

Submission ID #: 71257

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Title: Tunnel Handling and Environmental Enrichment for Repeated Whole-Body Plethysmography in Mice

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Author Questionnaire

- 1. Microscopy:** Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**
- 2. Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes**
- 3. Filming location:** Will the filming need to take place in multiple locations? **No**
- 4. Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **Yes**

Current Protocol Length

Number of Steps: 21

Number of Shots: 39

Interviews

Videographer: Obtain headshots for all authors available at the filming location.

Authors: Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**

Note: The first and second statements are slightly edited.

INTRODUCTION:

~~**REQUIRED:** Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Manuela Sgai:** Our research focuses on how handling refinement improves respiratory measurements and animal welfare during longitudinal studies in mice.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~**Describe the research gap or limitation in existing methods and how this protocol addresses them.**~~

- 1.2. **Manuela Sgai:** Traditional handling increases stress-related variability in respiratory measurements over time, whereas tunnel handling and environmental enrichment effectively mitigate this stress.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~**What does this protocol allow researchers to study or measure?**~~

- 1.3. **Lina Shehadeh:** This protocol allows researchers to obtain stable, reproducible respiratory measurements across repeated longitudinal sessions.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~**Describe the most important consideration or challenge when performing this protocol.**~~

- 1.4. **Manuela Sgai:** Consistent tunnel handling and habituation are essential for achieving stable respiratory measurements.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

1.5. Lina Shehadeh: Future studies can evaluate this approach across strains, disease models, and additional physiological endpoints.

1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Testimonial Questions (OPTIONAL):

Videographer: Please capture all testimonial shots in a wide-angle format with sufficient headspace, as the final videos will be rendered in a 1:1 aspect ratio. Testimonial statements will be presented live by the authors, sharing their spontaneous perspectives.

- Testimonial statements will **not appear in the video** but may be featured in the journal's promotional materials.
- **Provide the full name and position** (e.g., Director of [Institute Name], Senior Researcher [University Name], etc.) of the author delivering the testimonial.
- Please **answer the testimonial question live during the shoot**, speaking naturally and in your own words in **complete sentences**.

What problem in your field of research does your JoVE article address?

- 1.6. **Lina Shehadeh, Professor of Medicine – Division of Cardiology/interdisciplinary Stem Cell Institute – University of Miami, Leonard M. Miller School of Medicine:** (authors will present their testimonial statements live)

1.6.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

What is the key takeaway you hope viewers remember after watching your video article?

- 1.7. **Lina Shehadeh, Professor of Medicine – Division of Cardiology/interdisciplinary Stem Cell Institute – University of Miami, Leonard M. Miller School of Medicine:** (authors will present their testimonial statements live)

1.7.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Ethics Title Card

This research has been approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Miami

Protocol

***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

2. Environmental Enrichment for 6-Month-Old Wild-Type 129S1/SvlmJ Mice

Demonstrator: Manuela Sgai

- 2.1. To begin, place 1 compressed cotton square in each cage [1-TXT]. At every cage change, add a fresh cotton square to provide constant nesting material and transfer most of the existing nest structure intact to the new cage using both hands.[2].
 - 2.1.1. WIDE: Talent placing a compressed cotton square into a home cage. **TXT: Cotton square area and weight: 5 cm × 5 cm, ~2.5 g** ***Videographer:** Please obtain CU shot as well for this shot.*
 - 2.1.2. Talent transferring the intact nest into a clean cage using both hands.
- 2.2. If the accumulated nesting material exceeds approximately 10 grams, remove the oldest and most compacted portions from the bottom while preserving the external dome structure [1]. Add 1 fresh cotton square to the clean cage to support continued nest construction and maintenance [2].
 - 2.2.1. Talent removing the oldest compacted nesting material while preserving the dome structure.
 - 2.2.2. Talent placing a fresh cotton square into the clean cage.
- 2.3. Replace the nesting material only if it is visibly wet with urine contamination covering more than 25 percent of the surface or if the nest has been destroyed or not rebuilt within 48 hours [1]. At each cage change, transfer the intact nest carefully to a clean surface using both hands without disturbing the structure [2].
 - 2.3.1. Talent inspecting the nesting material and replacing it when the specified criteria are met.
 - 2.3.2. Talent transferring the intact nest onto a clean surface using both hands.
- 2.4. Photograph the nest from directly above with the camera positioned 30 to 40 centimeters perpendicular to the surface to visualize the overall structure and crater formation [1]. Photograph the nest from the side at nest height to document the wall integrity and dome characteristics [2].
 - 2.4.1. Talent photographing the nest from directly above.
 - 2.4.2. Talent photographing the nest from the side at nest height.
- 2.5. Score the nest using the standardized 5-point Deacon scale, where 1 indicates the nesting material is largely untouched, and 5 indicates a dome-shaped nest with a clear

crater [1].

