Journal of Visualized Experiments

Method to Obtain Pattern of Breathing in Senescent Mice through Unrestrained Barometric Plethysmography

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59393R1
Full Title:	Method to Obtain Pattern of Breathing in Senescent Mice through Unrestrained Barometric Plethysmography
Keywords:	Apnea; frequency; minute ventilation; tidal volume; VCO2; augmented breath
Corresponding Author:	Lara R. DeRuisseau, PhD Le Moyne College Syracuse, NY UNITED STATES
Corresponding Author's Institution:	Le Moyne College
Corresponding Author E-Mail:	deruislr@lemoyne.edu
Order of Authors:	Candace N. Receno
	Jacob Russell
	Caitlin M. Cunningham
	Lara R. DeRuisseau
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Syracuse, NY, USA

TITLE:

Method to Obtain Pattern of Breathing in Senescent Mice through Unrestrained Barometric Plethysmography

AUTHORS & AFFILIATIONS:

Candace N. Receno[1], Jacob Russell[1], Caitlin M. Cunningham[2], Lara R. DeRuisseau[1]

[1]Department of Biological Sciences, Le Moyne College, Syracuse, NY, USA
[2]Department of Mathematics, Le Moyne College, Syracuse, NY, USA

Corresponding author:
Lara R. DeRuisseau (deruislr@lemoyne.edu)
Tel: (315) 445-5463

Email addresses of co-authors:
Candace N. Receno (recenocn@lemoyne.edu)
Jacob Russell (russeljd@lemoyne.edu)
Caitlin M. Cunningham (cunninc@lemoyne.edu)

KEYWORDS:

Apnea, frequency, minute ventilation, tidal volume, VCO_2, augmented breath

SUMMARY:

Unrestrained barometric plethysmography is used to quantify the pattern of breathing in awake mice. We show that 15 s segments under a standardized protocol display similar values to an extended duration of quiet breathing. This methodology also allows for the quantification of apnea and augmented breaths during the first hour in the chamber.

ABSTRACT:

Unrestrained barometric plethysmography (UBP) is a method for quantifying the pattern of breathing in mice, where breathing frequency, tidal volume, and minute ventilation are routinely reported. Moreover, information can be collected regarding the neural output of breathing, including the existence of central apneas and augmented breaths. An important consideration for UBP is obtaining a breathing segment with a minimal impact of anxious or active behaviors, to elucidate the response to breathing challenges. Here, we present a protocol that allows for short, quiet baselines to be obtained in aged mice, comparable to waiting for longer bouts of quiet breathing. The use of shorter time segments is valuable, as some strains of mice may be increasingly excitable or anxious, and longer periods of quiet breathing may not be achieved within a reasonable timeframe. We place 22-month-old mice in a UBP chamber and compare four 15 s quiet breathing segments between minutes 60–120 to a longer 10 min quiet breathing period that takes 2–3 h to acquire. We also obtain counts of central apneas and augmented breaths prior to the quiet breathing segments, following a 30 min familiarization period. We show that 10 min of quiet breathing is comparable to using a much shorter 15 s duration. Additionally, the time leading up to these 15 s quiet breathing segments can be used to gather data regarding

apneas of central origin. This protocol allows investigators to collect pattern-of-breathing data in a set amount of time and makes quiet baseline measures feasible for mice that may exhibit increased amounts of excitable behavior. The UBP methodology itself provides a useful and noninvasive way to collect pattern-of-breathing data and allows for mice to be tested over several time points.

INTRODUCTION:

UBP is a common technique for the assessment of breathing patterns[1–4]. In this method, mice are placed in a closed chamber where pressure differences between the main chamber (where the animal is housed) and a reference chamber are filtered through a pneumotachograph to obtain values. The resulting UBP setup is noninvasive and unrestrained and allows for respiratory measures to be assessed without the requirement of anesthesia or surgery. Additionally, this technique is suitable for studies requiring multiple measurements in the same mouse over time. Variables such as breathing frequency, tidal volume, and minute ventilation can be quantified with this method, during a single trial or over several trials. Whole-body UBP also provides measures of peak flows and respiratory cycle duration. Together, these parameters quantify the pattern of breathing. The recorded breathing traces also make it possible to review the data and count the number of central apneas displayed within a given time period. This count can be used alongside an analysis of tidal volume and inspiratory times to gauge other alterations in the pattern of breathing.

While several noninvasive plethysmography techniques exist for the direct assessment of pulmonary physiological parameters, whole-body UBP allows for a way to screen for respiratory function with minimal undue stress to the mouse. Head-out plethysmography, which utilizes tidal midexpiratory flow measures and is also noninvasive, relies on restraint, like many other types of plethysmography (e.g., double-chamber plethysmography). While these methods have been used in rodent models to measure airway responsiveness[5], the use of neck collars or small restraint tubes may take mice (vs. other species) longer to acclimate to and return their breathing to resting levels.

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. There are numerous ways to collect an air-breathing segment, serving as one area of variation between experimental designs. One example includes averaging the final 10–30 min of data following a previously defined set of time within the chamber[1], while another method involves waiting until the mouse is visibly calm for 5–10 min[6]. The latter can take 2–3 h to achieve and in some cases, a trial may need to be abandoned if the mouse is not calm for long enough. This concern is an especially important consideration for strains of mice where observed behaviors are more anxious and excitable[7]. These mice may take longer to adjust to the chamber environment and only remain calm for short bursts of time. Limiting the time devoted to baseline collection standardizes the chamber time for each mouse.

It is crucial that experimenters obtain a suitable baseline that encompasses resting behavior values in the mouse but also occurs in a timely manner. Hence, the goal of this report is to provide a description of methods used to obtain short quiet baseline values for breathing parameters in mice. Moreover, we report that apneas and augmented breaths can be quantified during the first hour in the chamber.

PROTOCOL:

All procedures were approved by the Le Moyne College Institutional Animal Care and Use Committee. All use of animals was in agreement with the policies described in the Guide for the Care and Use of Laboratory Animals[8].

NOTE: **(Critical)** Prior to experimentation, obtain all necessary approvals and training required for animal use. It is important the experimenters are familiarized with the mouse behaviors and activity levels, including signs of sleep, distress, and/or movement artifact vs. normal sniffing and breathing.

1. Whole-body barometric plethysmography chamber

1.1. Read the appropriate user manuals for the barometric plethysmography chamber, including connectors, O-rings, etc., and create a standard protocol file to define analyzers (i.e., metabolic) and parameters specific to the software.

1.2. Make sure all hoses and tubes are connected to the chamber. Connect a gas flow tube (flow-in) and a vacuum tube (flow-out) directly to the barometric plethysmography chamber.

NOTE: The inflow must be attached to the opening marked **bias flow**.

1.3. Attach CO_2, O_2, and N_2 gas tanks to the gas mixer. Make sure all gas tanks are in the open position prior to experimentation.

2. Calibration of the barometric plethysmograph chamber

2.1. Calibrate a high and a low flow of gas by selecting the **7700-Amplifier Setup** under the **Hardware** tab of the barometric plethysmography software.

2.2. Set a vacuum (flow out of the chamber) appropriate for the experimental design, and set gas analyzers (~0.1 L/min).

NOTE: The outflow rate must remain the same throughout the calibrations and experiment for accurate metabolic recordings.

2.3. Set a low flow of air by removing the flow tube from the chamber and turning off the vacuum.

2.4. Record the zero flow by entering a **0** into the **Low Unit** cell. Double-click the **Low Cal** cell, change the time to 3 s, and hit **Measure**.

2.5. Reattach the flow tube and allow gas (20.93% O_2, balanced N_2) to flow through the barometric plethysmography chamber from the gas mixer.

2.6. Convert the inflow from liters/minutes into milliliters/second. Click the **High Unit** cell and enter the value in milliliters/second. Double-click **High Cal**, change the time to 3 s, and click **Measure**.

2.7. Leave the **7700-Amplifier Setup** tab open to calibrate the metabolic analyzers to the barometric plethysmography software.

3. Metabolic analyzer calibration

3.1. In the gas mixer program, set the gas mixer to release a flow of gas containing 20.93% O_2 and 79.2% N_2.

3.2. On the metabolic analyzers, set the O_2 calibration level to 20.93% and the CO_2 to read 0%. Turn the dial back to **Sample** once the appropriate values are entered.

3.3. Set the high O_2 percentage. Click on the **ABCD-4** tab of the barometric plethysmography software and then enter **20.93** under **High Unit** of the C2 line. Under **High Cal**, change the time to 3 s and hit **Measure**.

3.4. Set the low CO_2 percentage. Enter **0** under **Low Cal** of the C3 line, and then change the time to 3 s and click **Measure** under **Low Cal**.

3.5. In the gas mixer program, change the O_2 value to 10% and the CO_2 value to 5%. Wait several minutes for the gas flow to adjust to these values. On the metabolic analyzers, turn the adjustment knobs to calibrate CO_2 equal to 5% and O_2 equal to 10%. Be sure to turn the dial back to **Sample** once the values are calibrated.

3.6. Set the high CO_2 percentage. Ensure the analyzer readings are stable before inserting appropriate values into the O_2 and CO_2 on the barometric plethysmography software. Click **High Unit** under C3 and enter **5**. Change **High Cal** to 3 s and hit **Measure**.

3.7. Set the low O_2 percentage. Click **Low Unit** under the C2 option and enter **10**. Click **Low Cal**, input 3 s, and click **Measure**.

3.8. Change the gas values on the gas mixer back to 20.93% O_2 and 79.2% N_2. Wait several minutes for the chamber to adjust to these values. Repeat the steps 3.1–3.7 if the metabolic analyzers do not automatically read 20.93% O_2 and 0% CO_2, to ensure proper calibration.

3.9. Recheck the flow meters connected to the barometric plethysmography chamber. Adjust the air flow into and out of the chamber to rates appropriate for the experiment (typically, 0.1–0.3 L/min).

3.10. Once all settings have been applied to the barometric plethysmography software, click **OK** to begin recording.

4. Unrestrained barometric plethysmography

4.1. Record the mouse's weight and initial body temperature. Wait 10 min before placing the mouse in the chamber, to collect O_2 and CO_2 data from an empty chamber. Work in a quiet area familiar to the mice so noise and smells do not interfere with the data collection. Avoid any possible disruptions, including the opening and closing of doors or personnel moving in/out of the data collection room.

NOTE: This specific protocol employed 22-month-old male C57BL/6J mouse.

4.2. During the first hour, document the behaviors of the mouse and take detailed notes, including specific values of the flow in/out of the chamber.

4.3. After 60 min of chamber habituation, watch for segments of quiet breathing for the following 60 min. List any segments lasting at least 15 s in length without sniffing and grooming**.** Take body temperature measures every 10 min when using an implantable device.

4.4. At the end of the experiment, remove the mouse from the chamber and place it back in its cage. All equipment should be cleaned and wiped down thoroughly. If droplets of water remain, use pressurized air to remove them.

5. Analysis of pattern of breathing and metabolism

5.1. Open the barometric plethysmography review file and consult the recorded notes for the animal of interest.

5.2. Open the **Metabolic** panel in the software and take the average of the first 10 min of O_2 and CO_2, when the chamber was empty. Record these values as the FiO_2 and $FiCO_2$.

5.3. View the **Flow** panel of the barometric plethysmography software. Right-click **Analyze Attributes** and set appropriate parameters. Under the **Meta 1** tab, enter the FiO_2 and $FiCO_2$ from step 5.2, as well as the flow into the chamber under **Meta 2**, to calculate VO_2 and VCO_2.

5.4. For a pattern-of-breathing analysis, enter the times for the 15 s intervals of quiet breathing under **Open Data Parser Dialogue** from the **Data Parser** tab. Click **Parser View Mode** to only show the specific 15 s segments of interest.

5.5. Click **Save Parsed Derived Data**. Open the data file in a spreadsheet to obtain the binned data.

6. Analysis of apneas and augmented breaths

6.1. In the open review file, exit **Parser View Mode**. Go into the **Graph Setup** option under **Setup** > **P3 Setup** and select **Page View** under **Type**. Select **5** for the number of panes. Enter **-2** into the box labeled **Low** and **2** into the box labeled **High** for flow measures in milliliters/second. Apply the changes.

6.2. Scroll to the 30 min mark on the flow tracings panel.

6.3. Count apneas and augmented breaths for the 30–60 min after the mouse was placed in the chamber. Quantify periods of suspended breathing lasting longer than or equal to 0.5 s, indicative of an apnea. Augmented breaths are indicated by a sharp rise in the breathing trace above 1.25 mL/s followed by a sharp decrease below -0.75 mL/s.

REPRESENTATIVE RESULTS:

The results of UBP as an evaluation of pattern of breathing in 16 aged (22-month-old) mice performed under normal air gas (20.93% O_2 with balanced N_2) are reported. The analysis first included a comparison of a longer 10 min quiet breathing segment (which took over 2 h to obtain) versus the average of four short 15 s segments (quantified within minutes 60–120). A representative flow tracing of quiet breathing, where breathing is consistent with no active breathing behaviors, is provided in **Figure 1A**. When similar tracings are collected from animals, 100% of the breaths should be accepted by the software. However, **Figure 1B** represents breathing from a more active segment, where the mice are exploring the chamber, sniffing, and/or grooming. Tracings similar to that shown in **Figure 1B** are less likely to be accepted by the software and are not ideal for the type of breathing collection used and explained by this methodology. The parameters selected for the assessment of possible pattern-of-breathing differences between the two time points were breathing frequency (**Figure 2A**), tidal volume (VT, **Figure 2B**), minute ventilation (VE, **Figure 2C**), tidal volume/inspiratory time ratio (VT/Ti, **Figure 2D**), and minute ventilation/expelled carbon dioxide ratio (VE/VCO_2/g, **Figure 2E**), which were all calculated using the barometric plethysmography software and Drorbaugh and Fenn equation. Values reported for measures are within the range of what we have previously reported for the mouse model[6,9]. No significant differences ($p < 0.025$ was considered significant) were elucidated between groups; post hoc corrections for multiple comparisons of breathing frequency and VT data were accounted for with Bonferroni. These results show that the use of a simplified protocol using 15 s baselines provides similar results to that of a longer baseline protocol.

Further analysis was performed with each of the four 15 s baseline segments for frequency, VT, VE, VT/Ti, and VE/VCO_2/g (**Figure 3**). No significant differences ($p > 0.05$) between any of the time points were found. There were also no differences in the variability between any of the four time segments for any pattern-of-breathing measure. Additionally, we tested the variability of the segment of the 15 s group vs. the 10 min group and found no significant differences using

Levene's test when comparing the averaged group data.

The number of apneas and augmented breaths observed for each animal during minutes 30–60 of the UBP protocol are presented in **Figure 4**. These results show that aged animals showcase a high number of apneas and augmented breaths within a 30 min span (tracing shown in **Figure 1C**). The data are indicative of changes during the aging process, as these findings were observed in 22-month-old mice. To confirm interrater reliability for apnea and augmented breath analyses, Pearson correlation was calculated for two 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 for a breathing dysfunction stemming from a neural component.

FIGURE AND TABLE LEGENDS:

Figure 1: Representative flow tracings. (**A**) Flow tracing from a quiet baseline, where the mouse does not showcase any active behaviors such as sniffing or grooming. (**B**) Flow tracing from an active breathing period not included in our analyses, where mice are moving about the chamber and many breaths are not routinely accepted. (**C**) Flow tracing displaying an augmented breath followed by a period of apnea. A 5 s window is shown for all traces.

Figure 2: Breathing parameters are similar for calm breathing segments of 10 min and 15 s in 22-month-old mice. Barometric plethysmography was used to collect breathing data in aged mice ($n = 16$, 22 months old). Breathing data were calculated for mice during two different time points, namely the average of four 15 s calm intervals within the 60–120 min mark of the mouse being in the chamber and for 10 min of consistent calm breathing. (**A**) Breathing frequency (breaths/minute). (**B**) Tidal volume (VT; milliliters/breath). (**C**) Minute ventilation (VE; milliliters/minute). (**D**) Ratio of tidal volume to inspiration time (VT/Ti; milliliters/second). (**E**) Ratio of minute ventilation to carbon dioxide expelled, normalized to weight ($VE/VCO_2/g$). There are no statistically significant differences between the groups after post hoc corrections ($p > 0.025$). Values of >3 SD above the mean were considered outliers and removed from the data set. Data are presented as mean ± SD.

Figure 3: Comparison of four 15 s intervals. Breathing data were calculated in calm breathing mice ($n = 16$, 22 months old) for four separate 15 s intervals within the 60–120 min of chamber placement. (**A**) Breathing frequency (breaths/minute). (**B**) Tidal volume (VT; milliliters/breath). (**C**) Minute ventilation (VE; milliliters/minute). (**D**) Ratio of tidal volume to inspiration time (VT/Ti; milliliters/second). (**E**) Ratio of minute ventilation to carbon dioxide expelled, normalized to weight ($VE/VCO_2/g$). There are no statistically significant differences between the time segments ($p > 0.05$). Outliers are defined as >3 SD above the mean and removed. Data are presented as mean ± SD.

Figure 4: Apnea and augmented breath counts in mice. Apneas (≥0.5 s without breathing) and augmented breaths (ABs; a sharp increase in inhalation over 1.25 mL/s followed by a sharp exhalation below -0.77 mL/s) were counted in aged mice ($n = 16$, 22 months old) between 30–60

min. The counts were analyzed over 30 min and the total for that time period are reported. Data are presented as mean ± SD.

Figure 5: Schematic of the unrestrained barometric plethysmography (UBP) setup. The overall UBP setup should be similar to that described in the figure. Flow measurements must be measured for the gases entering and leaving the chamber, and the gas composition must be known for data interpretation.

