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TITLE:

Low-cost Protocol of Footprint Analysis and Hanging Box Test for Mice Applied the Chronic Restraint Stress

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SHORT ABSTRACT:

The low-cost protocol consisting of footprint analysis and hanging box test after restraint stress is useful for evaluating the movement disorders of mouse model.

LONG ABSTRACT:

Gait disturbance is frequently observed in patients with movement disorders. In mouse models used for movement disorders, gait analysis is important behavioral test to determine whether the mice mimic the symptoms of patients. Motor deficits are often induced by stress when no spontaneous motor phenotype is observed in the mouse models. Therefore, gait analysis followed by stress loading would be a sensitive method for evaluating the motor phenotype in mouse models. However, researchers face the requirement of an expensive apparatus to obtain quantitative results automatically from gait analysis. For stress, stress loading by simple methods without expensive apparatuses required for electric shock and forced running is desirable. Therefore, we introduce a simple and low-cost protocol consisting of footprint analysis with paper and ink, hanging box test to evaluate motor function, and stress loading defined by restraint with a conical tube. The motor deficits of mice were successfully detected by this protocol.

INTRODUCTION:

Movement disorders are defined as disturbances of the nervous system showing an excess or paucity of voluntary or automatic movements¹. In particular, gait disturbance is frequently documented among patients with movement disorders²⁻⁴. Therefore, gait analysis is a suitable behavioral test for the validation of animal models of movement disorders. In mice, automated gait analyses have been performed for walking at natural speed⁵ and at adjustable speeds by treadmill^{6,7}. These analyses provide quantitative results of gait automatically. An alternative method to detect gait disturbance is called footprint analysis. After labeling the bottoms of the feet with ink, mice walk on paper, and the footprints are analyzed. Initially, Vaseline and powdered charcoal were used to visualize the footprint⁸, and then were replaced by Ink on polygraph paper⁹ and photographic developer on photographic paper¹⁰. A cheaper and less toxic method using ink and paper than the other methods remains to date¹¹. Footprint analysis

is less expensive compared with automated analysis⁵⁻⁷ and would be useful to evaluate the movement disorders in mouse models for the researchers without abundant research funds.

The hanging box test is a kind of four limb hanging tests using wire cage lid¹² and wire mesh screen¹³. The box is an apparatus with rotatable mesh lid on the top of box along a center bar. In addition to gait analysis, the test can be inexpensively and easily performed. Therefore, we conducted the hanging box test to evaluate grip strength and balance, in addition to the footprint analysis in this protocol.

Stress induces the symptoms of movement disorders^{14,15}. Motor deficits are often induced by several chronic stresses even when no spontaneous motor phenotype is observed in the mouse models of a movement disorder¹⁶⁻¹⁸. Restraint is one of the commonly used methods for stress loading in mice, because the animal is not physically harmed¹⁹ and cost is less compared with other methods such as electric shock with dedicated apparatus and forced running with use of a treadmill. Restraint by a tube, which is performed by confining a mouse in a holed 50 ml conical tube, is easier than other methods such as wire mesh strainer, taped limb, and wrapping of animal with gauze (reviewed in ref. 20). In this paper, we summarize the protocols of footprint analysis and the hanging box test after restraint by a tube. This protocol would help us to use mouse models of movement disorders without spontaneous motor phenotype.

PROTOCOL:

All animal experiments were conducted in a humane manner. The Institutional Animal Experiment Committee of Jichi Medical University approved the study. The study was conducted in accordance with the Institutional Regulation for Animal Experiment and Fundamental Guideline for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the MEXT of Japan. Mice used in this protocol have been described previously²¹.

1. Hanging Box Test

1.1. Record the weight of each mouse. Mark the tail by marking pen for individual discrimination (e.g., a line, double lines, and triple lines).

NOTE: Growth curves are used for an index of general health²².

1.2. Place the mice in the experimental room at least 30 min before the behavioral test. Set the hanging box, which consists of a clear box (25 x 25 x 40 cm) with a rotatable mesh lid on the top (**Figure 1**). The mesh lid can be rotated along a central bar so that the top is flipped 180 degrees.

1.3. Put a mouse in the center of the mesh lid. Carefully turn the mesh lid up side down.

1.4. Measure the fall latency (hanging time) of the mouse from the mesh lid.

NOTE: If the mouse does not fall within 5 min, record the latency as 5 min.

1.5. Return the mouse to the home cage. Clean the hanging box with 70% ethanol after every test.

2. Footprint Analysis

NOTE: Following the hanging box test, perform the footprint analysis.

2.1. Set up the Runway (Figure 2A).

2.1.1. Cut a piece of white paper (29.7 cm x 42 cm x 0.09 mm) longitudinally into three lengths of equal width. Set a piece of the white paper (9.9 cm x 42 cm) on the table.

2.1.2. Put the dark goal box at the distal end of the paper. Put other boxes (approximately the same length as that of the paper) with the walls on both sides of the runway, preventing the escape of mice.

2.1.3. Put black ink and red ink into separate Petri dishes (35 mm in diameter).

2.2. Training session.

NOTE: Perform the training session only at 4 weeks of age.

2.2.1. Put a mouse on the proximal end of the paper (Face the head toward the goal box). Let the mouse walk from the proximal end to the goal box. Remove the mouse from the goal box. If the mouse stops on the paper, gently push the mouse to the goal box by finger.

2.2.2. Hold the mouse by grasping the scruff between the thumb and forefinger to limit the movement of forelimbs. Then, grasp the back and the tail between the ball of the thumb and the other fingers to limit the movement of hindlimbs.

NOTE: Insufficient holding of a mouse results in blots of ink on clothing.

2.2.3. Immerse the bottoms of forelimbs in red ink and the bottoms of hindlimbs in black ink. Immediately put the mouse on the proximal end of the paper (Face the head toward the goal box). Let the mouse walk from the proximal end to the goal box. If the mouse stops on the paper, gently push the mouse to the goal box by finger.

2.2.4. Remove the mouse from the goal box. Go to the test session.

2.3. Test Session.

2.3.1. Following the training session, set up the runway for footprints with a new cut piece of

white paper.

2.3.2. Hold the mouse by grasping the scruff between the thumb and forefinger to limit the movement of forelimbs. Then, grasp the back and the tail between the ball of the thumb and the other fingers to limit the movement of hindlimbs.

2.3.3. Immerse the bottoms of forelimbs in red ink and the bottoms of hindlimbs in black ink. Immediately put the mouse on the proximal end of the paper. Let the mouse walk from the proximal end to the goal box.

NOTE: Because mice prefer the dark, walking becomes steadier as the mouse approaches the dark goal box. If the mouse stops on the paper, gently push the mouse to the goal box by finger. Then, if reliable footprints are not obtained for analysis (see step 2.4. Analysis of footprints for details) because the mouse stopped, retry the test session.

