (step 2.1)[10].

Note: This should normally take place the evening before the training session. The 1.5:1 ratio between male flies to female flies assures that all females will be mated.

1.6.2 On the day of the experiment, separate the mated females from the males by aspirating the males out of the vial using a mouth aspirator. Perform this right before the beginning of each training day.

2. Experimental steps

2.1 Setting up the experience phase

2.1.1 Start the experiment as close as possible to the beginning of the light phase in the incubator. Place the trainer females (both virgin and mated) in the behavior chamber.

Note: It is recommended to perform the experiment in a behavior chamber, or any secluded environment that is quiet and set to 25 °C and 60-65% RH. Keeping humidity over 50% is very important, since courtship and mating behaviors depend on pheromone sensing, which is known to be sensitive to humidity.

2.2 Exposing male flies to rewarding and non-rewarding experiences

2.2.1 Arrange the glass vials containing the single male flies on microcentrifuge racks, positioning the vials along the margins of the rack.

Note: This arrangement allows the experimenter to easily observe the pairs of flies that undergo copulation without taking the vials out of the rack.

2.2.2 Prepare (as described in step 2.2.1) two groups of 100 glass vials containing single male flies on separate racks for males undergoing rewarding and non-rewarding mating experiences (*i.e.*, rejected and mated).

2.2.3 Aspirate trainer females and add them to each vial containing the male flies. Set the timer for 1 h. Starting with the rejected group, add a mated female to each vial. Then, move on to the males to be mated and add a virgin female to each vial.

2.2.4 Watch the mating encounters closely to make sure that the males interacting with virgin females mate successfully within the first hour and that the males interacting with the previously-mated females do not mate. To monitor the rejected cohort over the course of 1 h, set a second timer for 15 min and watch the males every 15 min.

2.2.5 Discharge the males from the rejected cohort that managed to mate and males from the mated cohort that did not end up mating by the end of the first training session.

2.2.6 Finish the first training session by aspirating out the previously-mated female trainers into a regular fly food vial, and keep them for the next conditioning session. Follow this step by gently aspirating the females from the mated male group.

2.2.7 Let the flies rest for 1 h.

2.2.8 Repeat steps 2.2.3-2.2.7 twice.

2.2.9 Repeat the training session in steps 2.2.3-2.2.8 each day for three more days.

2.3 Recording consumption (CAFE)

2.3.1 At the end of the last conditioning session (fourth day), aspirate the single males and group them into the capillary feeder vials. Use a soft plug to cover the capillary feeder vial while inserting the flies, and replace it quickly with the ready-made capillary feeder plug after all the flies have been inserted. Group 6-8 flies from each of the experimental groups into a vial, creating no less than 8 vials per condition.

2.3.2 Wet the plugs by gently adding 5 mL of water to the top of the plug. Be careful while adding the water to avoid spillage into the vials. Add equal amount of water to each vial.

Note: This step is done to reduce evaporation from the capillaries. Therefore, it is repeated every day for the rest of the experiment. Depending on the relative humidity, the following days may require less water to be added to the plugs.

2.3.3 Prepare two separate food solutions by adding ethanol or water to the stock food aliquots to create a final concentration of 15%.

2.3.4 To fill the capillaries with the two food solutions, create 5-µL liquid food drops on a laboratory film strip and allow the solution to fill the capillary until it reaches the 5-µL mark. Make sure to use the clear end of the capillary, which is close to the 5-µL black mark line.

Note: To fill several capillaries at once, it is possible to connect 6-8 capillary adaptors together and use them as an insert to hold the capillaries.

2.3.5 Tap the capillaries gently on the paraffin strip to position the food solution exactly at the black mark. Seal the capillaries by dipping the end of each capillary into small drops of mineral oil. This step minimizes potential evaporation of the food solution.

2.3.6 Insert two ethanol-containing capillaries and two non-ethanol containing capillaries through the four adaptors/holders in the plug until the exposed sides of the capillaries barely peek from the interior of the vial (1 mm or less from the plug surface).

Note: Keeping the opening of the capillaries close to the wet plug reduces evaporation and allows easy access to the food, as flies normally sit on the plugs and climb down on the exposed capillary to feed.

2.3.7 In addition to vials that contain experimental flies, prepare a mock vial without flies. This control serves to assess natural evaporation.

Note: Try to maintain relatively equal positioning of the capillary opening compared to the plugs, as differences can lead to variable evaporation and to misleading consumption data. Maintain adequate humidity during the experiment and position the capillary openings as closely as possible to the wet plug, as low humidity and large distance from the wet plug can both result in uncontrolled evaporation.

2.3.8 Record the relative position of the liquid in each of the capillaries with respect to the black mark line in millimeters using a ruler, and write these values in a notebook.

Note: If the solution is positioned exactly at the black line, the value should equal zero, and if it is above or below, it should be positive or negative values, respectively. The initial levels of the liquid set the reference points for the final overnight consumption values.