2.5.1. Talent assessing and recording the nest score using the standardized 5-point Deacon scale.

3. Tunnel Handling Implementation

3.1. Use clear polycarbonate tunnels measuring either 100- or 130 millimeters in length with a 50-millimeter internal diameter and smooth, rounded edges [1]. Place 1 tunnel permanently in each home cage as part of the cage enrichment, leaving it untouched during handling events [2].

3.1.1. Talent presenting the specified polycarbonate tunnel.

3.1.2. Talent placing the tunnel into the home cage.

3.2. At each cage change, wipe the tunnel interior with a small amount of used bedding from the previous cage before placing it in the clean cage [1].

3.2.1. Talent wiping the tunnel interior with used bedding before placing it into the clean cage.

3.3. Wash the tunnels with the same disinfectant agent used for cage sanitation if disinfection is required between uses [1]. Rinse the tunnels thoroughly and allow them to air dry before returning them to the cage [2].

3.3.1. Talent washing it with the disinfectant agent.

3.3.2. Talent rinsing the tunnel and placing it aside for air drying.

3.4. Implement a conditioning period of at least 1 to 2 weeks for naïve adult mice before initiating the whole-body plethysmography, or WBP (*W-B-P*) sessions [1]. Place the clear polycarbonate tunnels permanently in the home cage and allow 3 to 7 days for passive familiarization [2].

3.4.1. Talent placing mice into home cages for the conditioning period.

3.4.2. Talent placing the polycarbonate tunnel into the home cage.

3.5. After passive exposure, perform daily gentle handling sessions by allowing the mice to enter the tunnel voluntarily [1] and lifting the tunnel horizontally for 30 to 60 seconds before returning the mouse to the cage [2].

3.5.1. Talent allowing the mouse to voluntarily enter the tunnel

3.5.2. Talent lifting the tunnel horizontally.

3.6. Use tunnel handling as the primary handling method by lifting the tunnel horizontally with 1 hand supporting the base when the mouse is inside the tunnel [1]. If the mouse is outside the tunnel, position the tunnel opening within 5 to 10 centimeters of the mouse and allow 10 to 30 seconds for voluntary entry without chasing or forcing [2]. If the mouse does not enter within 30 seconds, encourage movement toward the tunnel

by cupping a hand behind the mouse without making contact [3]. If the mouse does not enter after 2 attempts within a maximum total of 60 seconds, use the cupping method by allowing voluntary approach for 10 to 20 seconds and gently scooping the mouse with both hands while avoiding tail handling, and ensuring absence of stress defined by the absence of vocalization, escape attempts, or stress-associated defecation [4].

- 3.6.1. Talent lifting the tunnel with the mouse inside.
- 3.6.2. Talent positioning the tunnel near the mouse for voluntary entry.
- 3.6.3. Talent encouraging the mouse toward the tunnel with a cupped hand.
- 3.6.4. Talent gently scooping the mouse using both hands.
- 3.7. Transfer the mouse from the tunnel to cupped hands before placing it into the chamber [1]. Hold the tunnel horizontally and tilt the rear end upward approximately 10 to 20 degrees [2]. Allow the mouse to back out of the tunnel, rear legs first, onto cupped hands [3]. Lower both hands into the chamber and allow the mouse to step voluntarily onto the chamber floor within 5 to 10 seconds [4].
 - 3.7.1. Talent transferring the mouse from the tunnel to cupped hands.
 - 3.7.2. Talent tilting the tunnel upward.
 - 3.7.3. Shot of the mouse backing out of the tunnel onto cupped hands.
 - 3.7.4. Talent allowing the mouse to step into the chamber.

4. Whole Body Plethysmography Session

- 4.1. Calibrate the whole-body plethysmograph once daily before data acquisition [1]. With the chambers empty and sealed, use the **FinePointe** respiratory analysis software to select the **Mouse 2 sites** option for calibration of two-chamber racks, and confirm successful calibration when the box flow effective range exceeds 40 milliliters per second per site [2].
 - 4.1.1. Shot of the empty sealed chambers in the system for calibration.
 - 4.1.2. SCREEN: 71257_4.1.2_t2.mp4. 00:07–00:15; 00:56–00:59; 02:29–02:33. *Video Editor: Please emphasize the row “Box Flow” and their corresponding values in the timestamp 02:29–02:33.*
- 4.2. Perform all measurements in chambers optimized for a quiet, low-vibration environment with consistent lighting, strictly during the same circadian window between 8 AM and 10 AM [1].
 - 4.2.1. Shot of the experimental setup prepared for measurements during the specified time window.