2.3.4. Return the mouse to the home cage from the goal box. Clean the goal box with 70% ethanol after each test session. Air-dry the foot-printed paper.

2.4. Analysis of footprints

2.4.1. Obtain three measurements of each parameter (stride lengths of forelimbs and hindlimbs, front and hind base widths, overlap between forelimb and hindlimb, **Figure 2B**) with a ruler from foot-printed paper.

NOTE: Because footprints of proximal and distal ends frequently show large variations because of stopping or running, choose the part with a steady gait pattern of footprints. The middle part of the foot-printed paper will usually be suitable for the analysis.

2.4.1.1. For the stride length, measure the distances between the same parts of the paw (e.g., paw pad or toe).

2.4.1.2. For the front base width, draw a line between consecutive right (or left) front footprints. Then, measure the length of the vertical line from the pad of the left (or right) front footprint to the line drawn between the right (or left) footprints.

2.4.1.3. For the hind base width, draw a line between consecutive right (or left) hind footprints. Then, measure the length of the vertical line from the pad of the left (or right) hind footprint to the line drawn between the right (or left) footprints.

2.4.1.4. For overlap, measure the distance between pads of left (or right) front and hind footprints.

2.4.2. Average the three measurements for each individual. Use the individual average of each parameter for the statistical analysis.

2.4.2.1. For the stride length, use the average of the individual averages of the left and right strides.

2.4.2.2. For asymmetry of stride length, use the absolute value of the difference between individual averages of left limb and right limb stride length.

2.4.2.3. For the statistical analysis of the other parameters (front base width, hind base width, and overlap), use the individual average directly.

3. Restraint Stress Loading

3.1. Preparation of Restraint Tubes.

3.1.1. Make 16 holes (approximately 2 mm in diameter) in a 50 ml conical tube (30 mm in diameter x 115 mm in length) along the scale marks (5, 10, 15, 20, 25, 30, 35, 40 mL) and the backside of each scale mark by square drill (**Figure 3**). Make a hole on the tip of the 50 mL conical tube (approximately 5 mm in diameter) for breathing by cutting off the tip. Make a hole (approximately 4 mm in diameter) in the tube cap to pass the tail of mice.

3.2. Stress Loading

3.2.1. Place the mice in the experimental room.

3.2.2. Hold a mouse by grasping the scruff between the thumb and the forefinger. Enter the mouse into the restraint tube from the head. Pass the tail through the hole in the cap. Close the cap.

NOTE: Limit the forelimb movement, because mice reject entering the tube by the forelimbs.

3.2.3. Keep the mouse enclosed for 2 h on a desk at room temperature. Remove the mouse from the restraint tube and return to the home cage.

NOTE: The restraint tubes can be reused after a wash and dry.

4. Experimental Schedule (Figure 4):

4.1. Perform the hanging box test and the footprint analysis on the same day at 4 weeks of age (see step 1. Hanging box test and step 2. Footprint analysis for details) as a baseline measurement on all mice prior to the grouping into 'stress group' and a 'non-stress group'.

NOTE: About 8-10 mice in 2-3 litters may be suitable to use in an experiment. Footprint analysis at 4 weeks of age consists of training and test sessions.

4.2. Randomly divide the mice into a 'stress group' and a 'non-stress group'.

NOTE: When the mice are used consisting of several litters, divide littermates evenly into both groups. Number in each group consists of about 4-5 mice.

4.3. Apply the restraint stress to the 'stress group' 6 times over the course of two weeks (see step 3. Restraint stress loading for details).

NOTE: 6 times of restraint are applied every two weeks, followed by a hanging box test and footprint analysis from 6-12 weeks of age. Do not apply the restraint stress on the test day of the hanging box test and the footprint analysis.

4.4. Perform the hanging box test and the test session of footprint analysis on the same day at 6, 8, 10, and 12 weeks of age.

REPRESENTATIVE RESULTS:

The heterozygous male mice of *Atp1a3* (*Atp1a3*^{+/-}) that are the mouse model for rapid onset dystonia parkinsonism and wild-type littermates were used in this protocol. *Atp1a3*^{+/-} showed significantly shorter stride lengths of forelimb and hindlimb than those of the wild type at 4 weeks of age (**Figure 5A** and **Figure 5B**, open circle and square). 'Stressed' *Atp1a3*^{+/-} showed significantly shorter stride lengths of both limbs than those of 'non-stressed' *Atp1a3*^{+/-} at 8 weeks of age (**Figure 5A** and **Figure 5B**, closed and open circle). Asymmetries of stride lengths of both limbs were not significantly different in all groups of mice at all ages (**Figure 5C** and **Figure 5D**). The front base and overlaps of both limbs were also similar in all groups at all ages (**Figure 5E, G, H**). The hind base was significantly wider in the 'stressed' *Atp1a3*^{+/-} than that in the 'stressed' wild-type mice at 10 weeks of age (**Figure 5F**, closed circle and square). Thus, restraint stress caused motor deficits (short stride and wide base) of *Atp1a3*^{+/-}.

The hanging box test was performed to evaluate the grip strength and balance on the test day of footprint analysis. No significant differences in hanging time were observed in 4 to 10 weeks old mice (**Figure 6**). At 12 weeks of age, the hanging time of 'stressed' wild-type mice was significantly longer than that of the other groups (**Figure 6**, closed square). Restraint stress prolonged hanging time in wild-type mice only, but not in *Atp1a3*^{+/-}. Thus, the motor deficit of *Atp1a3*^{+/-} was distinguishable from wild-type mice by restraint stress.

Body weight is an index of general health²². We measured the body weight of mice on the test day of the hanging box test and footprint analysis, and no significant differences were observed in all groups of mice at all ages (**Figure 7**). Thus, restraint stress did not affect the general health of mice.

FIGURE AND TABLE LEGENDS:

Figure 1: Hanging box apparatus with a rotatable mesh lid. A mouse was placed in the center

of the mesh lid, and then the mesh lid was turned up side down. The fall latency of the mouse from the mesh lid is to be measured.

Figure 2: Footprint analysis. (A) Runway for footprint analysis. A foot-painted mouse (forelimbs: red ink; hindlimbs: black ink) was allowed to walk from the proximal end to the goal box. (B) Representative image of footprint and measurement of parameters. Three measurements of each parameter (forelimb and hindlimb stride lengths, widths of front and hind base, overlap between forelimb and hindlimb) were obtained from foot-printed paper.

Figure 3: Restraint stress by holed 50 mL conical tube. The restraint tube has a hole for breathing, a hole for passing the tail, and 16 holes for air circulation. Mouse was kept in the tube for 2 h at room temperature.

