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## Assessing functional performance in the mdx mouse model

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<b>Abstract:</b>	Duchenne muscular dystrophy (DMD) is a severe and progressive muscle wasting disorder for which no cure is available. Nevertheless, several potential pharmaceutical compounds and gene therapy approaches have progressed into clinical trials. With improvement in muscle function being the most important end-point in these trials, a lot of emphasis has been placed on setting up reliable, reproducible and easy to perform functional tests to pre-clinically assess muscle function, strength, condition and coordination in the mdx mouse model for DMD. Both invasive and non-invasive tests are available. Tests that do not exacerbate the disease can be used to determine the natural history of the disease and the effects of therapeutic interventions (e.g. forelimb grip strength test, two different hanging tests using either a wire or a grid and rotarod running). Alternatively, forced treadmill running can be used to enhance disease progression and/or assess protective effects of therapeutic interventions on disease pathology. We here describe how to perform these most commonly used functional tests in a reliable and reproducible manner. Using these protocols based on standard operating procedures enables comparison of data between different laboratories.
<b>Author Comments:</b>	As requested, we have also uploaded the manuscript with all adjustments made with Track changes in word.
<b>Additional Information:</b>	
<b>Question</b>	<b>Response</b>

Leiden, August 22, 2013

To: Elizabeth Sheeley

Subject: Resubmission of Manuscript

Dear Elizabeth Sheeley,

We would hereby like to resubmit our manuscript “Assessing functional performance in the *mdx* mouse” by van Putten et al. The comments of the reviewers were useful. We have addressed all editorial and reviewer comments, which can be found in the Editorial comments document.

Sincerely yours,

Maaïke van Putten

**TITLE:**

Assessing functional performance in the *mdx* mouse model

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**KEYWORDS:**

Duchenne muscular dystrophy, neuromuscular disorders, outcome measures, functional testing, mouse model, grip strength, hanging test wire, hanging test grid, rotarod running, treadmill running, standard operating procedure

**SHORT ABSTRACT:**

The primary outcome measure in clinical trials for neuromuscular disorders is generally improved muscle function. Therefore, assessing the effect of potential therapeutic compounds on muscle performance pre-clinically in mouse models is of great importance. We here describe several functional tests to address this.

**LONG ABSTRACT:**

Duchenne muscular dystrophy (DMD) is a severe and progressive muscle wasting disorder for which no cure is available. Nevertheless, several potential pharmaceutical compounds and gene therapy approaches have progressed into clinical trials. With improvement in muscle function being the most important end-point in these trials, a lot of emphasis has been placed on setting up reliable, reproducible and easy to perform functional tests to pre-clinically assess muscle function, strength, condition and coordination in the *mdx* mouse model for DMD. Both invasive and non-invasive tests are available. Tests that do not exacerbate the disease can be used to determine the natural history of the disease and the effects of therapeutic interventions (e.g. forelimb grip strength test, two different hanging tests using either a wire or a grid and rotarod

running). Alternatively, forced treadmill running can be used to enhance disease progression and/or assess protective effects of therapeutic interventions on disease pathology. We here describe how to perform these most commonly used functional tests in a reliable and reproducible manner. Using these protocols based on standard operating procedures enables comparison of data between different laboratories.

## INTRODUCTION:

Duchenne muscular dystrophy (DMD) is the most common neuromuscular disorder affecting 1:5000 newborn boys. This severe and progressive muscle wasting disease is caused by mutations in the *DMD* gene that disrupt the open reading frame and prevent the synthesis of functional dystrophin protein. Muscle fibers lacking dystrophin are vulnerable to exercise-induced damage. Upon exhaustion of the muscle's regenerative capacity, and due to chronic inflammation of damaged muscle, fibers are replaced by connective tissue and fat, subsequently leading to a loss of function. Generally, DMD patients lose ambulation of the lower limbs early in the second decade. Later, also the muscles of the arms and shoulder girdle are affected and patients often develop thoracolumbar scoliosis due to asymmetric weakening of the muscles supporting the spinal cord. Assisted ventilation is generally required in the late teens or early twenties. Respiratory and heart failure lead to death in the third or fourth decade <sup>1</sup>.

Although the causative gene has been discovered over 25 years ago <sup>2</sup>, there is no cure available for DMD. However, improved health care and the use of corticosteroids have increased life expectancy in the Western world <sup>3</sup>. With the use of animal models like the *mdx* mouse, major steps forward into the discovery of potential therapeutic strategies have been made. The *mdx* mouse is the most commonly used DMD mouse model. It has a point mutation in exon 23 of the murine *Dmd* gene and consequently lacks dystrophin <sup>4</sup>. Over the last couple of years, many proposed strategies have progressed into clinical trials <sup>5-9</sup>. In these trials, improvement of muscle function is the primary endpoint, underlying the importance of testing the benefit of compounds on muscle function in mice during the pre-clinical stage of testing.

Like DMD patients, also the dystrophin negative muscle fibers of *mdx* mice are vulnerable to exercise-induced damage and their muscle function is impaired compared to C57BL/10ScSnJ wild type mice. This impairment can be assessed with a variety of functional tests. Some of these tests are non-invasive and do not interfere with muscle pathology (e.g. forelimb grip strength, hanging tests and rotarod running). Therefore they can be used to monitor the natural history of the disease or determine the effects of compounds on disease progression. To get an in-depth picture of the influence of compounds on muscle function in *mdx* mice, a functional test regime that does not interfere with disease progression consisting of all of these test can be used <sup>10</sup>.

Alternatively, forced treadmill running can be used to intentionally exacerbate disease progression and test the protective capacities of compounds <sup>11</sup>. The treadmill can also be used as outcome measure in which running time till exhaustion is measured <sup>12</sup>, or as a tool to fatigue *mdx* mice so that they perform less well in a subsequent functional test ensuring larger differences in performance between treatment groups <sup>13</sup>. When choosing functional tests, their effect on disease progression should be kept in mind especially when testing dystrophic mice like the *mdx* mouse <sup>14</sup>.

We here describe in detail how to perform the most commonly used functional tests in a reliable and reproducible manner based on available standard operating procedures from the TREAT-NMD network <http://www.treat-nmd.eu/resources/research-resources/dmd-sops/>.

## PROTOCOL:

The experiments described here were approved by the Animal Ethics Committee (DEC) of the Leiden University Medical Center (LUMC). Mice were bred by the animal facility of the LUMC and kept in individually ventilated cages with 12-h light dark cycles. They had *ad libitum* access to water and standard chow.

When performing any of the functional tests described below, experimental conditions have to be strictly controlled to reduce variation. Preferably, age and gender matched mice should be used, as performance differs between age and genders. Mice belonging to the same litter should be randomized over the experimental groups. Animals should be tested by the same operator, who is blinded to the experimental groups. Tests should be performed on the same time of day and weekday, same room to equalize odors, noises etc <sup>14</sup>. Large variation between individual mice and time points can be observed for all functional tests, therefore 6-8 mice per experimental group should be used. Functional test performance can also largely differ between different inbred wild type strains. Therefore, experimental and control wild type mice should always have corresponding backgrounds (in case of *mdx* mice use the C57BL/10ScSnJ wild type strain). All data described here have been obtained with the C57BL/10ScSnJ wild type strain, which we refer to as wild type from here on. The tests described here can be used longitudinally from at least 1 to 19 months of age in *mdx* and wild type mice. