Vet Review Comments:
1. The video shows the custom made box which was fabricated without adherence to the building material standard of the Guide. Wood is not an acceptable building material according to the 8th edition of the Guide.

The custom box depicted in the revised video is constructed of materials that adhere to the 8th edition of the guide (see Video 4:58-5:21).

2. The video demonstrates the insufficient cleaning of the custom box with an inappropriate cleaning agent. A 70% solution of alcohol is not an acceptable cleaning solution to assure disinfection of the box containing rats and aspen chip bedding. Documenting the cleaning procedure in this manner will lower the acceptable standard of sanitation. I do not believe that JOVe would like to provide the citation for lowering the sanitation standard.

The function of wiping the apparatus with 70% isopropanol is to control odors between sessions. Reference to "cleaning" the apparatus with isopropanol have been removed from the video and text and replaced with references to the odor control function of this step. Because the frequency and agent used for sanitization of the apparatus could vary based on a number of factors that are not related to the methods described in this protocol (e.g., heterogeneity in the microbiological status of the animals under study) we have directed the reader to clean and sanitize the apparatus with an appropriate agent as needed.

Editorial Comment: Regarding the following comments from this reviewer pertaining to data, please note that only representative results are required for publication in JoVE. However please ensure that any claims presented are fully substantiated in the video by data or references to published works. While we do not require a hypothesis statement for JoVE videos, please take this opportunity to ensure that the goal of the protocol is properly established in the video.

1. A hypothesis statement is not given in this research paper.
The goal of the protocol is to describe prenatal ethanol exposure methods and methods for quantification of social behaviors. These goals can be achieved without an explicit hypothesis statement.

2. Data is not demonstrated in in this video.
Data were presented in the original video. Representative results for the critical groups are shown from 9:47 to 10:20.

3. At 7:12 in this video there is an unsubstantiated claim that two groups could be distinguished using behavior testing, but data is not given.

This claim is not unsubstantiated. In addition to the representative results

depicted in the video at 9:47 to 10:20, we reference published articles using these methods that support this claim in the manuscript (see e.g., ref 1).

Reviewers' comments:

Reviewer #1:

1) Lines 174-175. Should this read "Follow steps 1.5.1-1.5.6…"?
This error has been fixed.

2) Line 209: How long does the breeding phase typically last? Is there a cut-off if a female does not become pregnant within 4-5 days (1 cycle)? What is the interval between the pre-pregnancy and pregnancy phases? It appears that the pregnancy phase begins immediately after the pre-pregnancy phase, but please note this specifically.

A female rat is placed with a proven male breeder for up to five days. She is removed on the morning that a vaginal plug is observed in the bedding below the hanging cage. If no plug is observed after five days, the female is removed from the study.

The period during which breeding is conducted is dependent on the number of male breeders on hand and the number of female rats to breed. We typically maintain about one male for every three females to be bred. The entire process normally takes up to two weeks to complete. Thus, the interval between the end of pre-pregnancy drinking and the start of pregnancy drinking can range from one day up to two weeks. During this time, females are not consuming 5% ethanol.

These aspects of the method have been included in section 1.6.

3) Lines 222-223: There is no explicit mention that the volume of alcohol/saccharin that the dams are drinking during the pregnancy phase should be recorded. This information is included in the video and is specifically mentioned in the pre-pregnancy drinking phase section, but should be added here for clarity.

We thank reviewer 1 for pointing out this omission. We have added this to section 1.7.1.

4) Line 232: While culling to an even sex ratio (5 males:5 females) is implied from the diagrams in the video, it is not mentioned explicitly in the manuscript. It would be helpful to indicate whether or not the male:female sex ratio is considered as part of the experimental methodology.

We sex the rat pups when we cull a litter to ten and we attempt to have five pups of each sex. However, on occasion when there is a disproportionate number of one sex, we will keep all of the sex with the smaller numbers represented, but still cull the litter to ten with the other sex.

We have modified section 1.7.5 in response to this suggestion.

5) Line 243. Instructions are to use a custom built chamber exactly as that used by these investigators. Should there be a comment that a similar chamber would be acceptable as long as it meets the basic requirements (type of floor and walls, open front, etc) or must it be exactly this chamber?

It is not necessary for the materials or dimensions of the custom chamber to match. We have simply described what we use in our laboratory. We do recommend that the dimensions provide sufficient space to identify genuine social interaction. That is, it should be large enough that the animals are not always in close proximity to one another. We have included a "NOTE" to this effect in section 2.1.1.