Figure 4: Experimental schedule of the hanging box test and the footprint analysis with the chronic restraint stress loading. Footprint analysis and the hanging box test were performed at 4 weeks of age. Then, the mice were split into 'stressed' and 'non-stressed' groups. For the 'stressed' group, the restraint stress was applied 6 times in 2 weeks until 12 weeks of age. For both groups, footprint analysis and the hanging box test were performed once per 2 weeks until 12 weeks of age.

Figure 5: Representative results of footprint analyses. Footprint analysis was performed with 4-week-old *Atp1a3*^{+/-} (a3) and wild-type (wt) mice (N = 19 and 17, respectively). Then, *Atp1a3*^{+/-} and wild-type mice were split into 'stressed' and 'non-stressed' groups. Footprint analysis was conducted for 'non-stressed' *Atp1a3*^{+/-}, 'non-stressed' wild-type, 'stressed' *Atp1a3*^{+/-}, and 'stressed' wild-type mice from 6–12 weeks of age (open circles, N = 9; open squares, N = 8; solid circles, N = 10; solid squares, N = 9, respectively). (A) Stride length of forelimb. (B) Stride length of hindlimb. (C) Asymmetry of forelimb strides length. (D) Asymmetry of hindlimb strides length. (E) Width between forelimbs. (F) Width between hindlimbs. (G) Overlap between left forelimb and hindlimb. (H) Overlap between right forelimb and hindlimb. Data are the mean ± SD. Statistical analyses (*t*-test for 4-week-old mice and pairwise *t*-test with the Holm adjustment method for 6-12-week-old mice) were performed by R²³. # *p* < .05, for 'non-stressed' *Atp1a3*^{+/-} and 'non-stressed' wild-type mice. * *p* < .05, for 'stressed' *Atp1a3*^{+/-} and 'stressed' wild-type mice. + *p* < .05, for 'non-stressed' and 'stressed' *Atp1a3*^{+/-} mice. **Figure 5A-F** has been modified from ref. 18 with permission of Elsevier.

Figure 6: Representative results of hanging box tests. The hanging box test was performed with *Atp1a3*^{+/-} (a3) and wild-type (wt) mice at 4 weeks of age. Then, *Atp1a3*^{+/-} and wild-type mice were split into 'stressed' and 'non-stressed' groups. Hanging time of 4-week-old *Atp1a3*^{+/-} (open circles, N = 19) and wild-type (open squares, N = 17) mice and hanging time of 6–12-week-old 'non-stressed' *Atp1a3*^{+/-} (open circles, N = 9), 'non-stressed' wild-type (open squares, N = 8), 'stressed' *Atp1a3*^{+/-} (solid circles, N = 10), and 'stressed' wild-type (solid squares, N = 9) mice were plotted on a logarithmic scale. Data are the mean ± SD. Statistical

analyses (t -test for 4-week-old mice and pairwise t -test with the Holm adjustment method for 6-12-week-old mice) were performed by R²³. * $p < .05$, for 'stressed' *Atp1a3*^{+/-} and 'stressed' wild-type mice. \$ $p < .05$, for 'non-stressed' and 'stressed' wild-type mice. + $p < .05$, for 'non-stressed' *Atp1a3*^{+/-} and 'stressed' wild-type mice. Data has been reprinted from ref. 18 with permission of Elsevier.

Figure 7: Representative results of growth curves. Body weights were measured of *Atp1a3*^{+/-} (a3) and wild-type (wt) mice at 4 weeks of age (open circles, N = 19; open squares, N = 17, respectively). Then, *Atp1a3*^{+/-} and wild-type mice were split into 'stressed' and 'non-stressed' groups. Body weights between 6 and 12 weeks of age of 'non-stressed' *Atp1a3*^{+/-} (open circles, N = 9), 'non-stressed' wild-type (open squares, N = 8), 'stressed' *Atp1a3*^{+/-} (solid circles, N = 10), and 'stressed' wild-type (solid squares, N = 9) mice were measured. Data are the mean \pm SD. Statistical analyses (t -test for 4-week-old mice and pairwise t -test with the Holm adjustment method for 6-12-week-old mice) were performed by R²³. Data has been reprinted from ref. 18 with permission of Elsevier.

DISCUSSION:

The footprint analysis and the hanging box test are simple and inexpensive behavioral tests for the motor function of mice. The neurobehavioral phenotypes in several mouse models have been successfully detected by these tests. For example, shortened stride length in amyotrophic lateral sclerosis²⁴, increased length of asymmetrical stride in ataxia-telangiectasia²⁵, increased length of overlap in Huntington's disease²⁶ and dystonia²⁷, and widened base in ataxia^{28,29}, Angelman syndrome³⁰ and dystonia³¹ were demonstrated by the footprint analysis. Additionally, shorter hanging time than that of the control mice was observed in mouse model of Duchenne muscular dystrophy³². Thus, both tests described in this paper would be useful for evaluating motor dysfunction of mice.

There is a critical step of this protocol to obtain the sharp footprints. It is very important to put a mouse on paper immediately after immersing the bottoms of limbs in ink to avoid drying. It is necessary to finish the immersing steps (steps 2.2.3 and 2.3.3) in a short time. A disadvantage of this protocol of footprint analysis is that the parameters obtained are limited to simple ones that do not include temporal information (e.g., duration of gait cycle and step sequence of each foot) and physical information (e.g., pressure of footprint based on touch-sensitive LED panel). When temporal and physical information is required, an automated gait analysis apparatus must be used. Alternatively, temporal information of gait pattern can be obtained by high-speed camera³³. Another disadvantage is that measuring the parameters on foot-printed paper is more laborious than automated gait analysis. For future application, the development of a program for semiautomated or automated data collection from foot-printed paper will be required to decrease the effort expended measuring parameters.

For the stress loading, total number and duration per session can be varied in this protocol. Restraint stress has been conducted with various durations and number of applications (e.g., single for 5 min³⁴, single for 24 h³⁵, 12 h x 5 sessions³⁶, and 6 h x 31 sessions³⁷, reviewed in ref.

20). To our knowledge, no systematic study concerns the effects of total number and duration per session on the motor function of mice. The activity of mice in the open field is affected by single stress loading of 1 h³⁸ or 2 h but not by that of 15 min¹⁹. Prolonged duration of stress loading may lead to severe motor deficits. For the number of stress loadings, the stance of mice becomes wider than that of non-stressed mice after 36 times of stress loading (1 h twice weekly), but not after 30 times³⁹. Chronic restraint stress (6 h per day for 31 consecutive days) aggravates rotarod performance in the mouse model of Parkinson's disease³⁷. Our results showed the hanging time of 'stressed' *Atp1a3*^{+/-} mice became shorter than that of the 'stressed' wild-type mice after 24 times of stress loading (at 12 weeks of age), but not after 18 times or less (at 4, 6, 8, and 10 weeks of age). Therefore, repeated stress loading is required for induction of certain motor deficits in mice, although the induction of motor deficits after a long period from the start of a single or several times of stress loading cannot be excluded. When mice do not show a neurobehavioral phenotype in this protocol, the recommendation is to increase the total number and duration of stress loading per session. By contrast, when the mice show gait abnormality from a single or several times of stress loading, duration and number can be decreased.

Finally, performance of walking and hanging can be affected by emotional behavioral characters (e.g., anxiety and activity) other than those of motor dysfunction. Mice with high anxiety and with hyperactivity often run (instead of walk) to the goal box. Mice showing depression-like behavior may walk with frequent stops. Therefore, an aberrant footprint pattern may not be due to motor deficits. Thus, to confirm the gait abnormality resulting only from motor deficits, performing additional tests that evaluate emotional characteristics (activity: open field test; anxiety: open field and elevated plus maze test; depression-like behavior: forced swim test) after this protocol as described previously¹⁸ is advisable.

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DISCLOSURES:

The authors have nothing to disclose.

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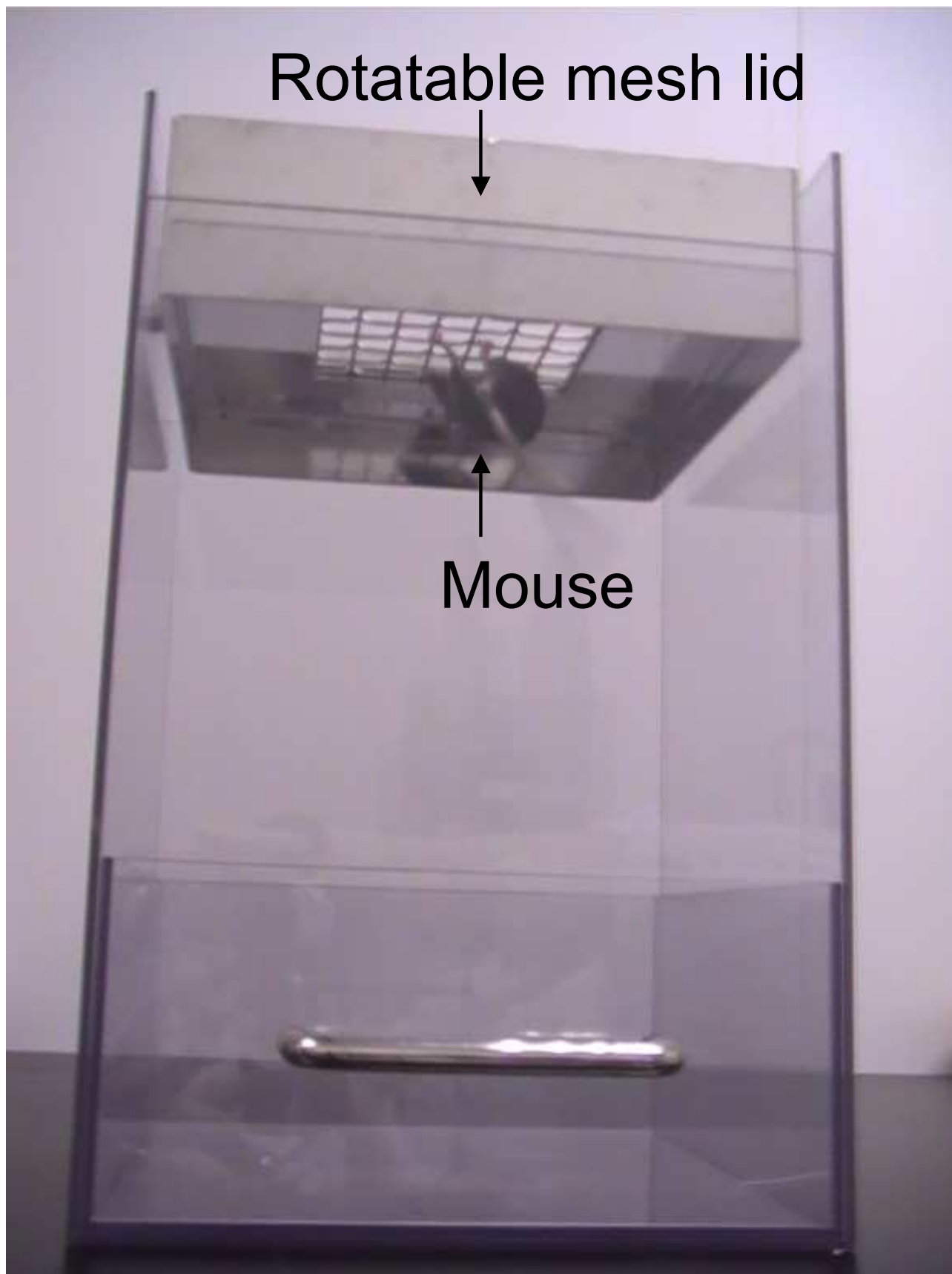


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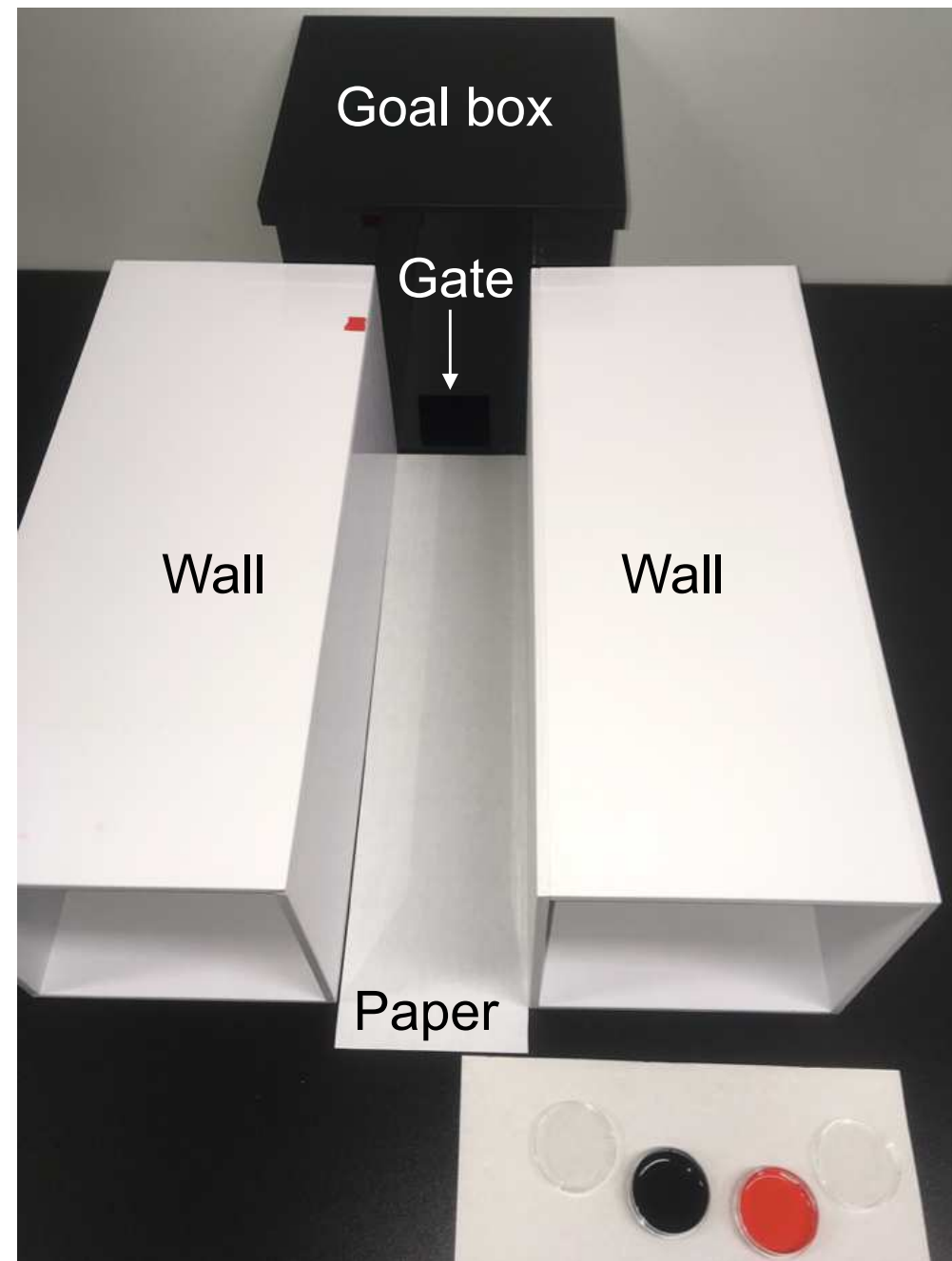
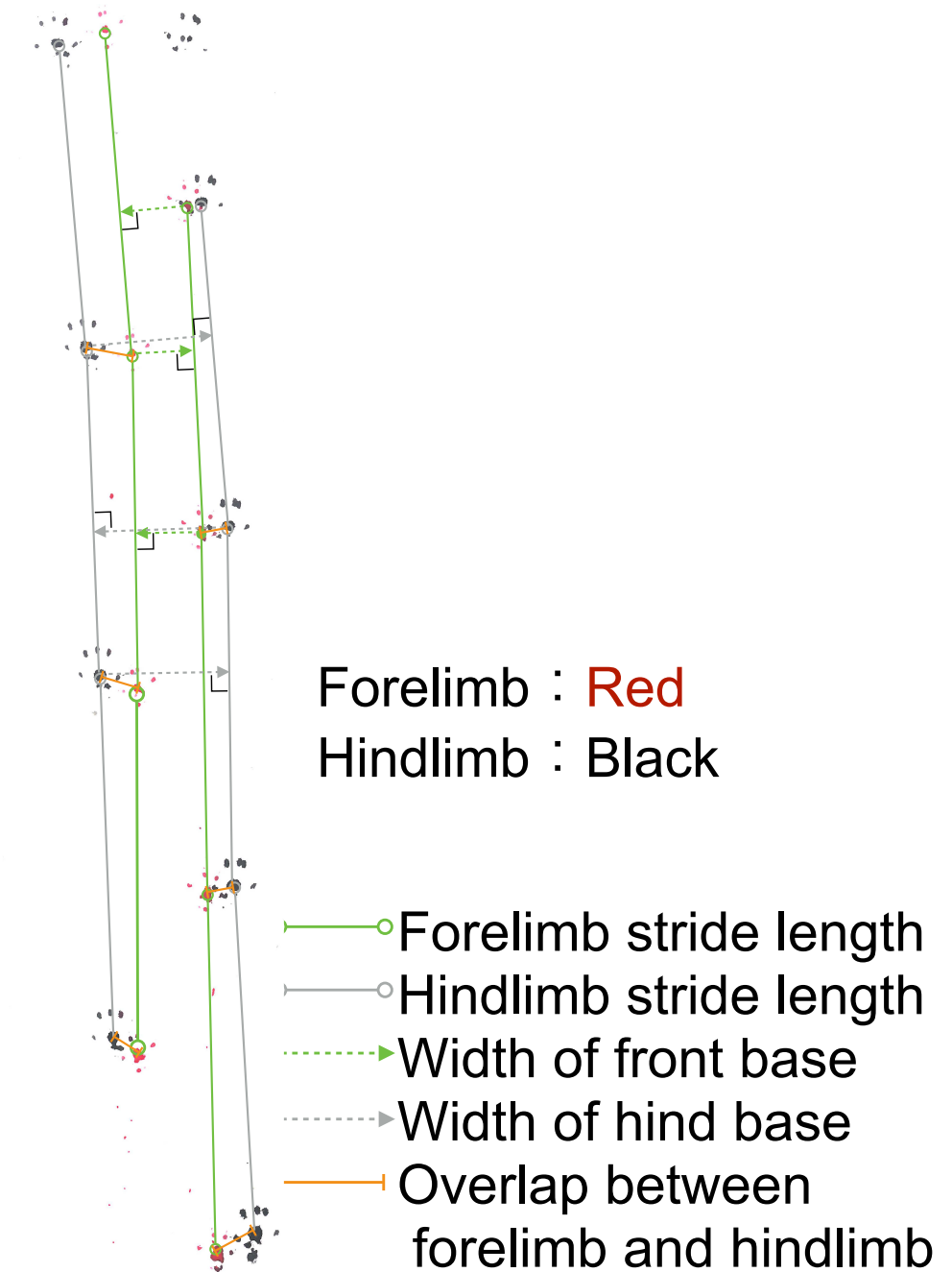
