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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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