DISCUSSION:

The protocol provides information regarding a quiet breathing baseline in mice, as well as collecting data about central apneas and augmented breaths. The representative results show that a 10 min quiet baseline has a similar pattern of breathing when compared to an average of four 15 s bouts for a cohort of old mice. Importantly, the 15 s bouts are not statistically different, nor do these groups have differences in variation from one another using Levene's test. These data demonstrate that even one short bout is sufficient for monitoring quiet breathing. However, it is entirely possible that analyzing individual variation within a mouse at 15 s vs. 10 min may result in different findings, as the 10 min bout could encompass minimal sniffing and grooming activities. However, using Levene's test for a comparison of individual mouse baseline segments provides a different analysis than the one described in this protocol. Overall, the design of this methodology uses 15 s breathing segments that can be acquired during minutes 60–120 in the chamber, versus having to wait for each mouse to achieve longer durations of quiet baseline.

The shorter duration required for baseline allows for more anxious/agitated strains of mice to be tested for quiet breathing. The use of a longer breathing segment (i.e., 10 or 2 min) lengthens the protocol duration, to a point where a trial may need to be abandoned if the mice do not display a quiet breathing trace within 3 h. Since many experimental designs also incorporate respiratory challenges (i.e., hypoxia), the extended time allotted for other gases highlights the need for baseline collection time to be standardized. The use of a single 15 s bout of quiet breathing helps to relieve the concern of working with mice (and strains of mice) that may be particularly excitable in the chamber. While working with barometric plethysmography, we found that ~10% of mice per study had to be excluded because of their inability to perform as little as 2 min of continuous quiet breathing within the chamber. The implementation of previous familiarization trials was unsuccessful in getting mice to calm down faster when placed in the chamber on the day of experimentation. However, because different strains, sexes, and ages of mice may all react differently to the chamber environment[10,11], it is possible that habituation techniques may be helpful[12,13] for some cohorts. Our familiarization trials consisted of placing the mice in the UBP chamber in the testing room for 1–2 h 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 in mice[12]. Additionally, Kabir et al. found that placing plastic cylinders similar in size to the barometric plethysmography chamber in the home cage was advantageous in getting rats to familiarize themselves with the setups prior to experimentation[13].

This protocol also uncovers possible respiratory dysfunction in mice via the quantification of

central apneas, indicative of neural control issues. Thirty minutes of observation prior to the baseline pattern-of-breathing collection showed that all 16 aged mice displayed a high number of apneic episodes and augmented breaths (represented in **Figure 1C**). The numerous apneas in this aged mouse cohort highlight the ability of this protocol to quantify another important breathing measure without adding additional time to experimentation. It should be noted that age and disease progression (if applicable) can affect the presence and number of apneic episodes.

In order to characterize quiet breathing, it is important to continuously observe the barometric plethysmography chamber and mouse throughout the duration of the protocol. For the quantification of quiet breathing, mice should be awake but not partake in any active behaviors such as sniffing, grooming, or exploring (represented in **Figure 1A**). Since patterns of breathing during sleep can differ from those in an awake animal[14,15], the collection of calm breathing during the awake state is critical. It is possible that longer segments of quiet breathing could include periods of sleep, which may not be desired depending on the experimental design. In this case, shorter segments of quiet breathing would be ideal to document, as the likelihood of data collection during sleep is reduced when active segments flank the short (15 s) quiet breathing segments. We have observed that longer segments of quiet breathing can be challenging to acquire in the mouse model, as mouse behavior in the chamber seems to be very different compared to that of rats. It is important both to critically observe mouse respiratory flow for appropriate breathing segments and to document animal behavior. In cases of reduced ventilation or unstable breathing, this method can still be utilized. In these instances, it is essential the experimenter is blinded to the cohort when selecting the 15 s segments. 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 that the animals meet the 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 room[13].

Measures of frequency, VT, VE, inspiratory and expiratory time, and VE/VCO_2 are all quantified using analyzers and the UBP software and are frequently reported in the literature. Particularly, VT and VE calculations use the Drorbaugh and Fenn equation[16], 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. 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 mind[5]. Additional components of the UBP setup that can impact variables calculated by the software include flow rates and the general calibration of the system. Confirm seals and gaskets are working properly (no leaks) and ensure proper connection of all equipment to the barometric plethysmography chamber (**Figure 5**). Flow rates in and out of the chamber should be kept consistent. Required flow rates can differ between UBP setups, so it is important to check these values prior to experimentation. The flow rate into the chamber should be enough to provide fresh air or gas

challenges in a timely manner. The flow rate should also be sufficient for allowing the metabolic analyzers to measure O_2 and CO_2 without having CO_2 build up within the chamber environment, which poses the risk of a changing pattern of breathing. Similarly, gas mixer/analyzer calibrations need to be regularly implemented to ensure that the metabolic parameters are accurately measured.

Other considerations for conscious UBP include reducing distractions within the experimental room while animals are being tested. Loud noises, different smells, and the presence of nonessential 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 for the mice. 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 10 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 that these actions occur minimally (and noting them when they do occur) is critical. Humidity can also affect the calculated tidal volume and minute ventilation, making it very important to confirm that the chamber and connecting tubes are dried before use. If necessary, the use of Drierite beads in sequence with the flow-in tubes can help remove all condensation in the air prior to chamber entrance. This step would be instituted in cases when the humidity has routinely been higher than levels listed in The Guide for the Care and Use of Animals[8] (30%–70%, ideally within 10% of setpoint). Humidity can also build up in the chamber due to the presence of 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. Moreover, the circadian cycle of mice and the time range of testing, as well as specific lighting conditions of the room, are important details to consider[13,17]. For instance, we typically test mice in lighting similar to their housing room (either light or dark cycle) and within a 3 h range[18]. Experimenters should also remain blinded to the animal groups during data collection and analyses to prevent differences in the selection of a 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. Ultimately, there are extraneous factors that may alter the flow tracings, and these concerns should be considered when performing UBP.

The UBP method is a technique used to characterize the pattern of breathing in mice. Baseline measures can be collected within 2 h when using a 15 s breathing segment. Here we report a method which can be performed with aged mice, who are often more agitated in the chamber than younger mice, suggesting that other anxious or active mouse strains could also be tested

with this protocol. The data collected from UBP is noninvasive and allows for testing over multiple time points, which is useful for studies about aging, drug therapy, and disease progression.

ACKNOWLEDGMENTS:

The authors would like to thank Angela Le, Sarah Ruby, and Dr. Marisa Mickey for their work maintaining the animal colonies. This work was funded by 1R15HD076379 (L.R.D.) and 1R15HD076379-A1S1 (L.R.D. to support C.N.R.).

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. DeRuisseau, L. R. et al. Neural deficits contribute to respiratory insufficiency in Pompe disease. *Proceedings of the National Academy of Sciences of the United States of America.* **106** (23), 9419-9424 (2009).

2. Ogier, M. et al. Brain-derived neurotrophic factor expression and respiratory function improve after ampakine treatment in a mouse model of Rett syndrome. *Journal of Neuroscience.* **27** (40), 10912-10917 (2007).

3. Ohshima, Y. et al. Hypoxic ventilatory response during light and dark periods and the involvement of histamine H1 receptor in mice. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology.* **293** (3), R1350-R1356 (2007).

4. van Schaik, S. M., Enhorning, G., Vargas, I., Welliver, R. C. Respiratory syncytial virus affects pulmonary function in BALB/c mice. *Journal of Infectious Diseases.* **177** (2), 269-276 (1998).

5. Glaab, T., Taube, C., Braun, A., Mitzner, W. Invasive and noninvasive methods for studying pulmonary function in mice. *Respiratory Research.* **8** (2007).

6. Loeven, A. M., Receno, C. N., Cunningham, C. M., DeRuisseau, L. R. Arterial Blood Sampling in Male CD-1 and C57BL/6J Mice with 1% Isoflurane is Similar to Awake Mice. *Journal of Applied Physiology (Bethesda, MD).* 10.1152/japplphysiol.00640.2018 (2018).

7. Receno, C. N., Eassa, B. E., Reilly, D. P., Cunningham, C., DeRuisseau, L. R. The pattern of breathing in young wild type and Ts65Dn mice during the dark and light cycle. *FASEB Journal.* **32** (1) (2018).

8. Committee for the Update of the Guide for the Care and Use of Laboratory Animals, Inistitute fpr Laboratory Animal Research, Division on Earth and Life Studies, National Research Council of the National Academies. *Guide for the Care and Use of Laboratory Animals, 8th edition*. National Academies Press. Washington, DC (2011).

9. Receno, C. N., Glausen, T. G., DeRuisseau, L. R. Saline as a vehicle control does not alter ventilation in male CD-1 mice. *Physiological Reports.* **6** (10) (2018).

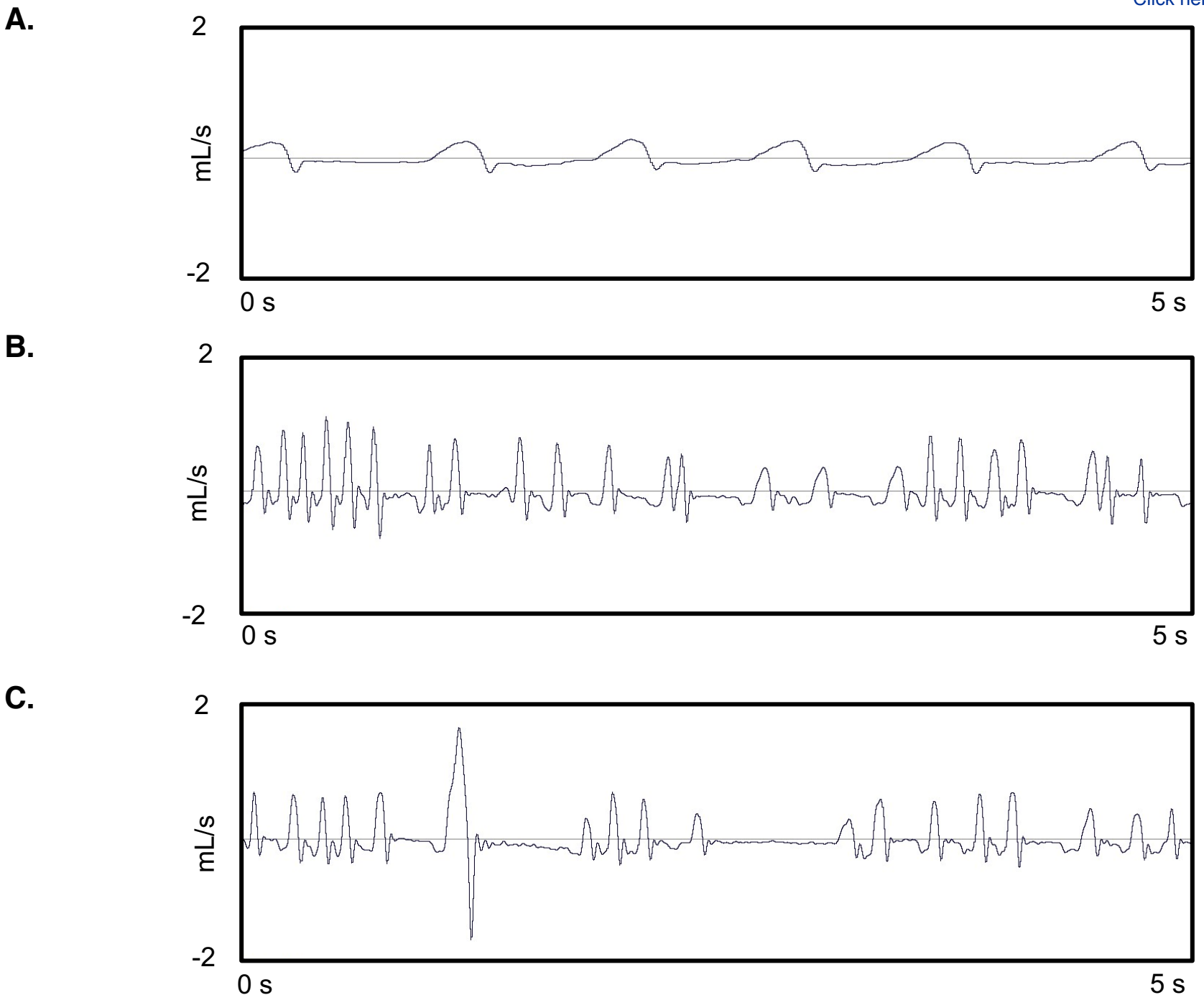
10. Shanksy, R. M. Sex differences in behavioral strategies: Avoiding interpretational pitfalls. *Current Opinion in Neurobiology.* **49**, 95-98 (2018).