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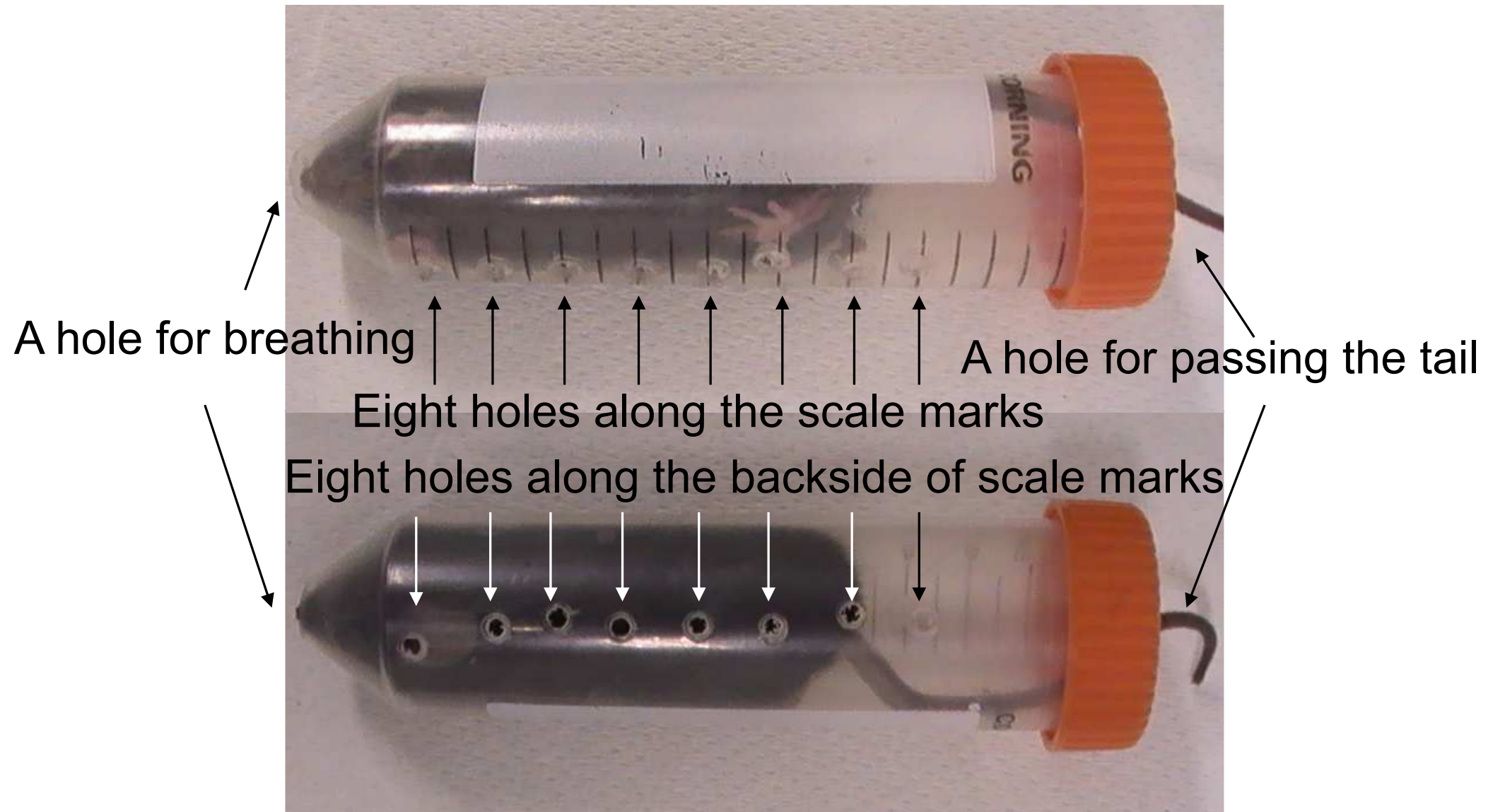
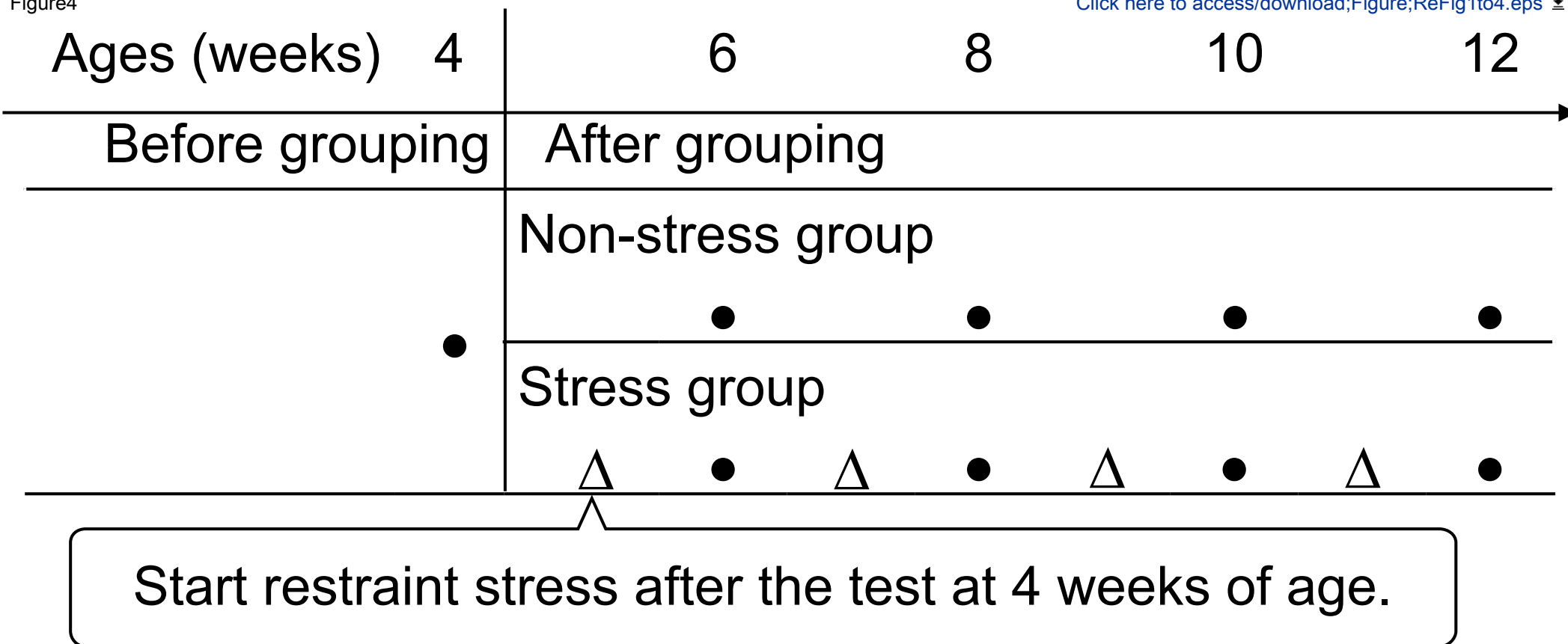


