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Title: Voluntary Group-Housing Compatible Fecal Sample Collection in Mice Using a Custom-Made Cage System

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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? **No**
- 3. Filming location:** Will the filming need to take place in multiple locations? **Yes**
If **Yes**, how far apart are the locations? 100 m
- 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. **No**

Current Protocol Length

Number of Steps: 38

Number of Shots: 52

Interviews

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

INTRODUCTION:

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

- 1.1. **Katharina Tillmann:** My research focuses on investigating laboratory animal behavior and welfare under different environmental conditions, especially housing systems and environmental enrichment.

- 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. **Katharina Tillmann:** Existing sampling techniques can disrupt the behavior under investigation if not voluntarily supported by the animals. This protocol minimizes that disruption.

- 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. **Katharina Tillmann:** This protocol allows researchers to study feces samples from up to 60 mice simultaneously without touching the animals.

- 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. **Katharina Tillmann:** The most important consideration is minimizing collection-induced stress while standardizing timing, handling, and sampling conditions to measure fecal corticosterone metabolites.

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

~~Are there any additional methods or analyses that can be performed following this procedure?~~

1.5. **Katharina Tillmann**: Additionally, following this protocol, fecal samples can be used to analyze microbiota, metabolic markers, parasites, inflammation, or hormones.

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.

Ethics Title Card

The study involved established breeding and husbandry practices only, together with non-invasive fecal sample collection

Protocol

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

2. Construction of the Fecal Collection Cage System

Demonstrator: Katharina Tillmann

- 2.1. To begin, cut parts 1 to 4 from 5-millimeter Plexiglass or acrylic glass according to the templates [1-TXT].
 - 2.1.1. WIDE: Talent cutting parts 1 to 4 from 5-millimeter Plexiglass or acrylic glass.
TXT: Refer to Fig 2A-C ***Videographer:** Please obtain CU shot as well for this shot.*
- 2.2. Cut off the bottom of the polycarbonate type II, or PTII (*P-T-two*) cage [1]. Reserve this part to serve later as the top cover of the fecal collection boxes [2].
 - 2.2.1. Talent cutting off the bottom of the polycarbonate type II cage.
 - 2.2.2. Talent setting aside the removed bottom of the polycarbonate type II cage.
- 2.3. Drill the holes and slots into the former bottom of the PTII cage [1].
 - 2.3.1. Talent drilling the holes and slots into the former bottom of the polycarbonate type II cage.
- 2.4. Next, drill the holes into the front and short side of the PTIII (*P-T-three*) cage [1].
 - 2.4.1. Talent drilling the holes into the front side and short side of the polycarbonate type III cage.
- 2.5. Use part 2 as a template to mark the corresponding drill holes on the short side of the PTIII cage [1] and on the long side of the PTII cage [2].
 - 2.5.1. Talent using part 2 as a template to mark the drill holes on the short side of the polycarbonate type III cage.
 - 2.5.2. Talent marking the corresponding drill holes on the long side of the polycarbonate type II cage.
- 2.6. Drill the marked holes [1]. Using the threaded screws, attach the PTIII cage, the PTII cage, and part 2 [2].
 - 2.6.1. Talent drilling the marked holes.
 - 2.6.2. Talent attaching the polycarbonate type III cage, the polycarbonate type II cage, and part 2 with threaded screws.
- 2.8. Slide the lower protrusions of parts 4a and 4b into the slots of part 1 [1], to form the

legs of the collection boxes [2]. Notes: This step is moved here to address author's comment.

2.8.1. Talent sliding the lower protrusions of parts 4a and 4b into the slots of part 1.

2.8.2. Talent showing the protrusions forming the legs of the collection boxes.

2.7. ~~Insert part 1 as the bottom of the PTII cage~~ the assembled parts into the PTII cage, with part 1 forming the bottom of the cage [1]. ~~Position parts 4a and 4b to hold part 1 in place and divide the sampling cage into separate collection boxes [2]. Fix parts 4a and 4b in place with a hot glue gun [2].~~

2.7.1. ~~Talent inserting part 1 as the bottom of the polycarbonate type II cage.~~ Talent inserting the assembled parts into the polycarbonate type II cage, with part 1 positioned at the bottom.

2.7.2. ~~Talent positioning parts 4a and 4b inside the cage.~~

2.7.3. Talent securing parts 4a and 4b with a hot glue gun.

2.8. ~~Slide the lower protrusions of parts 4a and 4b into the slots of part 1 [1], to form the legs of the collection boxes [2].~~ Notes: This step is moved before 2.7.

