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Handling Techniques to Reduce Stress in Mice

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TITLE

Handling Techniques to Reduce Stress in Mice

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SUMMARY:

This paper describes a handling technique in mice, the 3D-handling technique, which facilitates routine handling by reducing anxiety-like behaviors and presents details on two existing related techniques (tunnel and tail handling).

ABSTRACT:

Laboratory animals are subjected to multiple manipulations by scientists or animal care providers. The stress this causes can have profound effects on the animal well-being and can also be a confounding factor for experimental variables such as anxiety measures. Over the years, handling techniques that minimize handling-related stress have been developed with a particular focus on rats, and little attention to mice. However, it has been shown that mice can be habituated to manipulations using handling techniques. Habituating mice to handling reduces stress, facilitates routine handling, improves animal wellbeing, decreases data variability, and improves experimental reliability. Despite beneficial effects of handling, the tail-pick up approach, which is particularly stressful, is still widely used. This paper provides a detailed description and demonstration of a newly developed mouse-handling technique intended to minimize the stress experienced by the animal during human interaction. This manual technique is performed over 3 days (3D-handling technique) and focuses on the animal's capacity to habituate to the experimenter. This study also shows the effect of previously established tunnel handling techniques (using a polycarbonate tunnel) and the tail-pick up technique. Specifically studied are their effects on anxiety-like behaviors, using behavioral tests (Elevated-Plus Maze

and Novelty Suppressed Feeding), voluntary interaction with experimenters and physiological measurement (corticosterone levels). The 3D-handling technique and the tunnel handling technique reduced anxiety-like phenotypes. In the first experiment, using 6-month-old male mice, the 3D-handling technique significantly improved experimenter interaction. In the second experiment, using 2.5-month-old female, it reduced corticosterone levels. As such, the 3D-handling is a useful approach in scenarios where interaction with the experimenter is required or preferred, or where tunnel handling may not be possible during the experiment.

INTRODUCTION:

Mice and rats are essential assets to preclinical studies^{1,2} for multiple purposes, including endocrinal, physiological, pharmacological or behavioral studies². From the increasing number of studies involving animals, it arose that uncontrolled environmental variables including human interaction influence various outcomes in biomedical research³⁻⁵. This is responsible for significant variability observed across experiments and research laboratories^{4,5}, posing a major caveat in animal research.

Various approaches have been implemented with the goal of limiting the impact of environmental stressors and reducing reactivity to human interaction. For example, to limit the impact of environmental stressors, standardization of housing conditions and automated housing systems^{6,7} have been implemented across laboratories. Regarding interaction with human beings, commonly used approaches for handling and transporting animals had little regard for animal discomfort and stress. For instance, picking up animals by their tail or using forceps⁸ increases baseline anxiety⁹⁻¹¹, reduces exploration^{9,12} and contributes greatly to inter-individual variability within and across studies^{13,14}. As a result, other approaches were developed, such as the *cup handling* technique, which is applicable to mice and rats. In this approach, the animals are “cupped” out of their cage, and held by the experimenters with their hands forming a cup⁹⁻¹¹. Another useful alternative to tail handling involves the use of a polycarbonate tunnel to transfer mice^{9,10,15}. This approach eliminates direct interaction between the mouse and the experimenter. Both the cup and tunnel approaches showed efficacy in reducing anxiety-like behaviors and fear of the experimenter that can be exaggerated by aversive handling techniques, such as tail pick up/tail handling^{9,10}.

Therefore, increasing evidence demonstrates the usefulness of proper mouse handling for reducing variability between individuals^{9,11}, and improving animal welfare¹⁰. However, the techniques mentioned above are still faced with limitations. The cup handling technique has been implemented with schedules ranging from 10 days (10 sessions over 2 weeks¹⁶) up to 15 weeks¹⁷, which is a considerable amount of time for facility staff and experimenters. Additionally, the effectiveness of cup handling varies by strain⁹ and conventional cup handling in open hands may lead to naïve mice or particularly jumpy strains to jump from the hand^{9,18}. Tunnel handling results in more consistent and generally quicker results in gentling¹⁹. Tunnels are also used as home cage enrichment. They help animals habituate to handling quickly and provide the added benefits of enrichment. Tunnel handling, however, has limitations when transferring animals between apparatuses. Interestingly, Hurst and West⁹, and Henderson et al.²⁰ demonstrated that using gentle and brief manual handling to transfer animals from the tunnel to the apparatus does not

affect their phenotype.

To provide an alternative to existing methods, with achievable habituation in a short period of time, this article describes a novel technique that expands on the cup handling technique, therefore requiring no particular equipment. This approach uses milestones to gauge the level of comfort mice have with the handling process. It shows efficacy at decreasing mouse reactivity and stress (at the behavioral and hormonal levels), facilitates routine handling and contributes to reducing variability between animals. Details of this technique are provided here, and its efficacy at reducing anxiety-like behaviors, improving interaction with experimenters, and limiting peripheral stress-hormone (corticosterone) release are demonstrated in two separate studies (male and female mice), in comparison with tunnel handling (positive control) and tail handling techniques (negative control).

PROTOCOL:

Procedures involving animal subjects were approved by the CAMH animal care committee and conducted in compliance with the Canadian Council on Animal Care guidelines.

NOTE: The handling method described herein can be used in various mouse strains, including non-transgenic (C57/BL6, BalbC, CD1, SV129, etc.) and transgenic lines. It can also be used with young or old mice, noting that young adult (4-6 weeks old) mice tend to be slightly more active than adult or old mice, especially on day 1.

1. Experimental preparation

1.1. Prior to study initiation, as per ARRIVE guidelines²¹, randomly assign mice to each handling group (3D-Handling, Tunnel Handling or Tail Handling).

1.2. Identify the room to perform the handling. It can be performed in the housing room, or in a separate room. If the handling is performed in a separate room, which requires the animals to be moved on a moving cart, allow the animals to habituate to the new room for 20-30 min prior to initiation of the handling protocol.

1.3. For group housed-animals, use a temporary cage to house mice after the handling, before regrouping them all in their initial home cage. This reduces potential fights between animals prior to handling (particularly in males).

1.4. Work on a counter (preferably a cleared countertop) or in a biosafety cabinet, with the housing cage away from the animal being handled. Close proximity to the housing cage increases the risk of jumping. If animals are group-housed, jumping of the mouse being handled into the home cage may cause stress to cage-mates.

NOTE: Working in a biosafety cabinet limits the risk of mice jumping on the floor, and can be required in certain facilities. This technique can be used in a biosafety cabinet, making sure to

always perform all steps inside the biosafety cabinet, and avoiding mice walking on handler forearms.

2. DAY 1: 5 min per mouse

2.1. Gently open the cage and place the lid on the side, remove nesting materials, and other enrichment such as running wheels or shelters.

2.2. Introduce a gloved open hand to the home cage, slowly placing the hand along one side of the cage wall (the wall closest to the handler, **Figure 1A**).

2.2.1. Do not immediately try to pick up the mouse.

2.3. Remain immobile and allow the animal to habituate to the presence of the hand in the cage for about 30 s.

2.4. Attempt to pick up the mouse in the palm of the hand (i.e., avoid picking up the animal by its tail).

2.4.1. If the mouse is not easily picked up after 3 attempts, guide the mouse to a corner and cup with both hands.

2.4.2. Gently move the cupped hands towards the mouse to try to pick it up.

2.4.3. If unsuccessful after a maximum of 3 attempts with both hands, pick up the mouse gently by the base of its tail, and transfer it to your forearm or flat hand.

2.5. With the mouse in the hand, keep the hand as flat and open as possible.

NOTE: This provides a flat platform for the mouse to step onto, and limits the risk of bite.

2.6. Holding the hand open and flat with palm up, place the other hand adjacent to the hand holding the mouse and allow the mouse to move freely from hand to hand without any restraint (**Figure 1B**).

2.7. Let the mouse explore and move between hands for 1 min.

2.7.1. At this point mice may try to jump away. Position the hands such that if the mouse jumps, it will land on a countertop rather than the floor.

2.7.2. If a mouse looks like it is preparing to jump (moving towards the edge of the hand and rearing on hind legs), slowly place the other hand in front of it and try to guide it into walking onto this hand. Avoid sudden movements as it increases their risk of jumping.

2.7.3. If a mouse does jump, attempt to pick it up avoiding tail handling and resume the handling session. If the mouse stays on the floor or out of the hands for more than 10 s, add additional time to the handling session to make up for any time the mouse was out of the hands.