2.3.9 Set the vials back in the incubator for 24 h. Replace the capillaries at a constant time throughout the entire experiment.

2.3.10 Remove the capillaries gently from the vials and measure the levels of liquid with respect to the black marking line.

2.3.11 Carefully check the liquid level in the mock vial.

Note: If the levels dropped more than 2 mm, it indicates that evaporation is high and the consumption levels are not reliable.

2.3.12 Dampen the plugs gently with 2 mL of water and insert a new set of capillaries, as indicated in steps 2.3.3-2.3.10. Set the vials back in the incubator for 24 h.

2.4 Determining preference

2.4.1 Calculate the total consumption of each capillary by subtracting the final level from the initial level at the starting point.

2.4.2 Combine the values of both capillaries with the same food (*i.e.*, non ethanol capillaries separately from ethanol-containing capillaries).

2.4.3 Calculate ethanol preference by subtracting the amount consumed from the non-ethanol capillaries from the amount consumed from the ethanol-containing capillaries and divide the value by the total consumption values from all the capillaries of the same vial.

2.4.4 Average the preference indexes of all vials of the same experimental group.

$$PI = \frac{\text{Total E- Total NE}}{\text{Total Sum}}$$

Note: Non-ethanol 1 (NE1), Non-ethanol 2 (NE2), Ethanol 1 (E1), Ethanol 2 (E2), Total non-ethanol (Total NE) = NE1 + NE2, Total ethanol (Total E) = E1 + E2, Total sum consumption (Total Sum) = Total NE + Total E, and Ethanol preference Index (PI)

REPRESENTATIVE RESULTS:

Previously, Devineni *et al.* showed that when fruit flies are given the choice to consume ethanol-containing food, they display a strong preference for ethanol-containing food over non-ethanol containing food[6]. Shown here are some representative results we obtained when assaying the innate ethanol preference of naïve male flies that did not undergo the training protocol.

Naïve Canton S male flies were collected upon eclosion, aged until four days old, and assayed for their innate preference to consume ethanol over the course of four days (Figure 2). Analyzing the amounts consumed from ethanol- and non-ethanol-containing solutions shows that flies exhibit a robust preference for consuming 15% ethanol food over non-ethanol food (Figure 2).

Using opposing sexual experiences, we have previously demonstrated that male flies that were allowed to mate in groups ("mated-grouped") display lower ethanol preference than singly-housed males that were rejected by previously-mated females ("rejected-isolated"). We used several controls to uncouple the housing experience from the mating-related experience and showed that the differential preference results from the mating experience[5].

Using the training protocol described here, we generated singly-mated and rejected male flies that underwent similar housing and mating regimes. Single male Canton S flies were subjected over the course of four days to disparate mating experiences. One cohort of males was allowed to interact and mate with virgin female flies (single mated cohort), and the other cohort was trained with previously-mated female flies (single rejected cohort). Following the training phase, the single males from the two cohorts were grouped (8/vial) and placed in the capillary feeder vials; their voluntary ethanol consumption was recorded for four days (Figure 3A). The preference to consume ethanol was calculated from the consumption data using the formula in step 2.4 (Figure 3B). Positive values indicate preference for ethanol-containing food, and negative values indicate aversion to ethanol-containing food.

The present experiment supports our previous findings, suggesting that sexual experience, and not housing condition, modulates ethanol consumption. Successful mating increases internal reward levels, which in turn lowers ethanol consumption. Rejections, perceived as a lack of reward, lead to an increase in reward-seeking behavior (Figure 3B).

Figure 1. Schematic of the capillary feeder system (CAFE). (a) Schematics of the perforated vial with holes to equilibrate pressure and humidity. (b) Schematics of the cut-in-

half plug that serves as the base for the insertion of the capillaries. (c) Schematics of shape and location of the adaptors serving to hold the capillaries.

Figure 2. An example of innate preference to consume ethanol. Naïve wild-type males were grouped in groups of 8 males/vial, and their consumption of ethanol- and non-ethanol-containing solutions were recorded over the course of 96 h. The flies consumed a larger amount of 15%-ethanol containing food (**P < 0.01, ***P < 0.001, two-way repeated measures ANOVA with Bonferroni post-tests, n = 8). Data shown are the mean + SEM or mean – SEM.

Figure 3. Opposing mating experiences modulate ethanol preference. (a) Schematic of the combined protocol. Single virgin wild-type males are allowed to mate with virgin females or are subjected to three 1-h courtship-suppression training sessions (depicted as "T") spaced by 1 h of rest (depicted as "R"). Training is repeated for 4 days. At the end of each day, the flies are placed in the incubator (depicted as "O.N"). After four days of training, the males were placed in vials where they could choose to feed from capillaries containing food solutions with or without 15% ethanol. (b) Single rejected males exhibited higher ethanol preference than single mated males (**P < 0.01, two-way repeated-measures ANOVA with Bonferroni post-tests, n = 9, comparisons are between treatment groups across 3 days after the end of training). Data shown are the mean + SEM or mean – SEM.