2.8.1. ~~Talent sliding the lower protrusions of parts 4a and 4b into the slots of part 1.~~

2.8.2. ~~Talent showing the protrusions forming the legs of the collection boxes.~~

2.9. Slide the upper protrusions of parts 4a and 4b through the top cover, the former bottom of the PTII cage, to hold it in place [1].

2.9.1. Talent sliding the upper protrusions of parts 4a and 4b through the top cover to secure it.

2.10. Using one rod at the top and two rods at the bottom, thread the wooden chopsticks or rods through the holes in the protrusions to stabilize and fix the construction [1].

2.10.1. Talent threading the wooden chopsticks or rods through the holes in the protrusions with one rod at the top and two rods at the bottom. Notes: This shot is part of 2.9.1.

2.11. With a hot glue gun, close the remaining gaps or minor inaccuracies [1]. Use parts 3a, 3b, and 3c as sliding doors between the home cage and the collecting boxes [2].

2.11.1. Talent sealing the remaining gaps or minor inaccuracies with a hot glue gun.

2.11.2. Talent using parts 3a, 3b, and 3c as sliding doors between the home cage and the collecting boxes.

3. Animal Housing and Husbandry

- 3.1. Obtain the required number of mice.[1].
 - 3.1.1. Talent sitting with a cage containing mice on the workbench. **TXT: Mice Model: C57BL/6J**
- 3.2. House the animals under conventional conditions in adapted PTIII cages with a maximum of 3 adult mice per cage [1].
 - 3.2.1. Talent placing up to 3 adult mice into an adapted polycarbonate type III cage.
- 3.3. Maintain the animal room at approximately 22 degrees Celsius and 55 percent relative humidity under a 12-hour light and dark cycle with the lights turned on at 6 am [1].
 - 3.3.1. Shot of the animal room housing the cages with the lights turned on. **Notes: Use 4.1.1**
- 3.4. Provide a standard maintenance diet and water ad libitum [1].
 - 3.4.1. Talent placing the standard maintenance diet and water into the cage.
- 3.5. Add aspen wood bedding, paper toilet roll, red transparent plastic house, and paper towels as nesting material to each cage [1].
 - 3.5.1. Show the cage prepared with aspen wood bedding, a paper toilet roll, a red transparent plastic house, and paper towels.
- 3.6. Identify the mice primarily by their natural tail markings [1].
 - 3.6.1. Talent examining the natural tail markings of the mice for identification.

4. Animal Habituation and Training for Fecal Sample Collection

- 4.1. Place the animals in the cage system at least 2 weeks before the first planned sampling session [1].
 - 4.1.1. Shot of the animals in the cage system.
- 4.2. Habituate the animals to the collection boxes by removing the sliding doors for 1 night [1]. Allow the animals to freely explore the collection boxes [2].
 - 4.2.1. Talent removing the sliding doors from the collection boxes.
 - 4.2.2. Shot of the animals freely exploring the collection boxes.
- 4.3. Apply a small amount of reward to the wall opposite the entrance of each collection box [1].
 - 4.3.1. Talent applying a small amount of reward to the wall opposite the entrance of each collection box.

- 4.4. Perform 3 training sessions before the first sampling session [1]. Conduct the training under the same light conditions and at the same time of day planned for later sampling, ideally under red light shortly after the beginning of the dark phase [2].
 - 4.4.1. Shot of the animals in the collection setup during a training session.
 - 4.4.2. Shot of the training environment under red light shortly after the beginning of the dark phase.
- 4.5. On the first training day, leave the sliding doors open and allow the animals to discover and consume the reward voluntarily [1].
 - 4.5.1. Shot of sliding doors open and the animals entering the collection boxes and consuming the reward.
- 4.6. On the second training day, wait until an animal enters a collection box and starts licking the reward [1]. Carefully close the sliding door behind the animal [2] and release it after 10 minutes [3].
 - 4.6.1. Shot of an animal entering a collection box and licking the reward.
 - 4.6.2. Talent carefully closing the sliding door behind the animal. Notes: Use 4.6.1
 - 4.6.3. Talent opening the sliding door to release the animal after 10 minutes.
- 4.7. On the third training day, repeat the same procedure and gradually extend the time the animals remain in the collection boxes [1].
 - 4.7.1. Shot of an animal remaining in the collection box.