2.7.4. Take notes of the jump. Total number of jumps can be used to assess potential variability between animals.

2.8. After 1 min of handling with flat hands, relax the palm of the hand, and slightly cup the mouse in the hand, prior to gently rolling the mouse between hands (**Figure 1C**).

2.8.1. To “roll”, position the mouse in the palm of the hand, on a flat hand, perpendicular to fingers.

2.8.2. Slowly close the hand, placing the fingers on the back of the mouse.

2.8.3. Place the free hand directly under the hand holding the mouse.

2.8.4. Slowly turn/rotate the hand with the mouse to gently transfer the mouse to the other hand (180° flip).

2.8.5. Repeat this back and forth between hands.

2.9. Alternate from gentle rolling between hands and free exploration on open hands for 60 seconds, alternating between techniques about every 20 seconds.

2.10. Perform a “shelter test” (**Figure 1D**).

2.10.1. Let the mouse move to the edge of the hand then bring the 2 hands together.

2.10.2. Very slowly, cup them so the mouse fits inside a “shelter” formed by the hands. Leave an opening so the mouse can escape if needed.

2.10.3. Aim to keep the mouse in the shelter for 5-10 s, **without any restraint**.

2.10.4. Alternate between the shelter test, roll between hands and free exploration of open hands for another 60 seconds, being sure to perform the shelter step 3 or more times.

2.11. In all procedures described in 2.10, do not rush the process. If the mouse appears stressed (i.e., tentative to escape, jumps from the hands, avoiding contact with hands etc.) by being confined inside the hands, continue with rolling between hands and free exploration for 20 s, and then retry.

2.12. Milestone: Perform at least 1 successful shelter test of 10 s for completion of Day 1.

2.12.1. Consider a shelter test successful when the mouse stays in the hands. If the mouse pops its head out and returns to the shelter, it is still a successful test. If the animal entirely exits from the shelter, it is a failure.

2.13. Allow free exploration in hands for 30 s.

2.14. Gently replace the mouse in its cage. If group housed, place the mouse in the temporary cage until all cage mates are handled. Return the mice to their original cage by picking them up in the palm of the hand. Do not use a tail pick up.

2.15. Clean the bench top of potential feces and urine with 70% ethanol.

2.16. Rinse gloves thoroughly with 70% ethanol (or appropriate cleaning solution) or change gloves prior to handling the next mouse (it is possible to keep the same gloves for cage mates).

NOTE: It is recommended to perform the handling with a reasonable number of animals to avoid fatigue from the handler. Handling 24 mice takes around 2 h and it is recommended to not exceed 24 mice per handler. If more animals need to be handled, it is recommended either to have multiple handlers, or to split the handling procedures into subgroups, over multiple days.

3. DAY 2: 3 to 5 min per mouse

3.1. Attempt to pick up the mouse in the palm of the hand. At this stage, it should be already feasible and mice should not jump out of the hand.

3.2. Start with palm open as on Day 1, allowing the mouse to explore freely for 20 s.

3.3. Then, roll the mouse between hands a few times (4-5 times).

3.4. Perform the “shelter test” for 5 s.

3.5. Repeat the shelter test several times (~5-6) over a 2 to 3 min period.

3.6. During the same 2 to 3 min period, alternate with the roll between hands and free exploration of open hands step from day 1 to improve habituation.

3.6.1. Touch the mouse on its head and back (**Figure 1E**), 5-6 times. A sign of habituation is when the mouse lets you touch it without attempting to escape.

3.6.2. Perform a “Nose poke”: Try to touch the snout of the mouse, 2 to 3 times (**Figure 1F**).

3.6.2.1. If the mouse attempts to bite or shows obvious signs of stress at being touched, do not immediately attempt the nose poke again. Instead, alternate with flat hand exploration and roll. "Habituation" is reflected by the animal not running away or turning its head in cases of human contact.

3.7. In all procedures described in 3.5, do not rush the process. If the mouse appears stressed by being confined inside the hands or does not want to be touched, continue with rolling between hands for a 20-30 s and then retry.

3.8. Milestones: Perform at least 1 successful nose poke for 2-3 s for completion of Day 2.

3.9. Stop this session after about 3 min of handling if the animal reacts well to the "shelter", "head petting", "nose poke", and if the mouse appears to be willing to explore the hands without signs of stress.

3.10. If the mouse continues to exhibit signs of stress or is not reacting well to the "shelter test" or "nose poke" test, continue the session until reaching 5 min as in Day 1.

3.11. Replace the mouse in its cage, clean the bench top and gloves as in Day 1.

4. DAY 3: Around 3 min per mouse

4.1. On the third day, proceed through the same steps as in Day 2, for 2 to 3 minutes.

4.1.1. Pick up the mouse in the palm of the hand.

4.1.2. Transfer and roll the mouse between hands

4.1.3. Perform a shelter test.

4.1.4. Try to pet the mouse on the back and head.

4.2. Alternate between these steps over approximately 1 to 2 minutes.

4.3. Continue the procedure until the mouse is relaxed enough to sit in the palm of the hand without attempting to escape.

4.4. Before the end of Day 3, repeat the shelter test and nose poke test as a test of habituation.

4.4.1. If both tests can be completed on their first attempt, the habituation process is complete. Continue gently handling the mouse for 30 seconds to a minute.

4.4.2. If the mouse is initially resistant to either test, repeat steps 3.1. to 3.3 for 20-30 s before reattempting the nose poke and shelter test.

4.4.3. If the mouse remains resistant to these tests after 3 minutes, the third day may be repeated.

4.5. Milestones: Perform at least 2 successful shelter tests of 10 s each, and 2 successful nose poke test for completion of Day 3, and completion of the entire 3D-handling procedure.

4.6. Return the mouse to its cage, clean the bench top and gloves.

5. Optional approach for animals to be subjected to restrain for injection or gavage

NOTE: On Day 3, if the animal will be restrained for experimental purposes (oral gavage, intra-peritoneal injection, etc.), the mice can be subjected to the neck pinch test.

5.1. Grasp the nape of the neck between the thumb and forefinger (**Figure 1G**).

5.2. Lift the mouse 3-5 cm above the hand for 2-3 s.

NOTE: This is normally a non-natural position for adult mice, and if the mice remain near immobile, they are well habituated to handling and will be easy to restrain for experimental purposes.

5.3. Place the mouse back on in the flat hand, or if the mouse is reactive to the neck pinch, consider placing it on the experimenter's sleeve, cage lid or countertop

NOTE: If working in a biosafety cabinet, do not place the mouse on the sleeve or it could walk up and exit the biosafety cabinet. Prefer placing the mouse on the countertop inside the biosafety cabinet.

5.4. Leave the mouse to freely explore the experimenter's hand for 1 min.

6. Optional approach for additional days of handling

6.1. In the eventuality of a highly stressed mouse line, add additional days to decrease the reactivity and stress level of the animals, using the methods described in Day 2/3.

NOTE: Many factors can affect baseline stress of the animals including strain, presence of transgenic modification, age, sex and housing conditions. If these factors are not consistent between groups such as aged animals being tested against young controls or transgenic animals being tested against wild type controls, it is recommended that the same number of days of habituation are used for each group.

7. Tunnel handling

NOTE: This technique is applicable only to the Tunnel-handled mice. Tunnels are polycarbonate tubes approximately 13 cm in length and 5 cm in diameter.

- 7.1. Place the tunnel in the cage of the mouse.
- 7.2. Leave the tunnel in the cage for 7 days prior to handling.
- 7.3. Open the cage and place the lid on the side.
- 7.4. Gently guide the mouse into the polycarbonate tunnel (already in the cage).
- 7.5. Lift the tunnel from the cage, horizontally. If necessary loosely cover the ends of the tunnel to prevent the animal from jumping/falling out of the tunnel, potentially falling back in its cage or on the floor.
- 7.6. Move the animal in the tunnel away from the home cage and hold it away from any surfaces for 30 s.
- 7.7. Place the tunnel back in the home cage, allowing the mouse to exit the tube.
- 7.8. Wait for 60 s and then repeat steps 7.4-7.7 once.
- 7.9. Rinse gloves thoroughly with 70% ethanol or change gloves prior to habituating the next mouse.
- 7.10. Repeat this procedure for 10 consecutive days.

8. Tail handling

NOTE: This technique is applicable only to the Tail-handled mice. It is used to transfer mice from their cage to an apparatus, and vice-versa.

- 8.1. Open the cage and place the lid on the side.
- 8.2. Grasp the mice by the base of the tail between thumb and forefinger.
- 8.3. Lift the mouse from the cage.
- 8.4. In 2-3 s, transfer mouse to the experimenter's opposite forearm while maintaining a grip on the tail to avoid the mouse dangling.
- 8.5. When tail handling is required in the implementation of this experiment (e.g., before

blood draws for cortisol testing) animals are transferred to the experimenter's forearm by tail handling and held for 15 s before being returned to their cage.

