We would like to thank the Editor and Reviewers for their thoughtful critique of our manuscript. All comments have been addressed to improve the overall quality of the article.

**Editorial comments:**  
Changes to be made by the author(s) regarding the manuscript:  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have taken the time to proofread the manuscript for spelling and grammar

issues.   
2. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

Every use of the words “minute” and “second” has now been changed to SI

abbreviations. Other units have also been double checked.  
3. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

The protocol has now been edited to use the imperative tense for directions. Use of the terms “could be, should be, would be”, as well as most “Note” portions have been removed. When appropriate, the “Note” has been added to the Discussion section.  
4. 3.1: Is this set in the gas mixer program? Please specify.

This information has now been specified in Step 3.1 as an action in the gas mixer program.  
5. 4.1: Please specify the age, gender and strain of mouse used.

This has been added as a ‘Note’ in 4.1, as it is not an action needed to be done

by the reader.  
6. 4.3: For low long is the mouse observed for the breathing patterns?

Breathing patterns should be observed for one hour following a one hour habituation period.  
7. A schematic or picture of the plethysmography chamber used in this protocol may be provided to help the readers follow the protocol.

We agree a schematic would help the reader to identify all the components of the

setup and have now added one to the manuscript as Figure 5.   
8. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Less than 2.75 pages of the Protocol have been highlighted as essential steps.  
9. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting.

Only complete sentences have been highlighted and steps include actions

written in imperative tense.  
10. Figure 1 and Figure 2: Please use subscripts in chemical formulae (i.e., 2 in CO2) to indicate the number of atoms.

Subscripts have been added to Figures 1 and 2.  
11. Figure 4: Please change the time unit “sec” to “s”.

Figure 4 has been changed to meet SI abbreviations.  
12. Table of Equipment and Materials: Please sort the items in alphabetical order according to the name of material/equipment.

The Table of Equipment and Materials has now been sorted in alphabetical

order.  
  
**Reviewers' comments:**  
  
  
**Reviewer #1:**  
Manuscript Summary:  
The authors are applauded for undertaking this interesting investigation of Obtaining Pattern of Breathing in Conscious Mice through Unrestrained Barometric Plethysmography.

Thank you!  
  
Major Concerns:  
The study is important and holds important insights for examination of fragile mouse strains. This point is partially made to the reader, but could be emphasized. The primary concern with the current manuscript/protocol is that a novice user may lose sight of how to implement an abbreviated protocol. I recommend that you highlight how to determined "in house" methods (noting that local conditions could have an unpredicted influence - see 2 comments down). Then I believe you should recommend under what circumstances baseline methods should be established - e.g., different strains, sexes, ages, etc. Finally, give the end user additional insights on how they can implement the short duration assessments with confidence. In other words, what metrics/criteria can be used to convince a PI that the resting periods are valid? Ultimately, I think this comes back to how other reviewers of papers/grants are going to receive this practice.

Since local environmental conditions can impact the protocol and output, we have added more information to the manuscript about the factors to consider for each laboratory (Lines 766-789):

*“Other considerations for conscious UBP include reducing distractions within the experimental room while animals are being tested. Loud noises, different smells and presence of non-essential personnel in the room can all add to anxious behaviors exhibited by mice. Using smaller rooms as testing areas may help, but if this is not possible cardboard walls (with a small viewing window) can be set up surrounding the chamber to lessen distractions to the mouse. Electrical activity within the room should be kept at a minimum to prevent additional noise within the barometric plethysmography tracings. Therefore, it is important to take note of the flow tracings during the ten-min period when the software is collecting data from an empty chamber. These tracings should remain flat, and any interruptions or slight waves are signs of noise and should be addressed. Pressure changes from opening and closing doors, or from HVAC functioning can also add to erroneous fluctuations, and ensuring these actions occur minimally (and noting when they do occur) is critical. Humidity can also affect the calculated tidal volume and minute ventilation, making it very important to confirm the chamber and connecting tubes are dried before use. The use of drierite beads in sequence with the flow in tubes can help remove all condensation in the air prior to chamber entrance, if this action is deemed necessary. This step would be instituted in cases when humidity has routinely been higher than levels listed in The Guide for the Care and Use of Animals8 (30-70%, ideally within 10% of set point). Humidity can also build up in the chamber due to the animal. Although some humidity is normal, it may continue to build if the animal is excessively active or placed in the chamber for longer durations. If humidity levels reach maximum levels (99.99%), the chamber may need to be opened and wiped down during experimentation to maintain comparable breathing measures. The software accounts for changes in barometric pressure, ambient temperature, animal temperature and humidity. Best practice is to maintain the values within a reasonable range so that “apples to apples” are being compared across ages, strains and sexes.”*