6) Line 271. A more general issue:
In this paradigm, experimental animals are interacting with same-condition cage-mates. It would probably be useful to have a brief discussion about this choice. Many social behavior studies use a non-treated social stimulus animal as the partner for testing. Having a cage-mate (same prenatal condition) vs a novel social stimulus animal (typically a non-treated control but could be from the same prenatal condition) as the partner likely makes a significant difference for the interactions that occur and therefore in the behaviors observed in the experimental animal. Some discussion here would be very helpful for an investigator just getting into the social behavior area and trying to make decisions about testing conditions, and in fitting the data from this paradigm into the broader social behavior literature.

This is an excellent point and we have included several sentences addressing this issue in the revised Discussion section (lines 536 – 552).

Finally, it is important to note that social behavior using the methods described here can be influenced by the selection of the partner animal. In the representative results presented here animals were paired with familiar animals (cage-mates) from the same prenatal treatment condition[1,18]. The rationale for this selection was based largely on data demonstrating that housing control animals with ethanol-exposed rats alters social behavior in control rats[14], as well as similarly reliable unpublished observations from our laboratory. These effects can potentially complicate identification of and interpretation of PAE-related

alterations in social behavior. The methods described here for quantification of social behavior could, however, be applied to any variation on the source of the social partner, of which there are several possibilities including using a non-treated partner that comes from neither diet condition[1], using a partner animal from the same diet condition (as described here), or a partner animal from a different diet condition. Further, the familiarity of the partner animal can be manipulated to affect social interaction[1]. The selection of the partner condition and other variables related to social housing, sex, and exposure paradigm can be tailored to best meet the scientific goals of the particular laboratory while still utilizing the basic procedures for quantification of social behavior described here.

We have also added the following sentence in the representative results section on lines 396-397 to make it clear which partner condition is represented in the representative results.

All animals were paired with partners from the same prenatal treatment condition.

7) Line 303: How long is the social interaction session? Based on the data presented in Table 1, it appears that the session lasts for 5 minutes. This information is important for interpreting your representative results. Moreover, it would be useful to know the rationale for session length (i.e. is session length a critical factor for observing group differences?).

Approximately 12 minutes is recommended as a session duration. This is included as section 2.2.4.4. A 12 minute session was utilized for the representative results.

8) Line 324. Would it be useful to mention other possible programs to use for playback of the video and quantification of behaviors, and possible pros and cons of Matlab vs other programs (eg., Noldus, Anymaze, etc)?

Although other approaches to analysis can be used, including alternative software packages, it was our understanding that specific products and their associated trade names could not be mentioned in the article or video. Thus, we are hopeful that our general “NOTE” added in section 2.3.2 will help alert researchers to the possibility of alternative approaches and the important functions to consider when identifying a software package for analysis.

The added note reads as follows :
NOTE : Software packages capable of registering codes for observed events/behaviors and maintaining the relative timing and duration of these events would be adequate for the types of quantification described here.

10) Line 350: Are all of these results in male animals? Based on the previous sentence ("Robust alcohol-related alterations in the social behavior of female animals have not been observed .. by us…"), it would seem so. However, it should be explicitly stated here.

Yes, the representative results reported here are from male animals. We have stated this explicitly in the representative results of the revised manuscript.
(See also our response to reviewer 2's point 6).

Video Comments:
11) ~3:30: It might be nice to have an animation showing rat dams being assigned to either the EtOH or saccharin groups; this will make it clear that all dams have had EtOH experience prior to pregnancy.

We have modified the video to include a segment depicting assignment to drinking conditions. It begins at ~3:31.

Minor:
12) Lines 137-138: Consider revising "compared to" to "from"
We have made this modification

13) Line 182: It might be helpful to remind the reader that "1 h after the onset of the dark phase" corresponds to "(1000 hours)"
We have included this reminder.

14) Lines 222-223: Sentence is missing the word "is" before the word "matched"
This has been corrected.

15) Line 348: Delete the word "in" after the word "observed"
This error has been corrected.

Reviewer #2:

1. While the authors state that pre-pregnancy drinking should be at least two weeks, they need to provide a reasonable range (say, 2-4 weeks?).

The two-week interval of pre-pregnancy drinking is sufficient to obtain an accurate measure of voluntary drinking. We have modified the text in section 1.5, removing "at least" to specify that 2 weeks of pre-pregnancy drinking is needed to measure drinking levels.

2. Should the dams be first time mothers?

Yes, dams should be first time mothers. This is now stated in section 1.2 of the revised manuscript. Although it would seem that reusing rat dams as breeders would be both a practical and cost-effective approach, our experience with this paradigm over the past eight years indicates that voluntary drinking behavior from "first time moms" is the most consistent.