9. Elevated Plus Maze

9.1. Room setup

9.1.1. Place the maze in the middle of the room, under a digital camera equipped with a memory card.

9.1.2. Set up the light of the room at ~60 Lux using 2 standing lamps placed behind the maze.

9.1.3. Turn off any overhead lighting off to avoid direct light on the maze that creates reflection and disrupts the detection of the animals in the maze.

9.1.4. Once all the equipment is set up, transfer the animals to the room and let them acclimatize to the light settings and the new environment for 30 minutes.

9.2. Testing

9.2.1. Clean the maze with 70% ethanol to prevent smells from dust or from the animal that was tested previously.

9.2.2. Start the camera.

9.2.3. Use a piece of paper with the animal ID to record the ID on the video, prior to placing the animal in the maze (this will facilitate the proper identification of which mouse is being filmed on each video).

9.2.4. Use the appropriate handling technique to each animal to transfer it to the maze.

9.2.5. Place the mouse on the central platform, facing an open arm.

9.2.6. Allow the mouse to explore the apparatus for 10 min, undisturbed.

9.2.7. After 10 minutes, stop the camera.

9.2.8. Retrieve the mouse from the maze and put it back in its cage.

9.2.9. Clean feces and urine from the maze with 70% ethanol.

9.2.10. Once testing is complete with all mice, transfer videos from the memory card to a computer for video tracking.

9.2.11. Using automated animal tracking software, track the number of entries to the open and closed arms, and the time spent in open or closed arms (here Ethovision XT 14).

10. Experimenter Interaction (derived from Hurst and West⁹)

10.1. Room setup

10.1.1. Place a table in the middle of the testing room under a digital camera equipped with a memory card.

10.1.2. Set up the light at 50-70 Lux with 4 light bulbs placed in the corner of the room facing up to the ceiling. Turn off overhead lighting to avoid direct light on the maze that creates reflection and disrupt the detection of the animals in the arena.

10.1.3. Bring the animals to the room.

10.1.4. Let them acclimatize to the room for 30 minutes.

10.2. Experiment

10.2.1. Place the home cage under the digital camera.

10.2.2. Remove the lid.

10.2.3. Remove nesting material and other enrichment that might interfere with tracking of animals.

10.2.4. Start the camera.

10.2.5. Use the cage card with the animal ID to identify the animal on the video.

10.2.6. Place a hand in the home cage along the wall of the cage in the front right side.

10.2.6.1. Ensure that the handler's head is not blocking the camera to film the mouse.

10.2.7. Start a timer.

10.2.8. Keep the hand immobile for 2 minutes, and let the mouse explore the hand.

10.2.9. Remove the hand from the cage for 15 s.

10.2.10. Attempt to pick up the mouse using cupped hands and record whether the mouse flees.

10.2.11. Repeat the last step up to five times, every 5 seconds, or until the mouse allows itself to be picked up.

10.2.12. Record the number of attempts required to pick up the mouse.

10.2.13. Return nesting material and enrichment to the cage.

10.2.14. Clean gloves with 70% ethanol or change gloves before proceeding to the next animal.

10.2.15. After testing, transfer videos from the memory card to a computer.

10.2.16. Using automated video tracking software, divide the cage into four equal quadrants and record the time spent by the mouse in each quadrant (here, Ethovision XT 14).

11. Novelty Suppressed Feeding

11.1. Food deprivation

11.1.1. 3 days prior to the test, perform a full cage change, and single house the animals (single housing is preferable to perform the home cage testing.).

NOTE: Providing fresh bedding removes potential dust or little pieces of food accumulating in the bedding since last cage change.

11.1.2. The day before testing, weigh all animals around 6 pm.

11.1.3. Remove all food from the food hopper, and ensure that there are no pieces of food in the cage or in the bedding.

11.2. Room setup

11.2.1. Place the NSF chamber on a table.

11.2.2. Fill the chamber with a thin layer of corn bedding (or other bedding that is different from bedding used in animals home cage).

11.2.3. Set up the light at 70 Lux with 4 light bulbs placed in the corner of the table where the chamber stands, facing up to the ceiling. Turn off overhead lights to maintain low room lighting.

11.2.4. Place one pellet of standard chow used in the facility, on the side of the chamber facing the experimenter (≈ 10 cm from the wall).

523 11.3. Testing

524
525 11.3.1. In the morning after food deprivation, bring the animals to the room 30 minutes prior to
526 testing to let them acclimatize to the light settings and the new environment.

527
528 11.3.2. Weigh all animals in order to measure their weight loss based on the weight measured
529 the previous day. Animals should lose 8-12% overnight to be able to perform the task properly.

530
531 11.3.3. Sort the animals per weight loss, and screen them starting from the mouse that lost the
532 most to the mouse that lost the least weight.

533
534 11.3.4. Ensure that the chamber is filled with bedding and with a single pellet.

535
536 11.3.5. Place the animal on the opposite side of the chamber, away from the food pellet.

537
538 11.3.6. Start the timer immediately.

539
540 11.3.7. Let the mouse explore the chamber for up to 12 minutes.

541
542 11.3.8. Measure the latency to approach and feed (animal must bite and eat) on the food pellet.

543
544 11.3.8.1. Consider it to be an approach when the animal comes close to the pellet, smells it
545 and does not bite.

546
547 11.3.8.2. Define a bite as when the animal starts consuming the pellet.

548
549 11.3.9. Record the latency to approach and feed on the pellet in seconds.

550
551 11.3.10. Once the mouse has fed on the food pellet, remove the mouse from the chamber.

552
553 11.3.11. Discard the bedding but save the pellet that will be used for testing appetite drive
554 in the mouse home cage.

555
556 11.3.12. Reset the chamber for next animal and proceed with the next animal.

557
558 11.3.13. 15 minutes after completion of the test in the chamber, drop the pellet used
559 during the test, inside the home cage of the mouse, against the wall at the front of the cage.

560
561 11.3.14. Measure the latency to feed on the pellet when the pellet is in the home cage.
562 This is a measure for appetite drive.

563
564 11.3.14.1. It is preferable to remove the nesting material to ensure that the mouse sees the
565 pellet being dropped in its cage.

12. Serum Collection and Corticosterone Measurement

12.1. Handle animals for one minute using the assigned technique, 15 minutes prior to blood collection (this can be done with group housed or single housed animals, keeping in mind the risk for fights when regrouping mice).

12.1.1. For the tunnel handled mice, guide them to the tunnel, lift the tunnel from the cage for one minute, and replace the mouse in its cage.

12.1.2. For tail handled mice, grab the tail base of the mouse and remove the mouse from its cage. Transfer the mouse to the experimenters sleeve for one minute, and return the mouse to its cage by tail handling.

12.1.3. For 3D-handled mice, use cupped hands to remove the mouse from its cage. Hold the mouse in cupped hands for one minute, and return it to its cage.

12.2. 15 minutes after handling, proceed with blood collection from the submandibular vein²².

12.3. Firmly scruff the mouse such that the head of the mouse is securely immobilized.

12.4. Locate the site of puncture.

12.4.1. There is a small hairless dimple along the mandible of the face that can be used as a landmark to locate the puncture site. Drawing a line between the base of the jaw and this dimple the puncture site lies behind this dimple towards the ear by roughly 5 mm, just behind the hinge of the jaw.

12.5. Hold a clean 23 G needle perpendicular to the puncture site and use a quick firm lancing motion. The tip of the needle should penetrate to a depth between 1-2 mm, blood will flow immediately as soon as the vein is punctured.

12.6. Collect ~150 µL of blood in EDTA coated collection tubes and store on ice.

12.7. Apply slight pressure with a sterile gauze pad to the puncture site for 5 seconds or more to allow the blood to clot.