Fig 3 Sugimoto & Kawakami



- Footprint & Hanging box test at same day
- △Restraint stresses (6 times /2 weeks)

Fig 4 Sugimoto & Kawakami

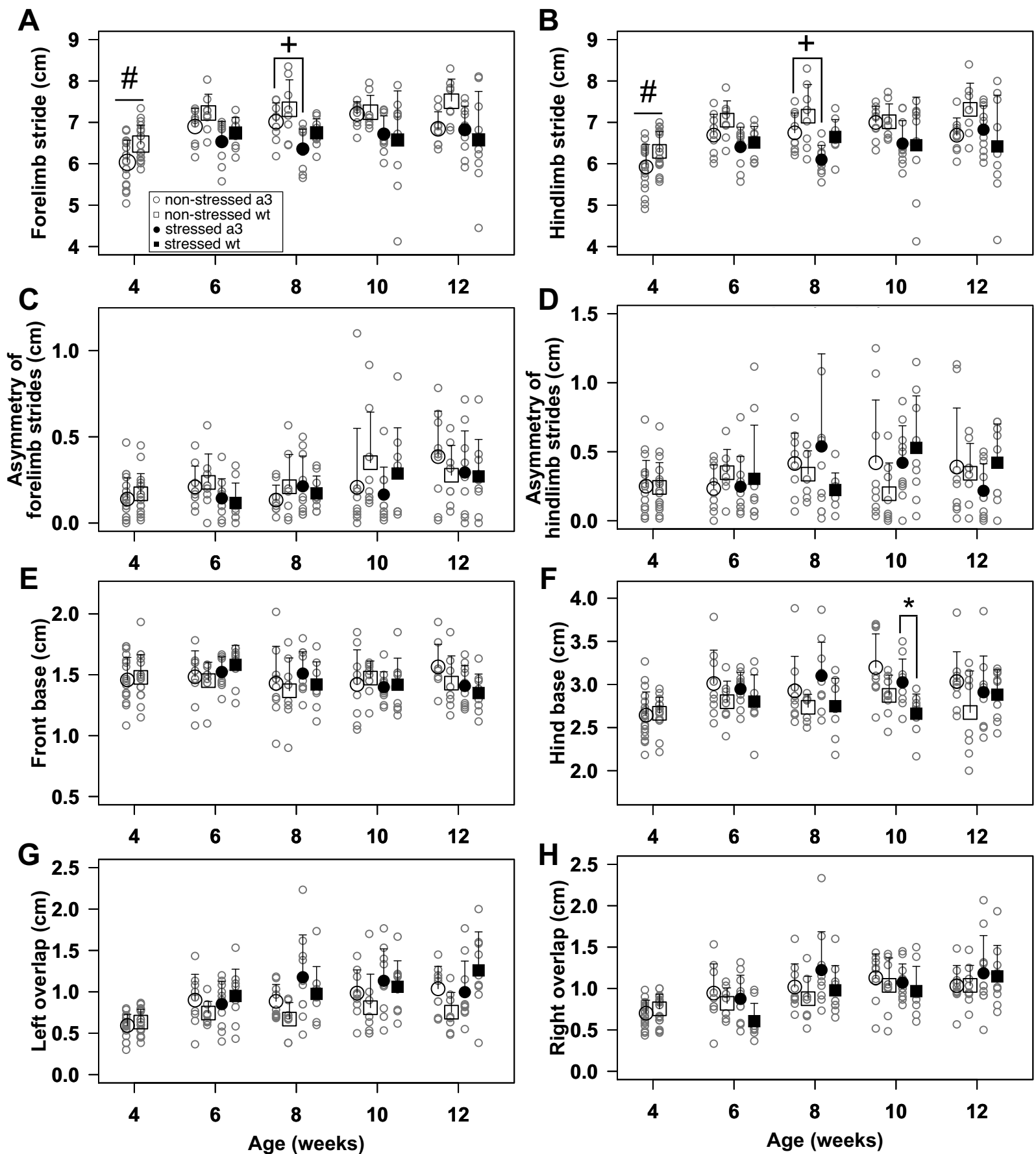


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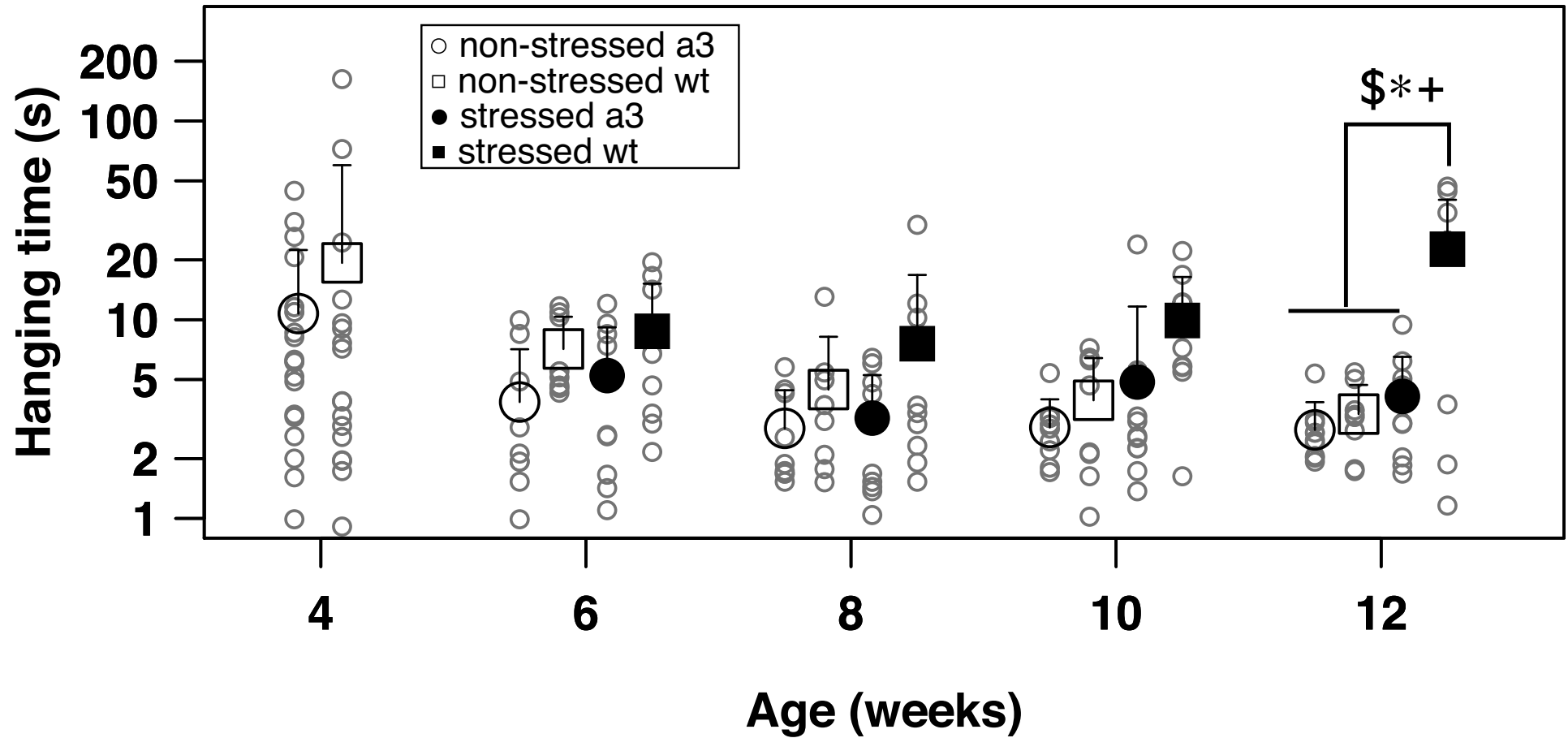