Table 1. An overview of the protocol depicting the order and time flow for the different steps.

DISCUSSION:

Here, we illustrate the details of an integrated approach to measure alterations in reward-seeking behavior, based on previous work described by Devineni *et al.*[6] and Shohat-*Ophir et al*[5]. The first section of the protocol uses different types of sexual interactions as the experience input, and the second section uses a two-choice feeding assay to assess the effect of experience on the preference to consume ethanol.

As shown by Devineni *et al.*[6], ethanol self-administration in flies is a robust behavior, characterized at first by low and variable preference that increases and becomes more consistent over time. The developing preference may reflect a process through which flies learn to associate the pharmacological effect of ethanol with its position, in spite of its unpleasant taste[6] and the aversive responses known to occur during initial exposure to ethanol vapors[11]. Changing the position of the ethanol-containing capillary on a daily basis results in a constant level of preference, strongly demonstrating the role of learning and suggesting that the flies leave chemical cues next to the preferred capillaries[12]. In addition, the shape, the slope, and the starting point of the preference curve can be slightly different between experiments, but the overall magnitude of difference between the groups of flies that are exposed to rewarding and non-rewarding experiences is preserved across experiments.

The question of whether flies display preference to consume ethanol for its caloric value or pharmacological properties is still under debate and was examined in the last few years by several independent studies (for a review, see Devineni *et al.*)[13]. Taking into account the results obtained from these studies, it is likely that the caloric value of ethanol contributes to the pharmacological effect of ethanol and is not the main reason for the development of preference to consume ethanol.

As this protocol reflects voluntary consumption in groups of flies, the technique is limited to cumulative changes in consumption over at least several hours and is not sensitive enough to reflect short temporal changes in consumption over short time frames (minutes). Other feeding assays, such as FLIC[14], flyPAD[15], and EXPRESSO[16], that can measure the frequency of feeding events and their bout length are technologically superior to the CAFE assay and can therefore provide high-content temporal data of feeding and ethanol preference. Still, compared to other simple two-choice assays that are based on measuring the ingestion of food mixed together with food dyes, the CAFE approach allows the accurate and uninterrupted monitoring of consumption data over several days[17,18].

Although described together, each section can be used separately in future applications. The training phase can be used for further downstream analyses of plasticity-related events using real-time PCR, RNAseq, proteomic, and metabolomic screens. Following the training phase, it is also possible to use FLIC[14], flyPAD[15], or EXPRESSO[16] to achieve higher resolution in the consumption data. The two-choice ethanol consumption assay can also serve to study the effect of other external stimuli, such as stress, on the internal state[19,20]. Additionally, using neurogenetic tools that control neuronal activity as an input experience and ethanol consumption as an output behavior, it can be used to dissect the neuronal circuits that encode internal reward.

Even though the described protocol is relatively simple and does not require sophisticated equipment, there are critical steps that require the most attention to ensure reproducible results. First, it is important to start the experiment with large male flies that eclosed from healthy fly bottles. Secondly, it is essential to make sure that the mated cohort indeed undergo mating and that the rejected cohort do not manage to mate. Lastly, it is important to maintain a well-humidified environment for the entire experiment, with an emphasis on the consumption part. This includes watering the plugs every day with equal amounts of water and keeping the capillary ends in close proximity to the wet plugs.

ACKNOWLEDGMENTS:
We thank U. Heberlein and A. Devineni for long-lasting discussions and technical advice. We also thank the Shohat-Ophir lab members, A. Benzur, L. Kazaz, and O. Shalom, for the help with demonstrating the method. Special appreciation goes to Eliezer Costi for establishing the fly systems in the lab. This work was supported by the Israel Science Foundation (384/14) and the Marie Curie Career Integration Grants (CIG 631127).

DISCLOSURE:
The authors have nothing to disclose.

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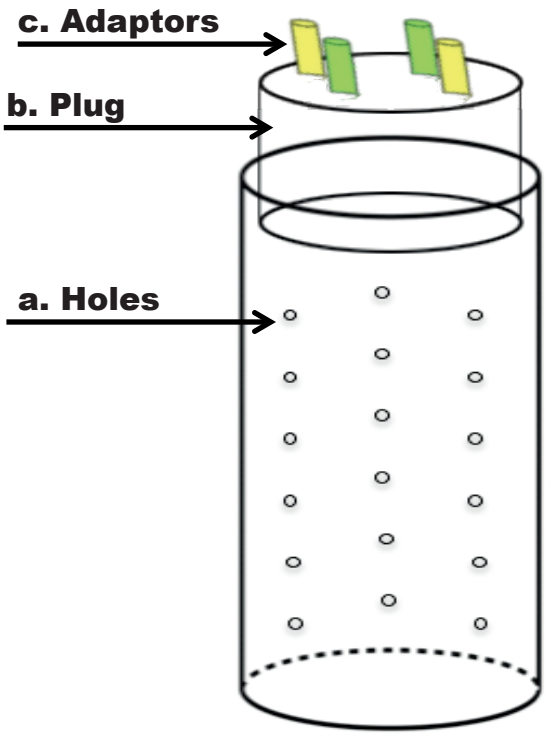


Figure.1

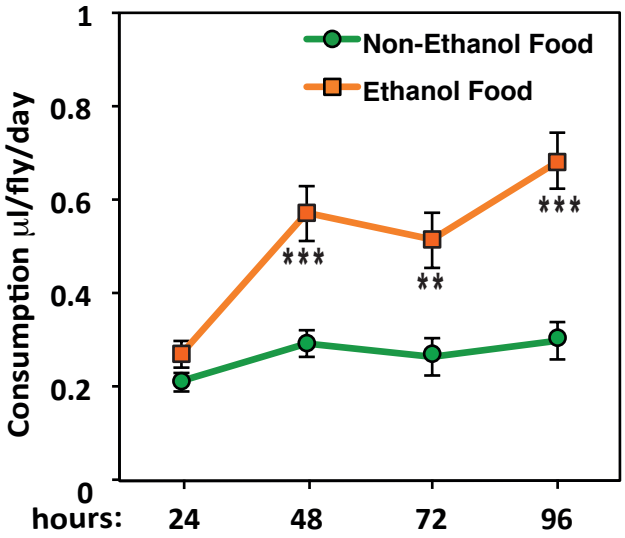
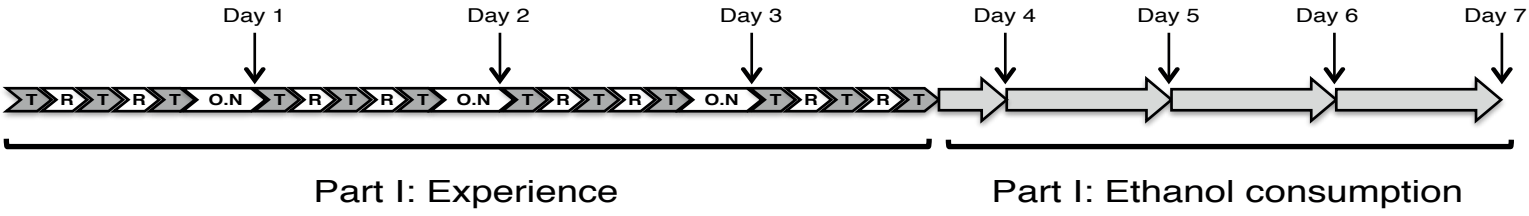

Non-Ethanol Food
Ethanol Food
Consumption µl/fly/day
1
0.8
0.6
0.4
0.2
0
hours:
24
48
72
96
***
**
***

Figure.2

A.


Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
T R T R T O.N T R T R T O.N T R T R T O.N T R T R T
Part I: Experience
Part I: Ethanol consumption

B.

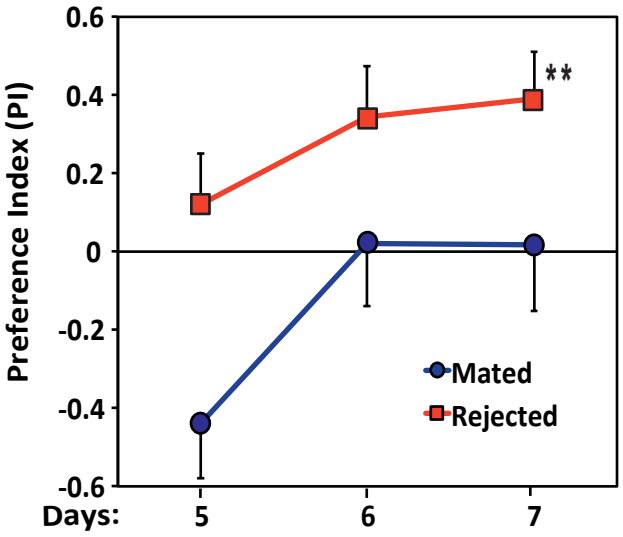

Preference Index (PI)
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
Days:
5
6
7
**
Mated
Rejected

Figure.3

Table 1

		Timing	Procedure	Reference section
Preparatory steps	Collecting flies	By day1	Preparing the capillary feeder system	1.1
			Preparing small glass food vials	1.2
			Making stock food solution for CAFE	1.3
		Days 1-4	Collecting experimental and trainer flies	1.4
			Generating mated female trainer flies	1.5
Experimental steps	Experience phase	Days 4-7	Setting up the experience phase	2.1
			Exposure to experience	2.2
	CAFE	Days 7-11	Recording consumption	2.3
	Analysis		Determining preference	2.4