12.8. Once blood has clotted, return the mouse to its home cage.

12.9. Centrifuge blood at 4 °C 3,500 x g for 10 minutes.

12.10. Decant the supernatant.

12.11. Store the supernatant at -20 °C for downstream analyses.

12.12. Measure corticosterone levels using a corticosterone ELISA kit following manufacturer's protocol.

12.13. Use a spectrophotometer to read the ELISA outcomes.

REPRESENTATIVE RESULTS:

Two separate studies were performed with C57BL/6 mice. Study #1 included 6-month-old males and Study #2 included 2.5-month-old females (N=36/study) from Jackson Laboratories (Cat #000664). Mice arrived in the facility at the age of 2 months. While Study #2 females were handled and tested two weeks after arrival, Study #1 males were only handled and tested at the age of 6 months (delay due to global pandemic shutdown). During this time, one mouse from Study #2 died, prior to starting handling experiments. The Study #1 male mice were cared for by animal facility staff. All mice were maintained on a 12 hour light/dark cycle (7:00 ON, 19:00 OFF), given access to food and water *ad libitum*. Their home cage was filled with recycled newspaper as bedding material, as well as nesting material. Mice were housed individually, in order to limit potential agonistic behavior in group-housed males during handling session or after procedures such as blood collection or behavioral testing. Mice were randomized into three groups: *tail handling*, *tunnel handling* and *3D-handling*, and handled in the open-room accordingly to the design of their respective group (**Figure 2**). The tunnel-handled group received the tunnel as an enrichment for 1 week prior to handling session. They were then handled for ten (10) consecutive days, prior to behavioral testing. One week after completion of the different handling sessions, behavioral testing commenced. On day 16, mice were tested in the EPM, and then in the NSF test on day 19. Two days later, mice were tested in the experimenter interaction test. Finally, on day 24, blood was drawn 15 minutes after a one-minute handling session of the same type as the initial handling.

For behavioral testing, tunnel-handled animals were transferred from their cage to the apparatus using the tunnel as much as possible. However, for the Elevated-Plus Maze experiment, the dimensions of the maze made it difficult to remove or place animals in the maze using the tunnel. In this case, animals were transferred from tunnels to cupped hands, and transported to the maze. 3D-handled mice were handled over the three days, concurrent with days 8-10 of tunnel handling (**Figure 2**). Tail handled mice were not habituated to handling but were tail handled during interactions with experimenters. During the time of the study, cage change was performed by the experimenter to ensure the use of the appropriate handling technique used for each group.

In the experimenter interaction test, animals were tested for their willingness to voluntarily interact with the experimenter and the ease of handling in an experimental context (**Figure 3**). ANOVA performed on the number of attempts to pick up the mouse from the cage showed a significant effect of the handling approach in Study #1 males ($F_{(2,31)}=6.36$, $p=0.004$), and in Study #2 females ($F_{(2,33)}=12.21$, $p=0.0001$). Scheffe's *post hoc* analyses revealed that the number of attempts required to pick up the mice was significantly reduced by both 3D ($p=0.0061$ in Study #1 males, and $p=0.0002$ in Study #2 females) and tunnel handling ($p=0.04$ in Study #1 males, and $p=0.003$ in Study #2 females), in comparison to the tail handled group (**Figure 3A**). ANOVA

performed on the time spent in the same quadrant as the hand showed significant effect of handling in Study #1 males ($F_{(2,31)}=5.38$, $p=0.009$), and in Study #2 females ($F_{(2,33)}=3.5$, $p=0.04$; **Figure 3B**). Scheffe's *post hoc* analyses showed that Study #1 male mice handled with the 3D-handling technique spent significantly more time in the same quadrant than the experimenter's hand, compared to tail-handled mice ($p=0.012$). There were no significant differences between handling groups in Study #2, 2.5 month-old females. Degree of interaction with the experimenter is further demonstrated by the combined heat-maps of the center points of the mice (**Figure 3C-E**). These illustrate how the 3D-handled male mice from Study #1 spent more time proximal to the hand, including areas near the hand, while tail handled mice had the least overall interaction with the hand.

The effects of the 3D- and tunnel handling were compared to tail handling in two tests of anxiety-like behaviors, the novelty suppressed feeding (NSF) test and the elevated plus maze (EPM). In the NSF test, ANOVA performed on the latency to approach showed an effect of handling technique used in Study #1 males ($F_{(2,31)}=3.5$, $p=0.04$). Scheffe's *post hoc* analyses in Study #1 males showed trends from 3D-handled mice ($p=0.08$), and from the tunnel-handled mice ($p=0.08$), with reduced latency to approach compared to tail handled mice (**Figure 4A**). No effects were observed in Study #2. ANOVA performed on the latency to approach in the mouse home cage (data not shown) showed no effect of handling ($p=0.88$ in Study #1 males, and $p=0.16$ in Study #2 females). ANOVA performed on the percent time in the open arms in the EPM revealed a significant effect of handling in Study #2 females ($F_{(2,33)}=3.5$, $p=0.04$). No effects were observed in Study #1 males ($F_{(2,31)}=2.1$, $p=0.1$; **Figure 4B**). Scheffe's *post hoc* analyses only revealed a trend towards increased time spent in the open arms in tunnel handled mice from Study #2, compared to tail handled mice ($p=0.07$). Regarding the percent entries in the open arms (**Figure 4C**), ANOVA revealed no effect of handling, neither in Study #1 males nor in Study #2 females ($F_{(2,31)}=1.12$, $p=0.33$; and $F_{(2,33)}=1.3$, $p=0.26$, respectively). Behavioral scores were summarized in a z-score, as in Guilloux et al.²³, informing on potential reduction of anxiety-like behaviors compared to tail handled mice (**Figure 4D**). ANOVA on the z-scores showed a significant effect of handling in Study #1 males ($F_{(2,31)}=5.6$, $p=0.008$) but not in Study #2 females ($F_{(2,33)}=1.07$, $p=0.35$). Scheffe's *post hoc* analyses showed that 3D-handling and tunnel handling significantly decreased z-score ($p=0.04$ and 0.01 , respectively), compared to tail handling, suggesting that both approaches reduces anxiety-like behaviors in Study #1 males.

Corticosterone levels after handling were also assessed 15 minutes after a brief handling session (**Figure 5**). ANOVA found a significant effect of handling in Study #2 females ($F_{(2,33)}=4.44$, $p=0.01$), but not in Study #1 males ($F_{(1,31)}=0.53$, $p=0.59$). In Study #2 females, *post hoc* analyses revealed a significant decrease in corticosterone levels in mice from the 3D-handling group compared to the tail handling group ($p=0.02$).

To determine if the handling techniques had a significant impact on the variability of data obtained, we applied Bartlett's test of homogeneity of variance. Our results found no significant difference in variability in the Study #2 female mice across measurements (% time EPM $B_{(2,33)}=4.95$, $p=0.087$; % Entries EPM $B_{(2,33)}=3.68$, $p=0.16$; NSF $B_{(2,33)}=0.20$, $p=0.91$; CORT $B_{(2,33)}=1.69$, $p=0.42$). However, in Study #1 male mice, there was a significant heterogeneity of

variance in the NSF test ($B_{(2,31)}=8.08$, $p=0.0175$) and in measured CORT levels ($B_{(2,32)}=11.63$, $p=0.0029$), but not in either of the measures for EPM (% time EPM $B_{(2,32)}=1.16$, $p=0.56$; % Entries EPM $B_{(2,32)}=2.79$, $p=0.25$). Using the F-test to compare two variances showed that in the NSF test variance was significantly reduced for Study #1 males by both the 3D ($F_{(1,21)}=4.22$, $p=0.04$) and tunnel handling techniques ($F_{(1,22)}=4.01$; $p=0.03$) in comparison to tail handling. For the concentration of CORT after handling, only 3D-handling significantly reduced variability ($F_{(1,20)}=9.65$, $p=0.0019$) in comparison to tail handling.

FIGURE AND TABLE LEGENDS:

Figure 1. Representative images of the 3D-Handling Procedure.

The images illustrate the 3D-handling procedure. A) Hand in cage: The experimenter's hand is placed in the cage and kept still, allowing the mouse to habituate to the presence of the hand in the cage. B) Flat hand: upon first removal from the cage, the mouse is placed on the flat palm of the hand. The mouse can freely walk around the palm and move between adjacent flat hands. C) Roll: Relax palm of the hand to form a loose "cup" around the mouse. Gently tilt the cup into the opposite hand the mouse should freely move to this hand, if not gently guide it into the other hand. D) Shelter: position the mouse at the edge of the hand then bring both hands together and very slowly form cup around the mouse. The mouse should not be restrained and an opening should be left so the mouse may escape. Hold for ~5-10 s and then open to flat hands. E) Head/Back Petting: While the mouse is exploring the flat palm of the hand, gently pet the mouse on the head and back. This habituates the mouse to the approach of the experimenter from above. F) Nose Poke: When the mouse appears to be habituated to handling, attempt to gently touch the mouse directly on the snout. If the mouse does not move its head away it is well habituated to handling. G) It is possible to perform a short (2-3 sec) neck pinch on the last day, to measure the habituation of the animals in the event of future interventions requiring contention. When habituated to handling, mice remain immobile during the neck pinch, while non-habituated mice will try to escape by rotating their tail to get freed from the contention.