- 4.3. Transport the home cages to the experimental room on a cart approximately 15 minutes before the session begins [1]. Allow the mice to acclimate to the experimental room while remaining in their familiar home cage and place the home cages near the plethysmography chambers [2].

4.3.1. Talent transporting the home cages on a cart.

4.3.2. Talent placing the home cages beside the plethysmography chambers.

- 4.4. Use tunnel handling to transfer each mouse from the home cage to cupped hands and then into the chamber [1]. Handle the mice calmly with minimal abrupt movements and transfer each mouse gently from the cupped hands into the chamber [2]. Seal the chamber lid within 5 seconds of placing the mouse [3].

4.4.1. Talent transferring the mouse from the home cage to cupped hands.

4.4.2. Talent placing the mouse into the chamber.

4.4.3. Talent sealing the chamber lid.

Author's NOTE: filmed using mice from our current colony, as the original study mice are no longer available. We attempted this step live during filming and have submitted the footage, but we'd appreciate the team's evaluation of whether it reads well on camera. If it doesn't hold up, Clip_01_TunnelHandling_Transfer.mp4, from 00:20–00:26, may serve as an alternative for this step.

If the videographer's footage doesn't match the shot descriptions, please use this alternate clip. Please contact the writer during RE if there are doubts

- 4.5. Start recording the respiratory signals using the respiratory analysis software, allowing 10 minutes for acclimation after placing the mouse into the chamber [1].

4.5.1. LAB MEDIA: Clip_02_RespiratoryMonitoring_CalmBehavior.MOV. 00:00 – 00:07

- 4.6. Continuously observe acclimation and log excessive movement, stress-related immobility, escape attempts, and absence of calm periods. Use initial data despite unreliable baselines, but flag sessions lacking calm acclimation periods for exclusion [1].

4.6.1. Talent recording the observed behaviors in the session logbook. **NOTE:** The VO for 4.6 and 4.7 are combined in concise format as 4.7 was not filmed

- ~~4.7. Recognize that sessions with excessive movement or stress may still yield valid initial data, even if baseline measurements are unreliable, and flag sessions without observable calm periods during acclimation for potential exclusion [1].~~

~~4.7.1. Talent reviewing the session observations.~~

Author's NOTE: None of our sessions in this study required flagging or exclusion (all sessions met acclimation criteria), so there was no footage to capture representing this. The narration for Step 4.7 can stand on its own, describing the flagging/exclusion criteria without an accompanying visual.

4.8. Observe the mice continuously throughout the 10-minute acclimation period, note overt stress indicators including defecation, urination, sustained escape attempts, or prolonged immobility, [1] and record the presence and approximate quantity of fecal pellets and urine in the chamber after each session prior to the analysis of acclimation-phase respiratory metrics [2].

4.8.1. LAB MEDIA: Clip_03_PostSession_ChamberCondition.MOV. 00:00 – 00:14.

4.8.2. LAB MEDIA: Clip_03_PostSession_ChamberCondition.MOV. 00:24 – 00:32.

Results

5. Results

5.1. The application of tunnel handling and environmental enrichment protocol across a 10-week longitudinal WBP study demonstrated progressive habituation and improved physiological stability [1].

5.1.1. LAB MEDIA: Figure 2 and Figure 1D.

5.2. Initial respiratory frequency, measured during 0–2 minutes following chamber placement, showed a progressive decline across the study period [1]. At baseline or week 0, the initial respiratory frequency averaged approximately 345 breaths per minute, consistent with handling-induced tachypnea observed during initial WBP exposure [2]. During early study sessions or weeks 2 to 3, the initial respiratory frequency measured approximately 374 breaths per minute, indicating persistence of acute stress responses during early acclimation phases [3]. By mid-study, the respiratory frequency decreased to 329 breaths per minute [4], and by late-study, it further declined to 275 breaths per minute [5].