Notes
Can be used in many experiments
Can be kept in 4 ^{o}C for several weeks
Keep in 4 ^{o}C after autoclave
Aged to 3-4 days before being used in the experiment
This step should take place the evening before each training session
This step should take place the morning of each training session
To be repeated in 3 consecutive training sessions over the course of 4 days
Dump the plugs with water, measure consumption and replace capillaries over the course of 4 days
This step is done each time before replacing the capillaries during the two choice assay

Excel Spreadsheet- Table of Materials/Equipment

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Name of Reagent/ Equipment	Company
Polystyrene 25 x 95mm Vials	FlyStuff
narrow plastic vials flugs	FlyStuff
Disposable Sterile Needle 18G and 27G	can be acquired by any company
10x75mm Borosilicate Glass Disposable Culture Tubes	kimble chase
calibrated pipets 5ul (microliter)	VWR
Mineral Oil	Sigma-Aldrich
Sucrose, Molecular Biology Grade	CALBIOCHEM
Yeast extract Powder for microbiology	can be acquired by any company
Ethanol	Sigma-Aldrich
standard pipette Tips (micro-pipets)	ThermScientific
IDENTI-PLUGS (Foam Tube Plugs)	Jaece
IDENTI-PLUGS (Foam Tube Plugs)	Jaece
virginator fly stock	bloomington drosophila stock center
Narrow Vials, Tray Pack (PS)	Genesee Scientific Corporation
Drosophila Media Recipes and Methods	Bloomington Drosophila Stock Center
propionic acid	Sigma-Aldrich
phosphoric acid	Sigma-Aldrich
Methl 4-Hydroxybenzoate	Sigma-Aldrich
Agar Agar	can be acquired by any company
corn meal	can be acquired by any company
Grandma's molasses	B&G Foods, Inc
instant dry yeast	can be acquired by any company

Catalog Number	Comments/Description
32-109	
42-102	
	1.20 X 38mm (18Gx1 1/2") , 0.40 X 13mm (27Gx1/2")
73500-1075	
53432-706	color coded white to contain 5 microliters
M5904	
573113	
32221	
T114R-Q	volume- 0.1-20 ul Ultra micro
L800-A	fits opening 6 to 13mm
L800-D	fits opening 35 to 45mm
#24638	
# 32-109BR	
http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/molassesfood.htm	
P5561	
W290017	
H3647	
not indicated	

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הפקולטה למדעי החיים
ע"ש מינה ואבררד גודמן
דר' גלית שוחט אופיר

Dear Editor,

Attached please find our revised manuscript and response to the editorial and peer review comments. I would like to extend our appreciations to the reviewers for their important remarks that improved the clarity and accessibility of this visualized protocol to the scientific community. Our response appears in blue below or next to the comments.

Editorial comments:

• JoVE reference format requires that DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.
Done.

• 1.5.1.1: Bloomington #24638 was removed from this step. Please add Bloomington #24638 to the Table of Material. Added to the material excel file.

•Formatting:
-Please spell out author first names and include a comma between Last, First name. Done
-Please include a space between numbers and units. Done

•Grammar:
-Please copyedit the manuscript for numerous grammatical errors. Such editing is required prior to acceptance.
-Line 68 – "using two choice voluntary ethanol consumption assay. We insist that this is not wrong, although this is two choice, it is still an assay.

-Line 103 – "other are needed. Changed to: should take place in a timely manner.

-2.2.1 - "…along the exterior raw of the rack. Changed to: margins of the rack.

-Line 419 – "Other feeding assays that can measure bouts of feeding, their length and temporal regulation like FlIC and EXPRESSO, which are more suitable to achieve high resolution analysis of feeding behavior." – reword to form a complete sentence. We changed the sentence to: Other feeding assays such as FlIC, Flypad and EXPRESSO that measure frequency of feeding events and their bout length, are technologically superior to the CAFE assay and can provide high content temporal data of feeding and ethanol preference.

-Line 423 – "the CAFE approach allow continues monitoring of flies accurate consumption" – several errors here. Changed to: continuous monitoring and accurate consumption of flies over.

-Line 425 – "flies behavior" changed to: interrupting their behavior.

-Line 430 – "as an approximate" – "approximate" not used correctly; "therefor" changed to: consumption assay serves as an estimation for internal reward. Therefor changed to: therefore.

-Line 433 – "the capillaries ends" change to: capillary ends.

•Additional detail is required:

-1.1.1 – Is this a standard fly vial? Yes, details are provided in the Excel material file.

-1.1.2 – Cut in half lengthwise or crosswise? Changed to: Cut across the vial plug.

•Branding: Line 138 "Bloomington", is there a generic term instead? Deleted and added to the "material excel file"
•Results:
-Figure 2: what do error bars represent? SE? SD? Standard Error
-Figure 3: there are no asterisks, were there no P<0.01 results? Fixed, see Fig. 2 and Fig. 3

Reviewers' comments:
Reviewer #1: *Manuscript Summary:* Details method for reward conditioning and measuring alcohol preference in flies *Major Concerns:* none *Minor Concerns:*

Line 56,61 - This manuscript describes - change to "We" changed to: we.