11. Kopp, C. Locomotor activity rhythm in inbred strains of mice: implications for behavioural studies. *Behavioural Brain Research.* **125** (1-2), 93-96 (2001).

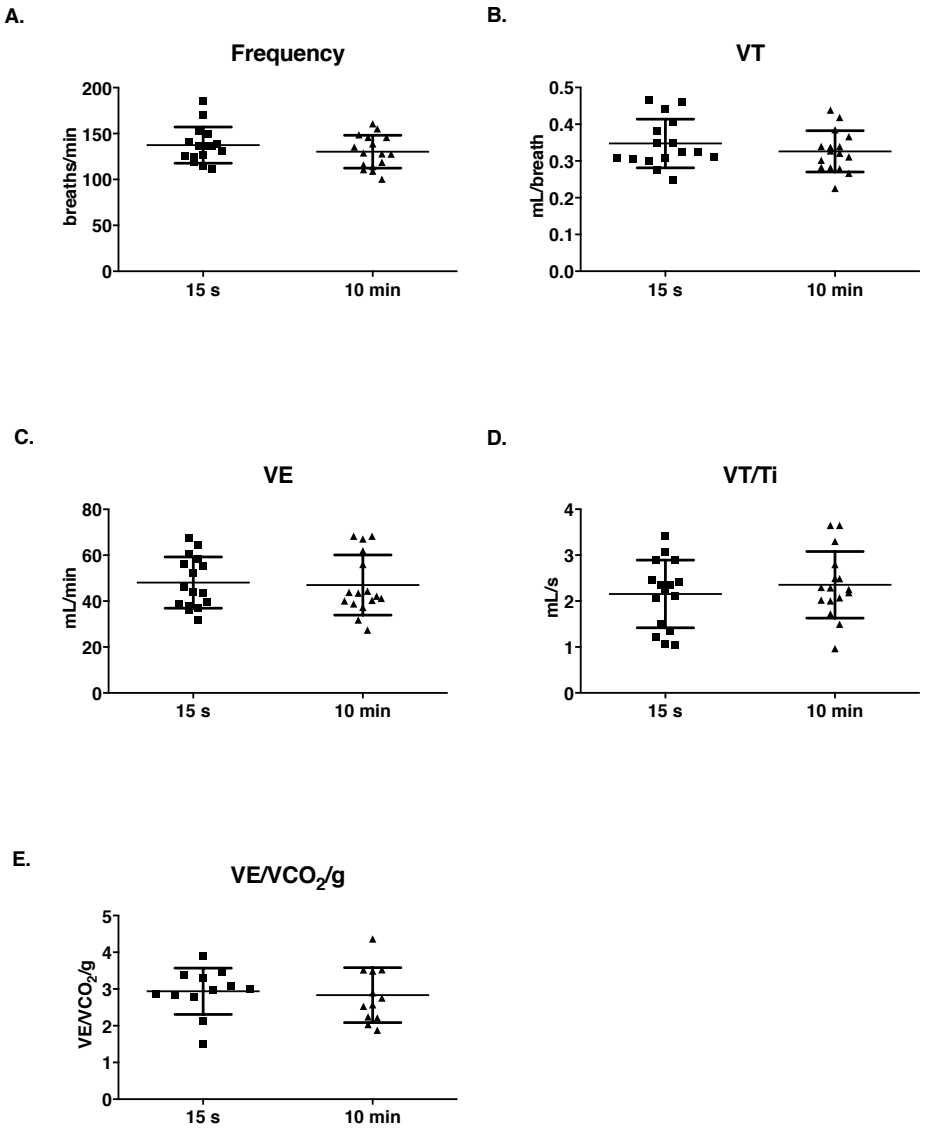
12. Teske, J. A., Perez-Leighton, C. E., Billington, C. J., Kotz, C. M. Methodological considerations for measuring spontaneous physical activity in rodents. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology.* **306** (10), R714-R721 (2014).

13. Kabir, M. M. et al. Respiratory pattern in awake rats: Effects of motor activity and of alerting stimuli. *Physiology & Behavior.* **101** (1), 22-31 (2010).
14. Terada, J. et al. Ventilatory long-term facilitation in mice can be observed during both sleep and wake periods and depends on orexin. *Journal of Applied Physiology.* **104** (2), 499-507 (2008).
15. Friedman, L. et al. Ventilatory behavior during sleep among A/J and C57BL/6J mouse strains. *Journal of Applied Physiology.* **97** (5), 1787-1795 (2004).
16. Drorbaugh, J. E., Fenn, W. O. A barometric method for measuring ventilation in newborn infants. *Pediatrics.* **16** (1), 81-87 (1955).
17. Seifert, E. L., Mortola, J. P. The circadian pattern of breathing in conscious adult rats. *Respiration Physiology.* **129** (3), 297-305 (2002).
18. Receno, C. N., Roffo, K. E., Mickey, M. C., DeRuisseau, K. C., DeRuisseau, L. R. Quiet breathing in hindlimb casted mice. *Respiratory Physiology & Neurobiology.* 10.1016/j.resp.2018.06.003 (2018).