3. Are the male and female breeders kept together for multiple days in a row? This method does not seem as accurate for knowing GD0 as placing them together for one night only.

Females are maintained with proven male breeders for up to five days and evidence of a vaginal plug used to confirm pregnancy and Gestational Day 1.

4. Why are weaned pups housed with animals from the same condition and not counter-balanced?

It is important to note that housing with same condition partners the approach we favor, but we have included a discussion of alternatives in the revised Discussion (final paragraph lines 536-552) to emphasize that the particular housing preparation can be selected by the investigator. Based on our experience and other published data, it is important to recognize that the particular housing configurations may negatively affect behavior in control animals (e.g., if paired with PAE rats), which may complicate interpretation. See also our response to reviewer 1's point 6.

5. Do the mirrored tiles in the cage increase anxious behaviors in the rats since it might look like there are more stranger rats than there really are?

All video recording is performed in the dark, so it is unlikely that the rats are able to see reflected light from the mirrors. Although we have not systematically compared behavior with and without the mirrors in the back of the apparatus, our early work was conducted with no mirrors and in our experience the presence of the mirrors does not appear to affect behavior.

6. Male rats were used in the experiments presented in the paper. Obviously, sex could be an important factor in the behavioral experiments. Are there any sex-specific behaviors or prevalence of certain behaviors between males and females?

There are effects that discriminate male and female animals, however, we have not observed effects of moderate PAE on social behaviors in female rats. We have added the following sentence to the Discussion section (lines 496 – 500).

It is important to point out that effects of PAE on social behavior in female rats have not been observed using the methods described here[1], although main

effects of sex have been reported for several dependent measures including anogenital sniffing (female > male), body sniffing (male > female), wrestling (male > female), and boxing (male > female).

7. What is the rationale for isolating the rats for 24 hours before the social interaction? Is it to increase social interaction?

Yes, the function of the isolation period is to motivate social interaction. This is stated in section 2.2.3 of the revised manuscript.

8. Monitoring BACs after alcohol consumption is know to be useful in interpreting variation of the behavioral (as well as neuroanatomical) outcomes. BACs were not measured in these experiments, and the only mentioning about BACs is in the Discussion (lines 394-397): "Because blood ethanol concentrations achieved with any protocol are important to quantify and communicate, measurements of maternal serum ethanol concentrations should be conducted periodically (e.g.,annually???)". The authors should clarify how they recommend to monitor BACs.

Collecting blood from rat dams is stressful and, therefore, a confound in prenatal ethanol exposure paradigms. For this reason, periodic measures of maternal serum ethanol concentrations must be conducted in a separate set of rat dams. We typically run a separate set of rat dams about once a year to confirm maternal serum ethanol concentrations. In the revised manuscript we indicate that assessment of BACs should be conducted at least annually in a separate set of rat dams for which the offspring are not used. It is also important to note that the peak ethanol concentration will come about 45 minutes to an hour after the introduction of the drinking tube.

In lieu of BACs from the dams producing experimental offspring, the most useful measure to correlate with outcome measures is a rat dam's daily ethanol consumption during pregnancy. However, limiting the range of rat dams used in a study to those whose voluntary drinking is within one standard deviation of the mean for the group will usually constrain any meaningful correlation between ethanol consumed and a given outcome measure.
We have included the following sentences on this issue in the revised Discussion on lines 446 – 458.

Because blood ethanol concentrations achieved with any protocol are important to quantify and communicate, measurements of maternal serum ethanol concentrations should be conducted periodically (e.g., annually) to ensure that comparable BACs are being achieved across breeding rounds. Due to the potential for interactions between prenatal stress and ethanol exposure these measures should be performed in a separate cohort of

females for which the offspring are not used in subsequent studies (see ref. [26]). It is important to recognize that peak serum ethanol concentrations will occur approximately 45-60 minutes after the drinking tube is introduced. In lieu of BACs from the dams producing experimental offspring, the most useful measure to correlate with outcome measures is a rat dam's daily ethanol consumption during pregnancy. However, restricting the range of drinking in rat dams to one standard deviation of the mean for the group would likely constrain any meaningful correlation between ethanol consumed and a given outcome measure.

9. Pups' weight should be taken on PD2-3. Should the number of pups/litter be recorded as well?

The number of live pups is recorded along with the litter birth weight to provide a mean pup weight for the litter. This is stated in section 1.7.4 of the revised manuscript.