92 - In this publication - "We" changed to: we.

229 - exterior 'row' of the rack Changed to: margins of the rack.

267 - 'Dampen' changed to: Wet the plugs.

*Additional Comments to Authors:*N/A

Reviewer #2:

Discussion Points -

The sole relationship to measuring fly reward rests on the assumption that ethanol preference is a definitive proxy for changes in reward states following natural reward. The authors should discuss this in the context of their previous findings.

Our previous study shows that there is an inverse correlation between naturally rewarding experiences and voluntary consumption of ethanol. We performed a series of experiments to show that the observed changes in the preference to consume ethanol reflect changes in reward states following different types of social experiences. This included experiments showing conditioned preference to a neutral cue associated with mating ethanol exposure, and the identification of a neuronal system (NPF/R) that connects experience input to behavioral output in the form of both ethanol consumption and conditioned odor preference. Altogether, these experiments suggest that voluntary consumption of ethanol is a valid tool to assess motivation to obtain drug rewards in flies.
In this manuscript, we chose to focus primarily on ethanol self administration, as a tool to assess changes in reward state. Still, it is highly recommend supplementing this approach with classical paradigms measuring reward directly such as conditioned odor preference.
Based on the remarks we chose to add a paragraph to the introduction that describes how our previous study with the NPF/R system couples both natural and drug reward.

What are "internal reward levels?" This term is discussed in a few places in the article, but it is not clear what the authors mean. Would motivation be a better term?

The phrase internal reward levels, is used to describe neuronal representation of rewarding experiences within the brain, based on the assumption that the brain encodes the value and intensity of the rewarding experience. As such, depriving animals from natural rewards is hypothesized to lead to reward deficit,

which is reflected by the motivation to obtain other drug or natural rewards. Based on the reviewer comments we used reward states to describe changes in reward levels.

I would like to see the authors come up with a better way to define what they are actually testing.

We are measuring changes in the preference to consume ethanol or the motivation to self-administer ethanol. This has previously been shown to model an addiction like behavior, which is affected rather by the hedonic/pharmacological effects of the drug than its caloric value. We changed the text accordingly.

Does the CAFE assay tube have food besides the control (no EtOH) capillary? It is a known fact that flies on the CAFE assay become starved after a few days, and in fact show decreased lifespan in this assay.

The CAFE assay as described in this manuscript does not contain food besides the two types of capillaries. From our experience, as long as the capillaries are replaced on a daily basis, and the number of flies in the vials does not exceed 8 flies, we do not observe flies being starved for the entire duration of the experiment.

This is important because EtOH is caloric, so the EtOH+yeast/sucrose solution is more caloric then the sucrose/yeast solution. Can the no EtOH solution be normalized for equal numbers of calories by using compounds with no-taste but that are caloric (maltodextrin, sorbitol?) This should be mentioned in the discussion in the least, because in my view it is a major flaw in the current assay.

The question of whether flies display preference to consume ethanol for its caloric value or pharmacological properties is still under debate and was examined in the last few years by several independent studies. The approaches to resolve the question included modulation of the food solutions to equalize the caloric value of the non-ethanol solution using some of the suggested compounds or varying the caloric value of the ethanol solution (for review see Devineni at al).
Early studies suggested that flies could survive longer on a diet consisting ethanol as a sole energy source in comparison to complete starvation (Van Herrewege & David 1980). On the other hand, the survival on ethanol as a sole energy source was found to be much less effective than isocaoloric concentration of sucrose, suggesting that the calories present in ethanol are weakly utilized by flies (Xu et al. 2012). Supporting evidence against the hypothesis of the caloric role of ethanol, comes from the observation that although flies modulate their sucrose consumption to maintain equal caloric intake over different concentrations of sucrose, this is not the case for varying concentrations of ethanol (Xu et al. 2012).
In addition, we showed that the preference to consume ethanol can be dissociated from general food consumption; although rejected and mated male flies display different levels of ethanol consumption, the overall food consumption of both cohorts is similar (Shohat-Ophir et al. 2012). To conclude, we cannot rule out the contribution of Ethanol's caloric value to the development of preference, it most likely that this property contributes to the hedonic/pharmacological role and is not the main reason for the development of preference.

The authors prioritized simplicity over accuracy in their choice to use the CAFÉ assay over others such as the FLIC or flypad (or the recently published EXPRESSO); now that the field has switched using high-resolution methods, it would be helpful to add a sentence about how this assay could be adapted to these new platforms, which have superior behavioral and temporal resolution. Added to the discussion.