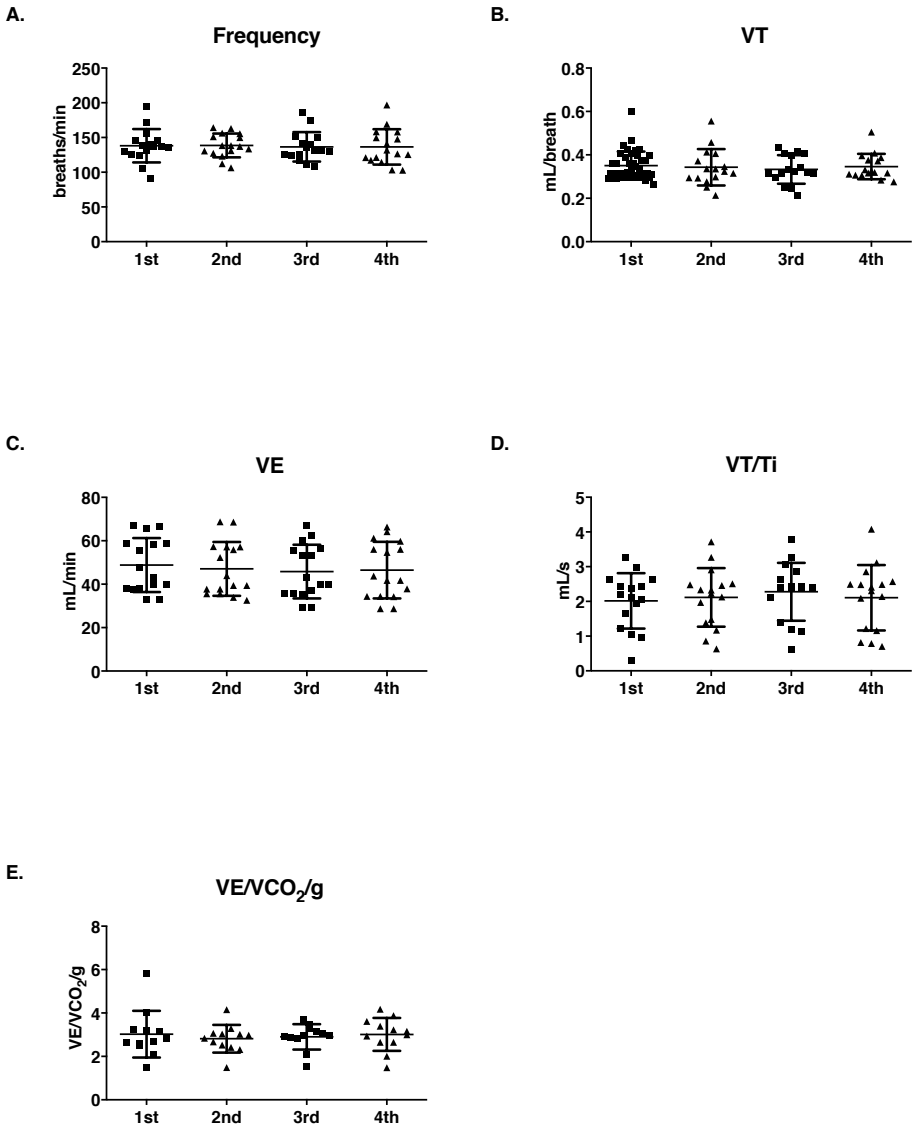
Figure



Figure

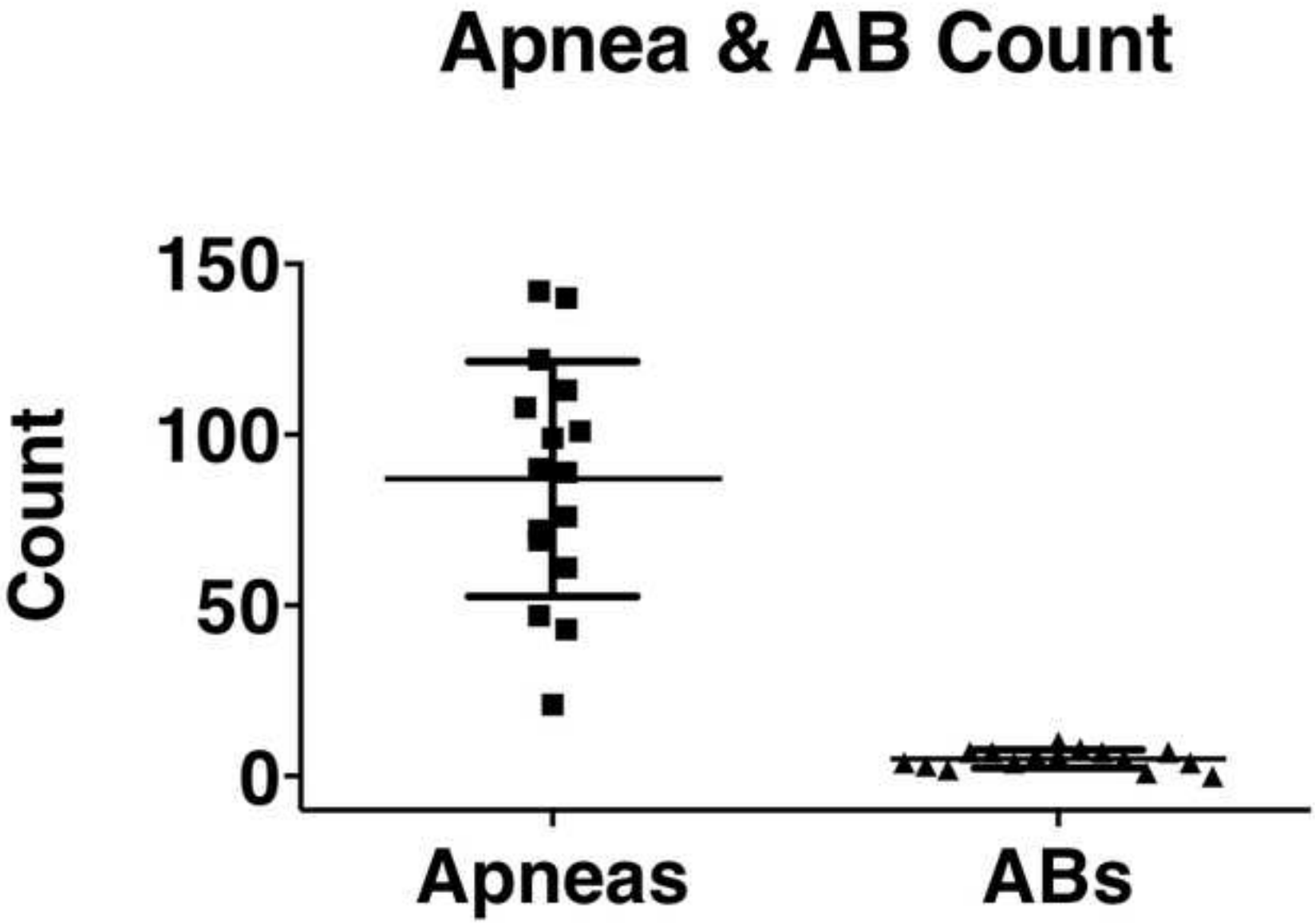


Figure



Figure

Click here to access/download;Figure;fig 4.tiff



Figure

Click here to access/download;Figure;fig 5.tiff

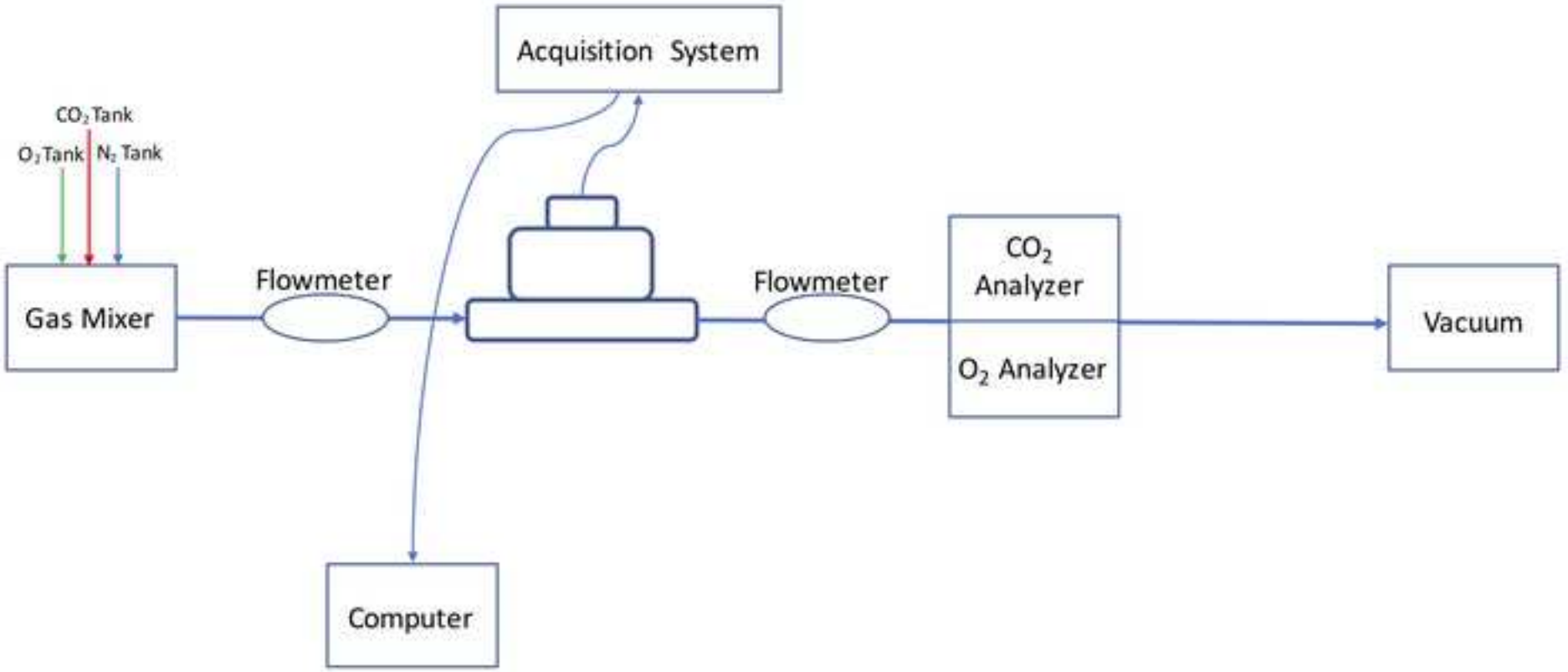


Table of Materials

Name of Material/ Equipment	Company	Catalog Number
Carbon Dioxide Analyzer	AEI Technologies	CD-3A
Carbon Dioxide Sensor	AEI Technologies	P-61B
Computer		
Drierite beads	PermaPure LLC	DM-AR
Flow Control	AEI Technologies	R-1
Flowmeter	TSI	4100
Gas Mixer	MCQ Instruments	GB-103
Gas Tanks	Haun	
Oxygen Analyzer	AEI Technologies	S-3A
Oxygen Sensor	AEI Technologies	N-22M
Polyurethane Tubing	SMC	TUS 0604 Y-20
Ponemah Software	DSI	
Small Rodent Chamber	Buxco/DSI	
Thermometer (LifeChip System)	Destron-Fearing	
Transducers	Validyne	DP45
Whole Body Plethysmography System	Data Science International (DSI)	

Comments/Description

must be compliant with Ponemah requirements

vacuum
need one per chamber and one for vacuum

100% oxygen, 100% carbon dioxide, 100% nitrogen - food grade, or pre-mixed tanks for nomal room air and gas challenges

any type of thermometer to take accurate body temperatures is appropriate, but the use of implantable chips allows for minimal disturbanc
need one per chamber

Includes ACQ-7700, pressure/temperature probes, etc.

:e to animal for taking several body temperature measurements while the animal is still in the UBP chamber

jove VISUALIZED EXPERIMENTS
1 Alewife Center 4200
Cambridge, MA 02140
Int: 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Obtaining Pattern of Breathing in Conscious Mice through Unrestrained Barometric Plethysmography

Author(s): Candace N. Receno, Jacob Russell, Caitlin M. Cunningham and Lara R. DeRusseau

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☒ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☒ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

jove
VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel: 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Lara R. DeRuisseau
Department:	Biological Sciences
Institution:	Le Moyne College
Title:	McDevitt Associate Professor
Signature:	Lara DeRu
Date:	10/25/2018

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

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 Animals[8] (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 consider[13,17]. For instance, we typically test mice in lighting similar to their housing room (either light or dark cycle) and within a three hour range[18]."

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 mind[5]."

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 equation[16], 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 room[13].”

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 environment[10,11], it is possible that habituation techniques may be helpful[12,13] 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 mice[12]. 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 experimentation[13].”

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 O_2 and CO_2 without having CO_2 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.