5. Collection of Fecal Sample

- 5.1. Plan the sampling time according to the biological question and the expected delay between plasma corticosterone and fecal corticosterone metabolite, or FCM (*F-C-M*) excretion [1].
 - 5.1.1. Talent preparing the sampling schedule.
- 5.2. Schedule sampling during a period of high fecal output, preferably at the beginning of the dark and active phase [1]. Perform all sampling sessions at the same time of day throughout the study to reduce variation caused by the diurnal rhythm of FCM levels [2]. Mark the animals individually before sampling using ear marks or individual tail markings [3].
 - 5.2.1. Talent preparing the sampling schedule for a period of high fecal output at the beginning of the dark and active phase.
 - 5.2.2. Talent scheduling all sampling sessions for the same time of day throughout the study. Notes: Use 5.2.1
 - 5.2.3. Talent marking the animals with ear marks or individual tail markings.

- 5.3. Prepare the collection boxes during the light phase by applying the reward to the back wall of each box [1].
 - 5.3.1. Talent applying the reward to the back wall of each collection box.
- 5.4. At the planned sampling time, open the collection boxes [1]. Wait until the animals enter voluntarily [2].
 - 5.4.1. Talent opening the collection boxes.
 - 5.4.2. Shot of the animals voluntarily entering the collection boxes.
- 5.5. Once a mouse has entered a collection box, carefully close the sliding door behind it [1].
 - 5.5.1. Talent carefully closing the sliding door behind the mouse.
- 5.6. After all animals have entered a collection box, record the identity and position of each mouse in a prepared sampling sheet [1].
 - 5.6.1. Talent recording the identity and position of each mouse in a prepared sampling sheet.
- 5.7. If more than 10 cages are sampled in one session, involve a second experimenter to support animal identification, door handling, and documentation [1].
 - 5.7.1. Talent and a second experimenter supporting animal identification, handling the sliding doors, and documenting the sampling session.
- 5.8. Keep the animals in the collection boxes for the planned sampling duration, typically 30 minutes to 2 hours, depending on the expected fecal output and study design [1].
 - 5.8.1. Shot of the animals in the collection boxes.
- 5.9. At the end of the sampling period, open the sliding doors and allow the mice to return to the home cage [1].
 - 5.9.1. Talent opening the sliding doors and allowing the mice to return to the home cage.
- 5.10. Close the collection boxes immediately after the mice have left to prevent re-entry and cross-contamination of samples [1].
 - 5.10.1. Talent closing the collection boxes after the mice leave.
- 5.11. Using long forceps, remove the fecal pellets from each collection box [1]. Transfer the fecal pellets into individually labeled collection tubes [2].
 - 5.11.1. Talent removing fecal pellets from a collection box using long forceps.
 - 5.11.2. Talent transferring the fecal pellets into individually labeled collection tubes.
- 5.12. Store the samples at minus 20 degrees Celsius until further preparation of the FCM assay [1].
 - 5.12.1. Talent placing the collection tubes into storage at -20 degrees Celsius.

5.13. Keep the animals in the PTIII home cage until the next cage change [1]. After the cage change, disassemble the cage system and wash it in a usual cage washer [2].

5.13.1. Shot of the animals remaining in the polycarbonate type III home cage.

5.13.2. Talent disassembling the cage system and placing it into a standard cage washer.

Results

6. Results

- 6.1. Females had higher FCM concentrations [1] than males across breeding conditions and sampling days [2].
 - 6.1.1. LAB MEDIA: Figure 3A–D. *Video editor: Highlight 3A and B.*
 - 6.1.2. LAB MEDIA: Figure 3A–D. *Video editor: Highlight 3C and D.*
- 6.2. Permanently bred females had higher FCM concentrations than males on Sundays [1] and on Wednesdays [2].
 - 6.2.1. LAB MEDIA: Figure 3B and D. *Video editor: Highlight the 'permanent' lines in the 'weekends' plots for 'females' and 'males'.*
 - 6.2.2. LAB MEDIA: Figure 3A and C. *Video editor: Highlight the 'permanent' lines in the 'cage change days' plots for 'females' and 'males'.*
- 6.3. Intermittently bred females also had higher FCM concentrations than males on Sundays [1] and on Wednesdays [2].
 - 6.3.1. LAB MEDIA: Figure 3B and D. *Video editor: Highlight the 'intermitted' lines in the 'weekends' plots for 'females' and 'males'.*
 - 6.3.2. LAB MEDIA: Figure 3A and C. *Video editor: Highlight the 'intermitted' lines in the 'cage change days' plots for 'females' and 'males'.*
- 6.4. Permanently and intermittently bred animals did not differ significantly within the individual sex and sampling-day combinations [1].
 - 6.4.1. LAB MEDIA: Figure 3A–D. *Video editor: Highlight the 'permanent' and 'intermitted' lines in all 4 plots.*