Additionally, we have discussed the metrics used to determine baseline so that others can use them to determine their own “in house” methods. These metrics include: ensuring mice are awake, stationary, and exhibit a breathing trace with 100% of breaths accepted by the software for analysis. If the breathing trace has increased activity within the same minute as the 15-s trace, we also use these data to verify the mouse is awake. This information is described in the manuscript at Lines 719-722 and Lines 733-736, which now read:

*“In order to characterize quiet breathing, it is important to continuously observe the barometric plethysmography chamber and mouse throughout the duration of the protocol. When quantifying quiet breathing, mice should be awake, but not partaking in any active behaviors such as sniffing, grooming or exploring (represented in Figure 4A)…*

*The software program should distinguish the breaths, with an acceptance rate of 100% during the 15-s period. We advise taking note of the breathing tracings in addition to ensuring animals meet behavioral criteria for baseline, since it is possible that stationary mice may still be anxious.”*

Minor Concerns:  
Line 89 - more specifically, what is meant by "biases", this could be confusing to the reader/viewer.

This commentary has been completely removed from this section, which now

reads:

*“Obtaining an optimal air breathing segment is an important consideration for*

*baseline comparisons. The increased use of commercially available plethysmography systems makes collecting pattern of breathing data possible in many laboratories. Importantly, pattern of breathing is variable throughout the collection period, particularly for mice. With that said, it is necessary to standardize baseline analysis as a means of ensuring that the training level of experimenters does not confound results.”*

Other information has been added and more appropriately phrased in Lines 792-797. We stress that experimenters should be blinded and have existing baseline

criteria in place prior to analysis. Whenever possible, the same person should do

all analyses for a given experiment. This section reads:

*“Experimenters should also remain blinded to animal groups during data*

*collection and analyses to prevent differences in selection of baseline. When possible, the same experimenter should collect all data and/or analyze all tracings in a given experiment. Steps to keep experimenters blinded to the animal groups, as well as randomization and testing during similar times of the day are crucial to the rigor of investigation.”*

Line 375-401 Excellent points. Additionally, there is a published record (Quindry, et. al.,) which indicates that room lighting and time of day impact these measures. I recommend including for the reader/viewer that these methodological considerations could be important to the current approach, unless demonstrated otherwise.

Time of day, ambient temperatures, and other environmental cues do influence

ventilation. We have added more information about these specifics to the

manuscript at Lines 789-792. Since Quindry, et. al. used a different setup and criteria for baseline and habituation, it would not be an appropriate reference to cite in this particular instance as the experimental designs between studies are not comparable. Also, the values they report are outside the ranges in this study. Instead, we use Kabir et. al. (2010) and Seifert & Mortola (2002) as references for differences stemming from room lighting and time of day for testing. These particular studies account for animal activity/locomotion during baseline breathing analysis. The addition reads:

*“Moreover, taking into account the circadian cycle of mice and the time range of testing, as well as specific lighting conditions of the room are important details to consider13,17. For instance, we typically test mice in lighting similar to their housing room (either light or dark cycle) and within a three hour range18.”*

The authors wisely limit their analyses to calculated/measured variables that are considered most reliable with these devices. That said, some users may be tempted to report the less well-regarded variables that are calculated by the software. Consider including some cautions about drawing outcomes or conclusions from those variables.

We thank you for acknowledging that our analyses only consider what is most reliably measured by unrestrained barometric plethysmography. We have also added commentary about the concern. Lines 745-754 now read:

*“Other variables that are calculated by the system should be used with caution. UBP is not a direct measure of pulmonary mechanics, thus variables related to airway resistance (e.g.: enhanced pause/Penh) should be interpreted with this caveat in mind5.”*

**Reviewer #2:**  
Manuscript Summary:  
This is an interesting manuscript from Dr. DeRuisseau's group that describes a useful technique for unrestrained plethysmography. Please see my comments below.  
  
Major Concerns:  
How is tidal volume actually measured here? Do the authors collect core temp to compute tidal volume according to the Drorbaugh and Fenn method or does their software provide an estimate of tidal volume using a correction factor? If so, this should be listed as a major limitation of the technique.

Tidal volumes were measured using the Drorbaugh and Fenn equation, which takes into account individual mouse body temperatures, ambient temperature, humidity and barometric pressure. Our system continuously collects ambient temperature, humidity and barometric pressure. Body temperature is typically collected via an implantable device, but could be achieved with rectal probes. We have added this information to the manuscript, and have also added the need for

a device to measure body temperature into the protocol. Lines 741-745 now read:

*“Particularly, VT and VE calculations use the Drorbaugh and Fenn equation16, which requires body temperature, ambient chamber temperature, humidity and barometric pressure. It is recommended to collect these measures throughout the experiment for the most accurate VT and VE values.”*

Lines 216-219 are very subjective reading " Breathing patterns should become fairly consistent during these time periods, although it is likely that the calm segments may be short in length". It is not clear to me in reading the manuscript if the investigators are watching for the animal to be calm or the breathing pattern to be calm. Waiting for the breathing pattern to become "calm" may bias the data in attempting to collect data in transgenic models, etc with breathing instability. If the authors suggest waiting for the animal to be calm, please clearly define what criteria must be met.