As flies find initial ethanol exposure aversive (Kaun et al., 2011) within the first 30 minutes following exposure, it is likely that this effect is contributing at least to Day1's variability in Figure 2. Should add to the discussion.

Devineni et al analyzed the role of different sensory modalities in the development of preference to consume ethanol and found that the taste of ethanol aversive (Devineni et al). Both this and the short-term

aversive response to ethanol intoxication can contribute to low preference and variability observed in the first day. Additional factors affecting the development of preference are: learning process associated with two choice assays, and the need to reach a consumption threshold that will facilitate the pharmacological effects of ethanol.

The timeline at the interface of the end of the courtship training and the beginning of the ethanol preference assay was confusing. In Figure 3, if one assumes the "Day" labels refer to the age of the fly as in part A, why in part B do we see ethanol preference for 4-day-old flies, which should not have begun to consume ethanol as they are in the first day of courtship suppression training? It seems that flies in B should be tested during days 8-11 on EtOH, as showed in the A schematic. Also, in Fig. 2, why are flies tested during the "aging" phase, 1-4 days? Changed accordingly.

The figures are very confusing and poorly constructed, the X axis needs to be clear. When are days compared to Fig. 3A? The authors should also add ul consumed, not just preference index, since they are doing CAFÉ assays.
Figures were changed and the requested data was added.

Line 162, what is meant with naïve?
Naïve means virgin males without prior social experience. We added an explanation in the text.

Line 167, what is meant with individually housed? Can't flies be housed in groups?
One can control the social experience of flies and house them either singly or together with other flies in a group (referred as grouped housed).
The conditioning protocol is based on single males that interact with either virgin or mated female flies. Prior to the conditioning session, flies can be collected and kept in groups or maintained singly from eclosion on.

Line 182, define "virginator stock" by genotype added to the "material excel file

Line 219, what is a behavior box?

A behavior box is a chamber that minimizes environmental variations and is routinely used in behavioral experiments. One can use even a carton box for this purpose, or build a behavior chamber with temperature, lighting and humidity control. We changes behavior box into behavior chamber.

- *Major Concerns:* CAFE assay is a very primitive tool given the FLIC and FLYpad assays published in 2014 Figures need to be better explained

We agree with the comment that the CAFE is very basic compared to the technologically advanced FLIC, FLYpad and Expresso systems. As such it requires manual recording and offers only basic consumption readouts compared to the high content data and high throughput features of the above-mentioned tools. Still, the simplicity of the system, being constructed from basic components that are available in every lab, and the simple but continuous readout, offer robust and reliable consumption data. We do not try to claim by any mean that this method is superior, but just to demonstrate visually for the first time the one of the behavioral paradigms we use in lab, that can be useful for others.

- *Minor Concerns:* Some editing and rephrasing necessary
- *Additional Comments to Authors:* N/A

Reviewer #3: *Manuscript Summary:* A simple way to measure alterations in reward-seeking behavior in Drosophila melanogaster" by Zer and others describes a simple set up to measure ethanol consumption preferences after previous mating or non-mating experience of male flies. The assay is thought to measure

experience dependent changes in internal state, e.g. reward. It requires a two step procedure.

Does the assay measure reward seeking? I am missing one proof of principle. For example dopamine is known as a "reward molecule" in Drosophila.

What happens when dopamine is altered? So far the assay measures only ethanol consumption preference and we do not know whether there is a relationship to reward.

The CAFE assay is analogous to the two bottle choice assays used in rodent studies for drug self-administration, and has been shown to reflect certain properties of addiction like behavior. When given the choice to consume food or food supplemented with 5-20% ethanol, flies display strong preference to consume ethanol that increases over time and can reach pharmacologically relevant concentrations. Flies would exhibit preference to ethanol even if it contains aversive compounds and show relapse like behavior by rapidly returning to high levels of ethanol preference after a period of two days deprivation Devineni and Heberlein 2009).

The link to reward comes from experiments showing that activation of NPF neurons inhibits ethanol preference, and perceived as rewarding in the well-established conditioned odor preference assay (measuring conditioned responses to a neutral cue associated with the activation). This inverse correlation suggest that rewarding experiences, reduce the motivation to self administrate ethanol.

From the presentation within the draft we do not know whether reward related behaviors are indeed altered. The increased "drinking" in non-mated males could be also due to training effects and not related to reward. Mating males have other things to do than drinking and have less drinking experience. Alternatively the assay could be renamed and the title of the manuscript changed.

Previously it has been shown that mating experience and ethanol intoxication is rewarding to flies using conditioned responses to a neutral cue associated with mating or ethanol intoxication (Kaun, Shohat-Ophir). In addition, as mentioned above we identified a molecular signature of both mating experiences and ethanol intoxication in the form of changes in NPF levels and shown that there is a causal link between the NPF system, ethanol consumption and perception of reward.