Figure 2. Experimental Design

After arrival in the facility, tail handled mice received no habituation. Tunnel-handled mice were habituated to the tunnels in their home cage for one week before the start of handling. Tunnel handled mice were handled with the tunnel handling technique for 10 days (First day of handling = Day 1), while 3D-handled mice were habituated for three days (Day 8-10). Mice were then subjected to the elevated plus maze (EPM) (Day 16), experimenter interaction test (Day 19), novelty suppressed feeding (Day 21), and a brief handling session followed by serum collection for CORT measurement (Day 24).

Figure 3. Impact of the three handling techniques on ease of handling and willingness to interact with experimenter.

A) Average number of pick up attempts required to remove a mouse from the cage. Study #1 Male (left panel, Tail Handling $N=12$, Tunnel Handling $N=12$ and 3D handling $N=11$) and Study #2 female (right panel, $N=12$ per group) mice from both tunnel and 3D-handled groups displayed a significant reduction in the number of attempts required to remove them from the cage compared to tail handled mice. B) Average amount of time spent by an animal in the same

quadrant of the cage as the experimenter's hand. Study #1 male mice handled with the 3D-technique showed a significant increase in time spent in the same quadrant as the experimenter's hand. C-E) Average heat-maps of mouse center-point by time rendered in Ethovision XT 14, visually demonstrated the increased exploration and interaction with experimenter of the Study #1 3D-handled male mice. Error bars indicate SEM. * $p < 0.05$, ** $p < 0.01$ compared to Tail Handled group.

Figure 4. Impact of the three handling techniques on anxiety-like behaviors

A) Latency to approach and feed on the pellet in the novelty suppressed feeding chamber in Study #1 male mice (Tail Handling N= 12, Tunnel Handling N=12 and 3D handling N=11) and in Study #2 female mice (N=12/group). Data from the Study #1 male mice in the 3D-handling and tunnel-handling groups showed a trend towards significant reduction of latency to approach the pellet. B) Means of % of time spent in the open arms of the elevated plus maze. There were no significant differences between groups in Study #1 males, and a trend towards more time in open arms by Study #2 females in the tunnel-handling group. C) Entries in the open arms: There were no significant differences between groups in Study #1 males, nor in Study #2 females. D) Z-score summarizing the anxiety-like behaviors. Using the data presented in A, B and C, a z-score was calculated using the Tail-handled mice as reference. Decrease in the z-score suggests a decrease in anxiety-like behaviors measured by the NSF and EPM tests. Study #1 male mice handled using the 3D- or tunnel technique showed a reduced anxiety-like phenotype compared to Tail-handled mice. Error bars indicate SEM. * $p < 0.05$ comparison to tail-handled group. t depicts trending level of significance ($p < 0.1$) compared to tail handled group.

Figure 5. Levels of corticosterone after handling

Serum was collected 15 minutes after a brief handling session and then CORT levels were measured by ELISA in both Study #1 male (Tail Handling N= 12, Tunnel Handling N=12 and 3D handling N=11) and in Study #2 female mice (N=12/group). Study #2 female mice handled via the 3D-handling technique showed reduced corticosterone levels compared to mice handled by the tail. ANOVA in Study #1 male mice did not reach significance for differences between groups ($p = 0.5$). Error bars indicate SEM. * $p < 0.05$ compared to Tail Handled group.

DISCUSSION:

This study and method development are based on the observation that handling techniques in mice are still overlooked by the scientific community, and that some labs are still reluctant to implement habituation or handling techniques to reduce stress and reactivity of their animals prior to experiments. While representing a time commitment, animal handling provides beneficial effects to the animals that may contribute to the success of the experiments to be performed and prevents experiments from having to be performed multiple times due to data variability or animal over-reactivity. The use of the 3D-handling technique decreased escape attempts in mice. It also increased interaction with the experimenter and decreased anxiety-like phenotypes in our 6-month-old male mice. Further, 3D-handling decreased data variability and decreased corticosterone levels in 2.5 month-old female mice after only 3 initial days of handling. This approach relies on gentle manipulations to habituate the mouse to handling by the experimenter facilitating smoother transport and easier intervention.

Something worth emphasizing from the 3D-handling technique is that the progression of handling methods occurs in response to the reactivity of the mouse, depending on the achievement of the milestones described above and in **Table 1**. Animals should have reduced reactivity to one handling step before progressing to the next steps. Attempting to progress too quickly to the “shelter” or “nose-poke” steps on animals that are not sufficiently habituated would likely result in increased stress and potentially reduce the effectiveness of the procedure. Similarly, the reactivity of the animal on each day of handling should be monitored and should be considered when deciding if additional handling days are required. If animals do not respond well to the shelter test on the first day, not meeting criteria for achieving the first milestone, the first day of handling could be repeated until completion of the milestone. Similarly, if animals fail to respond to the nose poke test on the second day, the second day may also be repeated. Another caveat to note with this approach is that the risk of mice jumping away is greater on the first day of handling, in particular in jumpy strains like C57BL6. Following the guidelines described above should reduce the risk of jumping, and provide ways to limit such behaviors. Duration of the handling and progression through the steps may vary depending on the strains, particularly if working with transgenic models known to exhibit anxious phenotypes.

Several factors can contribute to reducing the effectiveness of the presented 3D-handling technique. One such factor is the potential fear or hesitancy from the experimenter, in the event of the experimenter being not familiar with mouse handling, or being scared of mice. Therefore, the effect on the handler is also something to consider. However, the gradual increase in the level of interaction with the mice allows novice experimenters to develop confidence and greater skills at performing the handling technique as they proceed through the handling steps. The proposed steps/milestones (the shelter and nose poke tests) can help counter potential human variability in novice handlers, ensuring that animals reach similar levels of habituation. It has been reported that fostering positive human-animal interactions with animals had resulted in greater quality of life and compassion satisfaction in animal care staff²⁴. As such, the gentling from handling presents benefits to both the handler and the animal during any general interaction or intervention.

With its impact on decreasing the number of attempts to pick-up the mouse, in both 6-month-old males and 2.5 month-old females, the 3D-handling provides an alternative to tunnel handling or other techniques, facilitating easier transfer of animals from their cage to experimental apparatus. The 3D-handling technique also increased the interaction of 6-month-old male mice with the experimenter. This was not observed in 2.5 month-old female mice, but female mice remained easier to pick up, compared to tail-handled mice. This suggests that the 3D-handling technique may be more suitable for experiments requiring direct interactions between the animal and the experimenter, such as the Morris water maze (despite potential sex/age confounding factors discussed later). Others have developed and used manual handling techniques, consisting of picking up the animals with cupped hands, without additional manipulation¹⁰. While these techniques showed beneficial effects, data in the literature often present handling protocols with habituation periods exceeding 10 days^{9,16}. Additionally, cupped-handling without the refined interaction provided by 3D-handling may not be suitable for jumpy

strains that continue to jump out and away from the hands. While we did not do a direct comparison in this study to the cup method, the 3D-handling addresses this and relies on refined moves to foster interaction between the mouse and the handler. The study by Ghosal et al.¹⁶ used a cup-handling technique combined with massage for 5 days, and showed this technique limits the impact of stress on metabolic endpoints, highlighting the need for refined moves and interaction during handling for better efficacy. Based on this cup-massage technique, the 3D-handling uses additional interaction to habituate mice. Using the 3D-handling approach, handlers ensure that all mice reach a similar level of habituation by performing standardized moves and by adapting the duration of the procedure to each animal depending on its need (in the present study, all mice passed the milestones and finished the 3D-handling protocol in 3 days). This approach can be considered “personalized” to each mouse, so all animals reach the desired level of habituation on each day of handling. As mentioned earlier, if animals do not reach the milestones described in the protocol, this technique can be adjusted by increasing the number of days. This technique showed beneficial effects for reducing variability between animals in behavioral studies and physiological measurement (CORT levels), suggesting that this approach could contribute to the reduction of intra-study variability and reduce the impact of experimental error potentially driving biased results in preclinical studies.

Supporting results suggested that mice subjected to 3D- and tunnel handling exhibit reduced anxiety in the novelty suppressed feeding test, compared to tail-handled mice. Considering combined data from the NSF and the EPM, both approaches showed significant effects at reducing anxiety in 6-month-old male mice. This replicates the findings that animals habituated to tunnel handling had improved performance in tests for anxiety^{9,15} after 10+ days of handling, and further demonstrate the potential of the 3D-handling to exhibit similar effects. This also showed that 3D-handled 6-month-old male mice approach and voluntarily interact more with their experimenter than 6-month-old male mice subjected to tunnel and tail handling. Importantly, 2.5-month-old female mice subjected to 3D-handling had reduced levels of CORT, which is in agreement with previously published results⁹. The two studies (Study #1 in 6 month old males and Study #2 in 2.5 month old females) confirmed, in two different ways that the handling has beneficial impact on anxiety-like phenotypes (either on behavioral outcomes in Study #1, or on CORT levels in Study #2).