We have removed this sentence from the ‘Methods’ section and added

clarification about criteria for calm breathing. Since this technique also requires looking at the breathing pattern on the barometric plethysmography tracing, we

also discussed why this point is necessary. Lines 733-738 now read:

*“The software program should distinguish the breaths, with an acceptance rate of*

*100% during the 15-s period. We advise taking note of the breathing tracings in*

*addition to ensuring animals meet behavioral criteria for baseline, since it is*

*possible that stationary mice may still be anxious. A previous study reported that*

*although rats exhibited calm behavior, they still showed altered breathing*

*patterns (i.e. increased frequency) in response to controlled stimuli within the*

*testing room13.”*

Are the animals routinely allowed to acclimate to the room for any period of time before testing (for example, overnight)?

Mice underwent short (60-minute) familiarization trials prior to testing, although

we observed no differences in animal exploratory behaviors on the day of testing.

However, previous studies by Teske et al. (2014) and Kabir et al. (2010) report

successful familiarization techniques, which include a 24 h familiarization period

or placing cylinders similar to the chamber size in the home cage of animals. We

have added this information to the manuscript so that readers can make use of a

similar protocol if desired. Lines 652-709 now read:

*“However, because different strains, sexes and ages of mice may all react differently to the chamber environment10,11, it is possible that habituation techniques may be helpful12,13 for some cohorts. Our familiarization trials consisted of placing the mice in the UBP chamber in the testing room for one to two hours for several days prior to experimentation. While we observed no changes in animal behavior following this procedure, a previous study has shown that 24-h of habituation was needed to eliminate novelty effects resulting in spontaneous physical activity of mice12. Additionally, Kabir et al. found that placing plastic cylinders similar in size to the barometric plethysmography chamber in the home cage was advantageous to getting rats to familiarize themselves with the setups prior to experimentation13.”*

This may be my unfamiliarity with the setup, but the authors list 0.3L/min flow in and 0.1 L/min flow out. Doesn't this pressurize the system?

The UBP chamber has a pneumotach screen at the top of the chamber that

allows a known amount of air to leave the system, so the chamber does not over pressurize. However, we have changed the flows listed since each experiment may need some adjustments depending on the mouse size and baseline metabolic rate. Lines 758-763 now describe this information:

*“Required flow rates can differ between UBP setups, so it is important to check these values prior to experimentation. Flow rate into the chamber should be enough to provide fresh air or gas challenges in a timely manner. Flow rate should also be sufficient for allowing the metabolic analyzers to measure O2 and CO2 without having CO2 build up within the chamber environment, which poses the risk of changing pattern of breathing.”*

Am I correct in interpreting that apneas and augmented breaths are counted by hand? Are there specific criteria for defining these? I saw a 0.5s pause for apnea, but what about augmented breaths? If it is subjective, have the authors every quantified their inter-investigator error? How do the authors train new students or investigators to identify these breaths and distinguish an augmented breath from sniffing.

Yes, these measures are counted by hand. The specific criteria are: a period of

suspended breathing ≥0.5 s for apneas and a sharp rise in the breathing trace above 1.25 mL/s followed by a sharp decrease below -0.75 mL/s for augmented

breaths. This information has now been clarified in the manuscript in Step 7.3. In a subset of 9 mice, the Pearson correlation for inter-rater reliability is *r*=.99 for apneas and *r*=.86 for augmented breaths, indicative of high agreement between raters. New students/investigators are trained to identify augmented breaths and

distinguish them from sniffing using the criteria described. In our experience,

sniffing does not meet the same criteria as augmented breaths. New lab

members use previously collected flow tracings from mice to go through analysis

steps with a trained lab member, so that collection of augmented breaths and

apneas are consistent between investigators. Lines 509-513 now contain the information regarding the inter-rater reliability analysis.

*“To confirm inter-rater reliability for apnea and augmented breath analyses, Pearson correlation was calculated for 2 different investigators. A high degree of agreement between raters was found, as indicated by a value of r=.99 for apneas and r= .86 for augmented breaths. In future studies, an increased number of apneas compared to a control group would be telling of breathing dysfunction stemming from a neural component.”*

Minor Concerns:  
Please check the axes in figure 1. Panel E reads VE/CO2 and I think it should be VE/VCO2.

Figure 1 has been changed to be appropriately labeled.  
  
In Figure 3, what time frame is the count over? Are these from the 4 15s sampling periods? 150 apneas per minute seems like a lot. Are these not impacted by the animal moving or being agitated?

The frame count for Figure 3 is over the course of 30-minutes. This has been

specified in the manuscript and related figure legend. We still observe

augmented breaths and apneas even with animal locomotion and grooming.