When I have decided that I would like to use this assay, I would have questions related to the experimental strategy. I am not sure how alterations of the behavior would look like and how to plan my experiments in respect to the number of animals required to perform an experiment successfully. I need to know how many animals I have to take and how high the variance of the assay is. In general it would be nice to be briefly introduced into the assay as a whole. This could be done by modifying Figure 1, before mentioning all the details for the used vials. In addition it would be easier to understand when fig2 and fig 3 are pooled together. I would have liked to know the infos earlier, the infos in the REPRESENTATIVE RESULTS appear quite late.

Do we need a JOVE for it? Yes, it is important to describe such a tricky assay in detail! What I like about it is that it is a two-step assay that could be separated. However the second part is still highly variable. It would benefit from a second JOVE that complements this approach.
We feel that both parts can still go together.

How robust is the assay?

1.1.5 "Maintain the positions of the two types of the capillaries consistently…." Why? This should not matter for the choice of the flies, otherwise this raises questions regarding positional effects of the capillary and its influence on feeding.

When analyzing the consumption data along the days of the experiment, flies display constant consumption of non-ethanol containing food, and increase their consumption of ethanol containing food, resulting in overall increased preference over time. Altering the position of the ethanol-containing capillary on a daily basis resulted in a constant, rather than increasing, level of preference (Xu et al. 2012). This may reflect learning and memory processes that allow flies to reliably discriminate which capillaries contain the ethanol food and reinforce their preference, possibly by leaving chemical cuses next to the preferred capillaries.

Statistic is missing (Fig. 2) and a discussion of possible pitfalls. Done

The variance of the preference appears quite high, number of experiments and tested animals needs to be mentioned and discussed. We made changes to explain this better.

Are the data significant different from random choice? Done

Some details:
*Page4: The preparation for the CAFE set up refers to Fig.1a and Fig.1b. There is only one Figure 1. Figure 1: Experimental set up contains one picture.
Changed to Fig.1

*Step two: cut micro-pipets…what kind of pipets? This is difficult to understand. Explained in the "material excel file"

*Preparing small glass food vials: Bloomington cornmeal molasses recipe: I would like to have the recipe without looking somewhere else. A link to the recipe was added.

*2.1.1: Here it would be good to mention that the 12h/12h light cycle could be changed to a convenient starting time. Added accordingly.

*2.3.1 At the end of the last conditioning session (fourth day), aspirate the male flies into the capillary feeder vials and pool single males into groups of 6-8 flies it would be better to mentioned that single males are grouped to perform the second part of the assay. Done

*2.3.4 It is not clear what a capillary adapter is (maybe a picture?). This is indicated in the figure.

*2.3.7. In addition to vials that contain experimental flies, prepare a mock control vial without flies to assess natural evaporation…." Here it would be interesting to know how high the evaporation rate for the experimentar normally is…to assess the variance of the assay. Evaporation is usually up to 1-1.5 mm.

*Figure 2. Preference of EtOH intake: here we need statistics. How many N's and is the PI different from random? In addition it would be nice to see the total consumption of non EtOH versus EtOH solutions. Done

*Line 372: The present experiment supports our previous findings, suggesting that sexual experience and not housing conditions modulate ethanol consumption. I believe that this is true. However to support the experimental set up and robustness of the behavior a statistical analysis of the data is important for the procedure. Exist

*Fig3. In the pict there is no statistics. Done.

*For the controls: the evaporation control for the CAFÉ assay is rather vague, since it is not known how variable the assay in general is. When relative humidity is maintained around 60%, the evaporation reaches no more than 1mm in the mock vials.

*The pitfalls need to be discussed and the pro and cons in comparison to other techniques. Discussed

*It would be nice to know how the flies are set up that are used for the experiments: The following statement is weak:"start with healthy and large male flies eclosed from a well-populated fly bottle". We changed this sentence.

More general concerns: I am wondering whether it is technically possible to see within 200 vials every 15 min whether males mate. Is "mating" = copulation? There is a lot of literature out there that described that mating is more complicated than that.
Thank you for this remark; we meant copulation. Courtship ritual in flies is indeed more complicated, and the analysis of required recording the behavior and manually going over the movies and scoring the behavior (e.g. wing vibration, attempted copulation, licking and so on). Once copulation is achieved, it lasts for about 20min and is very obvious and easy to see by naked eye, therefor it is very easy to quickly watch all the vials and identify copulation events.

One more general comment: the introduction gives a reference of 3 - 35 papers for one sentence and the next 10 citations are in the discussion. This is an interesting format and might not reflect an in depths discussion of the pros and cons of the assay and the possible mistakes/ technical problems.
We moved most of the references to the discussion and shorten the list.

Major Concerns: N/A *Minor Concerns:* N/A *Additional Comments to Authors:* N/A

[**Editorial recommendation**: Please keep JoVE's protocol requirements in mind as you address the above comments - the protocol must contain sufficient detail in order to enable users to accurately replicate your technique. We recommend NOT removing any details from the protocol text.]

Bets wishes,

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