A possible contributing factor to the effect is the sex of the experimenter, in this case male. It has been shown by Sorge et al.²⁵ that the presence of male experimenters can lead to an increase in CORT and anxiety like behaviors in male but not female mice. This is in contrast with results from the present study. The major difference between this study and the study from Sorge et al.²⁵ is that the approach described here consists on habituating the mice to handling, by fostering positive (non-reinforced) interaction with the experimenter, while Sorge et al.²⁵ used naïve rodents that never interacted with human beings. One can expect that naïve mice could have a strong reaction against human experimenters if they do not learn that the experimenter does not represent a threat. However, the present study was only performed with a male experimenter, and future studies should investigate if such effects are reproducible with a female experimenter. Though isolating these factors is outside the scope of this paper, it is worth highlighting the importance of identifying such sources of variability when implementing

handling habituation, or in experimental design more generally.

The present study also confirmed efficacy of the tunnel-handling technique at reducing anxiety-like behaviors and CORT levels in mice⁹⁻¹¹. An additional benefit of this approach is that the tunnel can be left in the cage as enrichment²⁶, which may also contribute to a reduced stress/anxiety response, altogether contributing to improved welfare^{10,11}. In this case, the role of the experimenter is to manipulate the tunnel only, with each animal, for one minute. However, as described by Gouveia et al¹⁹, the tunnel does not necessarily need to remain in the home cage and instead can be presented to the animals only when required to transfer the animal, without causing additional stress. Both approaches, the tunnel and 3D-handling techniques, offer benefits that should be assessed by the lab and the experimenters in order to determine which approach is the most appropriate for their needs. In the present study, the tunnel was left in the cage, and the effects we observed on anxiety-like behaviors may be due to a combination of tunnel handling and enrichment.

While both provide beneficial effects, the 3D- and tunnel-handling techniques are not without limitations. A shared limitation is that it can be time consuming and potentially discouraging for animal facilities to implement such procedures. However, the added benefits are invaluable, improving animal welfare by reducing stress and improving interaction with experimenter and animal care providers (as described in Spangenberg and Kelling²⁷), and research reliability and reproducibility. Evidence from our facility suggests that this technique improves interactions between animals and husbandry staff, facilitating cage change and health monitoring. From other users in our facility, contention and overall manipulation are reported as being significantly easier with handled mice, consistent with our findings that mice handled with the 3D-technique are less likely to flee when being picked up and in our example, 6-month-old males are more prone to interact with their experimenter. Follow up studies could quantify such effects to demonstrate further the usefulness of the technique. Altogether, this 3D-handling approach, as well as the tunnel-approach, contributes to the rule of the 3Rs, particularly by refining routine animal interactions to minimize the stress in response to handling. Given the observed reduction in variability of data, this also has the potential to reduce the number of animals needed to obtain consistent results and refining the approach used to limit variability.

Another point of discussion based on the data presented is that this study was performed with animals being single-housed. Single housing was preferred as it limits the potential agonistic behaviors (particularly in male mice), that can contribute to inter-individual variability^{28,29}. For consistency between groups, all animal were single housed. It is also interesting to note that positive experimenter-animal interactions in rats in the form of rat tickling, was able to mitigate some of the effects of social isolation in single housed rats^{30,31}. It is possible that handling techniques involving direct contact between animal and experimenter, such as the 3D-handling technique or the cup-massage technique described by Ghosal et al.¹¹ could have a similar effect. Future studies could explore this question by comparing the effects of handling techniques in single and group housed animals. Past studies investigated the impact of cup and tunnel handling approaches with mice in a group-housed setting, and obtained similar results^{7,8}. This confirms that it is possible to use the handling protocols described herein with animals kept in single-house

or group-house conditions, keeping in mind the possibility of agonistic behavior when taking one animal out of the cage and placing it back in (particularly in male mice, or in aggressive mouse lines). In such cases, it is recommended to use a temporary cage before regrouping all the animals together.

To conclude, the proposed 3D-handling approach contributes to reducing reactivity and stress in mice. It also increases data reliability by reducing variability after 3 days of handling. Similar results are observable with the tunnel handling, in our case after 10 days of tunnel handling. In comparison with the tunnel handling technique, the 3D-handling technique provided the benefit of increasing interaction with an experimenter in our 6-month-old male mice, which can be critical in some cases. If the 3D- or tunnel handling technique were to be implemented in all animal facilities that would represent a major improvement for data generation and would greatly contribute to the reduction of animal use in research.

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DISCLOSURES:

The authors have no conflict of interest to disclose.

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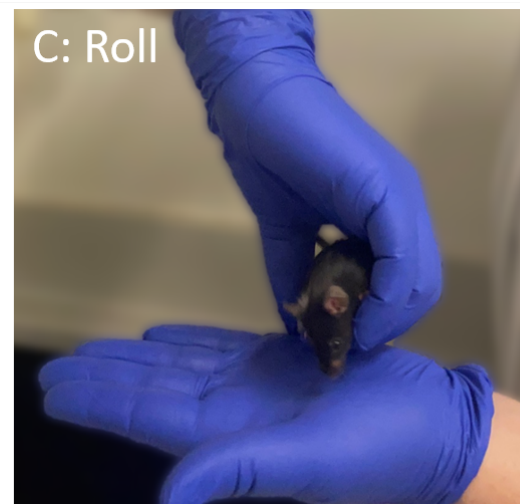
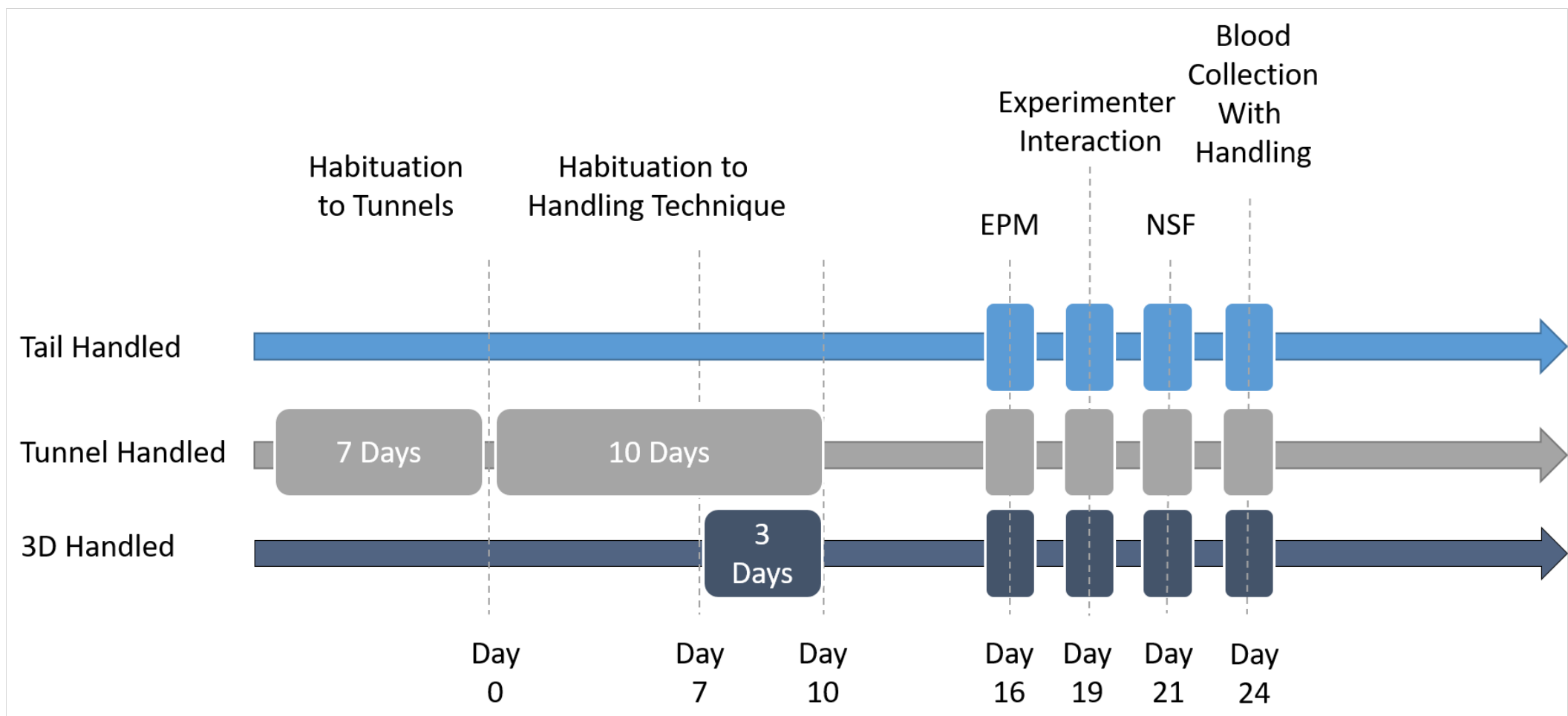