5.2.1. LAB MEDIA: Figure 2A

5.2.2. LAB MEDIA: Figure 2A. *Video Editor: Please emphasize the height of the bar labelled “Baseline (Week 0)” on the X-axis.*

5.2.3. LAB MEDIA: Figure 2A. *Video Editor: Please emphasize the height of the bar labelled “Early (Weeks 2-3)” on the X-axis.*

5.2.4. LAB MEDIA: Figure 2A. *Video Editor: Please emphasize the height of the bar labelled “Mid (Weeks 5-7)” on the X-axis.*

5.2.5. LAB MEDIA: Figure 2A. *Video Editor: Please emphasize the height of the bar labelled “Late (Weeks 8-10)” on the X-axis.*

5.3. Subsequently, the stabilized baseline respiratory frequency, measured during 8–10 minutes of the acclimation period, showed a progressive decrease across the study timeline [1]. Mean stabilized baseline respiratory frequency values were approximately 255 breaths per minute at week 0 [2], 235 breaths per minute during early-study [3], 198 breaths per minute during Mid-study [4], and 193 breaths per minute during late-study [5].

5.3.1. LAB MEDIA: Figure 2B

5.3.2. LAB MEDIA: Figure 2B. *Video Editor: Please emphasize the height of the bar labelled “Baseline (Week 0)” on the X-axis.*

5.3.3. LAB MEDIA: Figure 2B. *Video Editor: Please emphasize the height of the bar labelled “Early (Weeks 2-3)” on the X-axis.*

- 5.3.4. LAB MEDIA: Figure 2B. *Video Editor: Please emphasize the height of the bar labelled “Mid (Weeks 5-7)” on the X-axis.*
- 5.3.5. LAB MEDIA: Figure 2B. *Video Editor: Please emphasize the height of the bar labelled “Late (Weeks 8-10)” on the X-axis.*

1. Polycarbonate
Pronunciation link: <https://www.merriam-webster.com/dictionary/polycarbonate>
IPA: /ˌpɑːliˈkɑːrbənət/
Phonetic Spelling: paa·lee·kaar·buh·nuht
2. Disinfectant
Pronunciation link: <https://www.merriam-webster.com/dictionary/disinfectant>
IPA: /ˌdɪsɪnˈfektənt/
Phonetic Spelling: dis·in·fek·tuhnt
3. Naïve
Pronunciation link: <https://www.merriam-webster.com/dictionary/naive>
IPA: /naɪˈiːv/
Phonetic Spelling: ny·EEV
4. Plethysmography
Pronunciation link: <https://www.merriam-webster.com/medical/plethysmography>
IPA: /ˌplɛθɪzˈmɑːgrəfi/
Phonetic Spelling: pleth·iz·MAH·gruh·fee
5. Plethysmograph
Pronunciation link: <https://www.merriam-webster.com/dictionary/plethysmograph>
IPA: /plɛˈθɪzməˌgræf/, /pləˈθɪzməˌgræf/
Phonetic Spelling: pleh·THIZ·muh·graf, pluh·THIZ·muh·graf
6. Circadian
Pronunciation link: <https://www.merriam-webster.com/dictionary/circadian>
IPA: /sɜːˈkeɪdiən/
Phonetic Spelling: sur·KAY·dee·uhn
7. Acclimation
Pronunciation link: <https://www.merriam-webster.com/dictionary/acclimation>
IPA: /ˌækləˈmeɪʃən/
Phonetic Spelling: ak·luh·MAY·shuhn
8. Defecation
Pronunciation link: <https://www.merriam-webster.com/dictionary/defecation>
IPA: /ˌdefɪˈkeɪʃən/
Phonetic Spelling: def·ih·KAY·shuhn
9. Respiratory
Pronunciation link: <https://www.merriam-webster.com/dictionary/respiratory>
IPA: /ˈrespəreɪˌtɔːri/, /rɪˈspairəˌtɔːri/
Phonetic Spelling: RES·puh·ruh·tor·ee, rih·SPY·ruh·tor·ee
10. Physiological
Pronunciation link: <https://www.merriam-webster.com/dictionary/physiological>

IPA: /ˌfɪziəˈlɑːdʒɪkəl/

Phonetic Spelling: fiz·ee·uh·LAH·jih·kuhl

11. Habituation

Pronunciation link: <https://www.merriam-webster.com/dictionary/habituation>

IPA: /həˌbɪtʃuˈeɪʃən/, /həˌbɪtʃuˈweɪʃən/

Phonetic Spelling: huh·bich·oo·AY·shuhn, huh·bich·oo·WAY·shuhn

12. Tachypnea

Pronunciation link: <https://www.merriam-webster.com/medical/tachypnea>

IPA: /ˌtækɪpˈniːə/

Phonetic Spelling: tak·ip·NEE·uh