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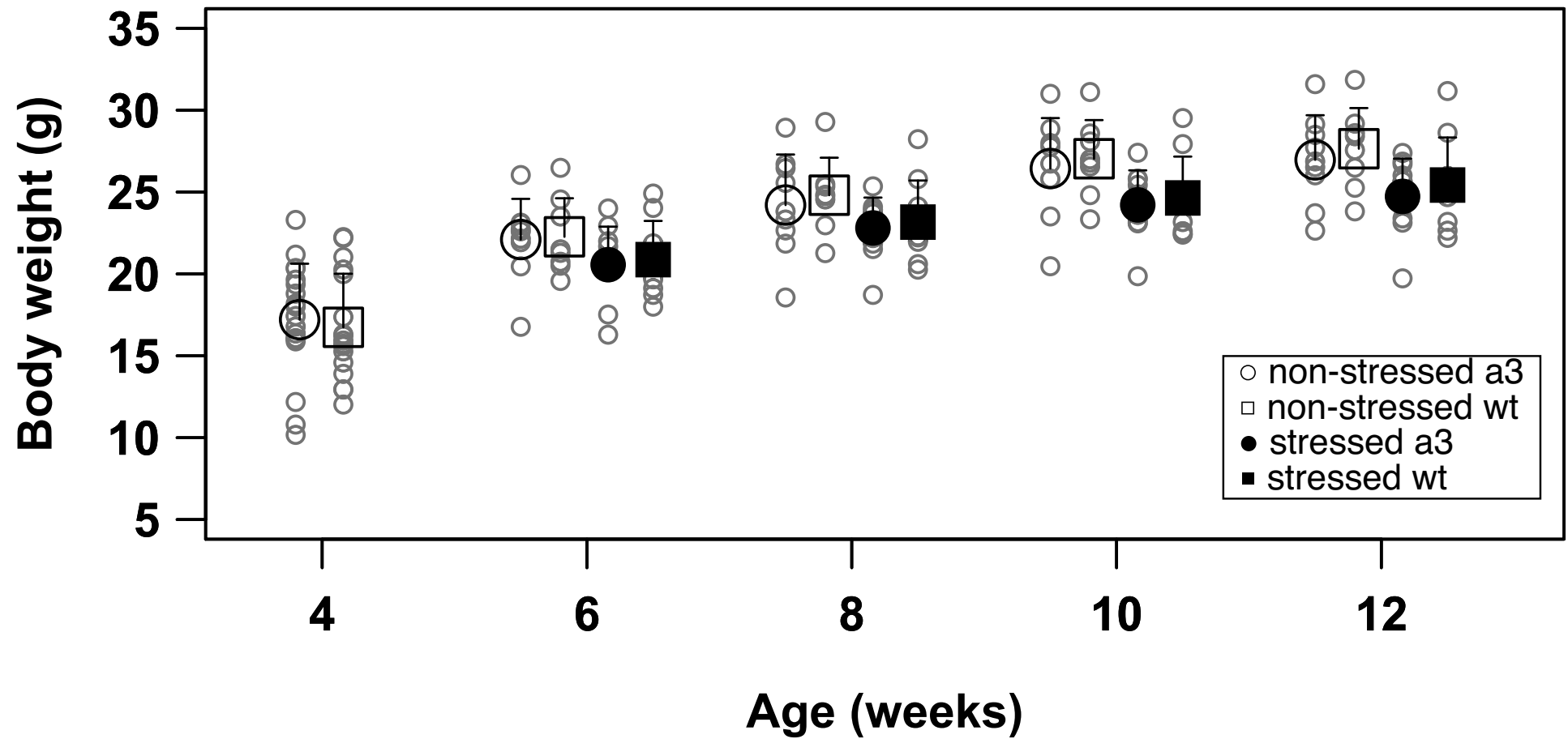


Fig 7 Sugimoto & Kawakami

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Hanging box	O' hara & Co.	http://ohara-time.co.jp/products/wire-hanging-test/	
Marking pen	ZEBRA	MO-120-MC-BK	
Goal box	O' hara & Co.	http://ohara-time.co.jp/products/balanced-beam-test/	Accessory for apparatus of balanced beam test
Boxes	O' hara & Co.	–	Side wall of runway
Black ink	Shin-asahi	–	
Red ink	Maruyamakogyo	BC-6	
Disposable Petri Dish	Corning	351008	Petri dishe (35 mm in diameter)
Askul Multipaper Super White J Monochrome A3	Askul	701-712	White paper (29.7 cm x 42 cm x 0.09mm)
50 mL Conical tube	Corning	430829	
Square drill	KAKURI Corporatio	DIY FACTORY (K32-0313)	



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Thank you for editorial comments. We have changed the manuscript based on the comments. We have answered all of the comments in the revised manuscript and have summarized our responses to the comments below.

We hope that the paper is now acceptable for publication in Journal of Visualized Experiment.

Sincerely,

Hiroki Sugimoto

Point-by-point response to Editorial and reviewer's comments (Our response in blue)

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: We have checked the issues by self-check and proofing tool of Word.

We changed "nonstressed" to "non-stressed" in the text and Figure 5-7,

"nonstress" to "non-stress" in the text and Figure 4,

"vaseline" to "Vaseline" in line 43,

"more steady" to "steadier" in line 144,

and "stride" to "strides" in line 292 and 293, and Figure 5C and D.

2. The highlighted protocol steps are over the 2.75 page limit (including headings and spacing). Please highlight fewer steps for filming.

Response: We have highlighted fewer steps as suggested.

3. Please do not highlight notes for filming.

Response: We have removed the highlight of notes as suggested.

4. We suggest move Step 1 to the end of the Protocol for clarity.

Response: We have moved Step 1 to the end of the Protocol as suggested. Then, we have changed numbering of step and Figure.

5. Figure 2: Please provide a short description in addition to the figure title in Figure Legend.

Response: We have added a short description as suggested, and we have renumbered Figure 2 to Figure 1 according to the comment 4.

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Response: We have added a short description as suggested, and we have renumbered Figure 4 to Figure 3 according to the comment 4. In addition, we have added a short description in Figure 3, and we have renumbered Figure 3 to Figure 2 according to the comment 4.



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