Figure 2



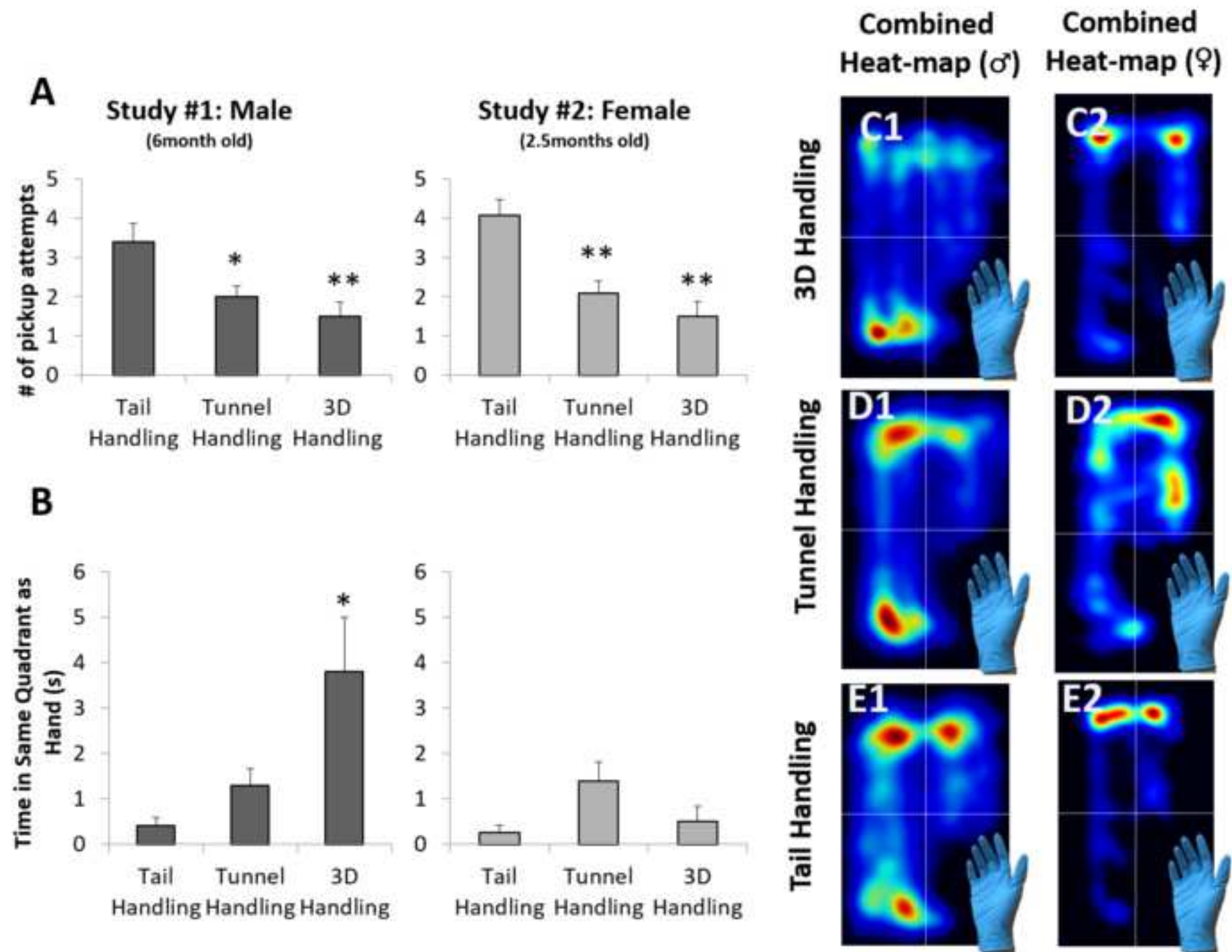
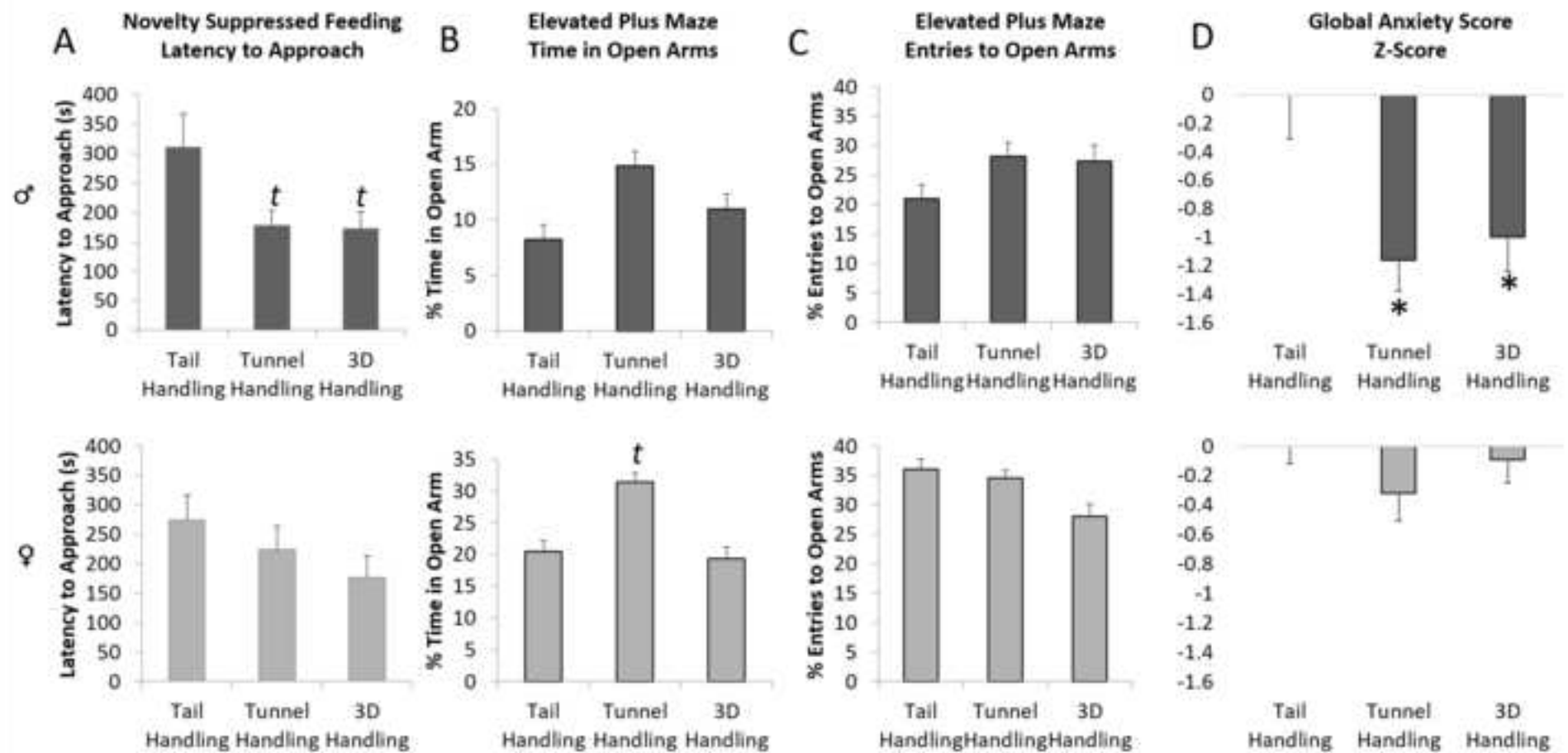
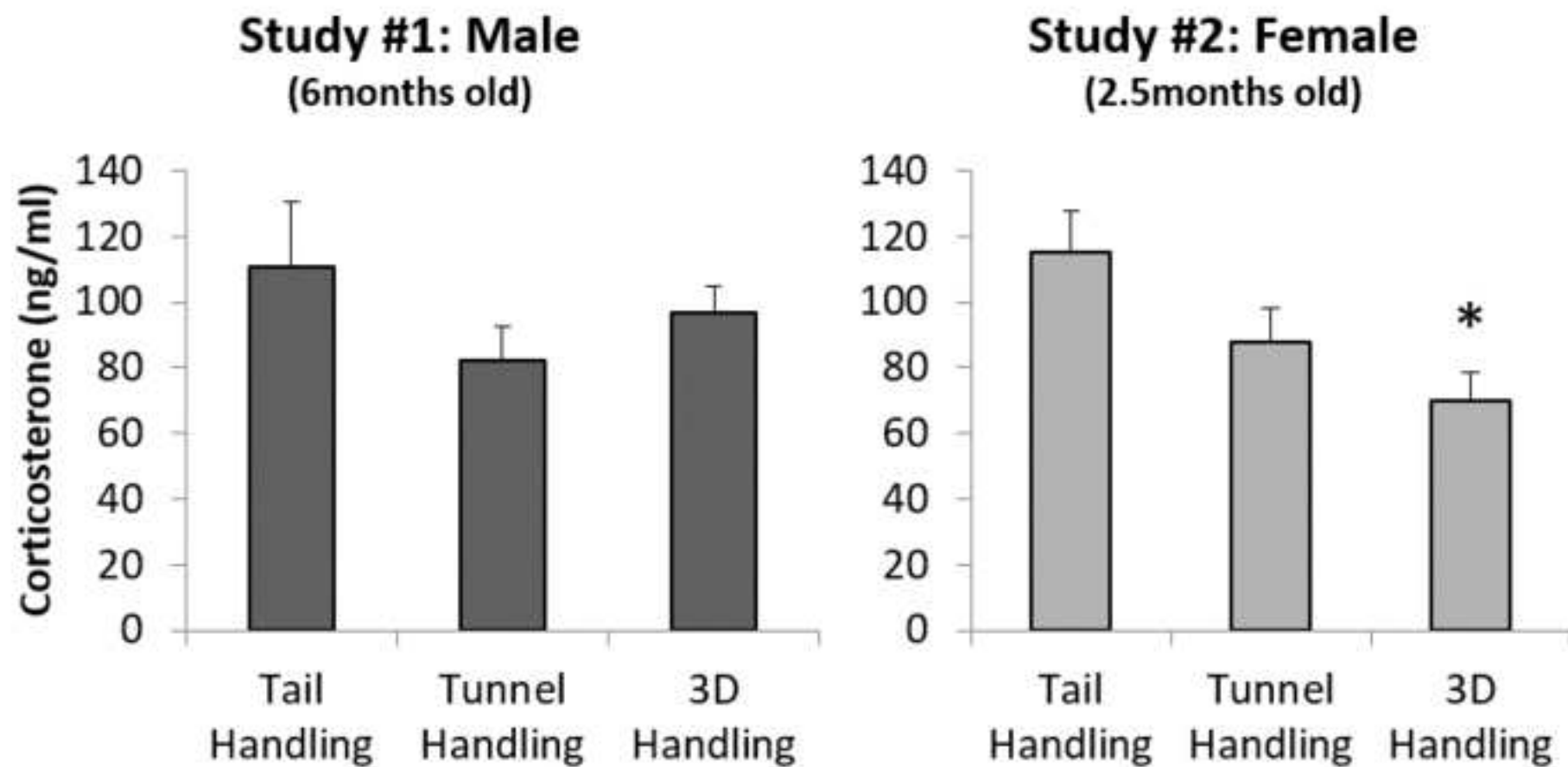


Figure 4





Day	Milestone	Time
1	1 or more shelter tests	10 s
2	1 or more nose poke tests	2-3 s
3	2 or more shelter tests, and 2 or more nose poke tests	5 s and 2-3 s



Click here to access/download
Table of Materials
JoVE_Materials.xls



Psychiatry
UNIVERSITY OF TORONTO

Date: August 6, 2021

Nam Nguyen, Manager of Review, JoVE

Dear Nam Nguyen, and reviewers,

Please accept our apologies as we missed the second vet review and did not include answers to these comments on our last resubmission. We have now included answers to these comments, as well as the new editorial comments sent to us on August 5th 2021.

Please find in the following pages, our answers to the editorial comments, and the vet review with our answers in blue. We also provide a revised manuscript to be considered for publication.

We remain available may you, the vets or the reviewers have further questions or comments.

Sincerely,

Thomas D. Prevot
Project Scientist



Editorial and production comments

Changes to be made by the Author(s) regarding the written manuscript:

1. Some minor changes are needed. Please see the comments in the attached manuscript.

>> We have addressed all comments in the attached manuscript, removing the references to commercial brands, changing the format of the reference on page 2 to use numbered reference and changing the title of the manuscript to match the video.

2. Please send us the rebuttal letter for the vet review comments.

>> Sorry we missed answering this on our previous rebuttal. We addressed each point in the following pages.

#	Time in the video	comment	Change in video required Yes/No	Change in text is sufficient Yes/No	Suggested Changes
1	3:29-4:14	Sound level changes significantly and is distracting	YES		Can the voice over be fixed? >> We addressed this issue by re-recording all sequences in the previous version.
2	3:29-4:14	The animal seems very habituated to the rolling and shelter test despite it being day 1. This will be misleading to individuals trying to emulate this handling style	YES		Can the video be reshoot with animals who it truly is day 1 for? >> We changed the video of this sequence. In the new video, the mouse appears more alert, trying to flee, as expected on day 1.
3	9:22	There is a claim that this method will increase the ease of animal husbandry however, the technique takes at least 3-5 minutes per mouse. Considering even a small vivarium has 5000 cages of 5 animal per cage, the time commitment is substantial, potentially limiting the acceptance and use of this technique.	YES		Remove the last reference to animal husbandry >> We re-recorded this part as well and removed the mention of facilitating animal husbandry. The sentence now says that "implementing this technique has the potential to facilitate routing handling"(no reference to

					husbandry anymore)

Vet Report

There are details not addressed in the video or text or contradictory between the two:

- Are these animals housed in ventilated or static caging systems?

>> *The mice of the present study were housed on static caging system. Other animals in our facility are housed in ventilated caging systems.*

- What enrichment was provided to these animals? The text states Yesterday's news was used for bedding but the video also shows a Nesting puck. Can this be more fully described in the text?

>> *Mice were provided with nesting puck to build nest. As this is now mandatory in all facilities, we did not include this in the methods, but added a mention at the very beginning of the result section (Line 626). It now says "Their home cage was filled with recycled newspaper as bedding material, as well as nesting material."*

- Is this handling technique done in an animal transfer station (ATS), biosafety cabinet (BSC), or in the open room? The video gives the appearance that the animals are in an open room, but most animals are usually handled in an ATS or BSC. Can this be better described in the text.

>> *This is an excellent comment that other reviewers also made. We do it in BSC, or in open room, depending on the study. For the present study, we did it in open room, and recorded it in open room. In the manuscript, we include option to work in the BSC (line 126-134). For the present studies, we now mention that the mice were handled in the open room (Line 629).*

- 438-440: the video shows animals housed 2 per cage but the text states that animals, at least males, were single housed. Figure 3 also shows 2 animals per cage with the heat map data. Can this be explained further or rectified?

>> *In the video, we showed animals group-housed as this is the most commonly used housing condition, and to avoid providing confusing messages on the video that would suggest that the handling technique needs to be done exclusively with single-housed mice. This is not the case, and we decided to show on the video that we can do it on group-housed mice. We were afraid that showing data from single-housed mice, and showed handling done in single housed mice in the video would make viewers think that mice need to be single housed for the handling to be efficient, which would decrease its adaptability and use in other facilities.*

In the manuscript, we also describe that animals can be single-housed or group-housed (line 122). In this study, because we provide details on behavioral and neuroendocrine effects, we decided to single house the animals, but the core technique to be demonstrated in the video is the handling and not the behavioral and neuroendocrine results. These results are only here to support the efficacy of the technique at reducing anxiety like behavior and improving interaction with experimenters. Therefore, we made the video more generic than the approach that we used in the paper.

Regarding the heatmaps, they are representative heatmaps from animals being single-housed. Mice were not group housed in this experiment.

- 455-456: Do you mean handling by the wrong method rather than bias of the facility staff?

>> Yes. Our facility staff is still transitioning from tail handling to tunnel or cup handling when doing the cage change. In order to limit tail handling of our tunnel or 3D handled mice during cage change, or more generically to limit the use of a different handling technique during the experiment, the experimenter changed all cages, using the appropriate handling technique relevant to each group. We changed the sentence to avoid any confusion. It now says: "During the time of the study, cage change was performed by the experimenter to ensure the use of the appropriate handling technique used for each group."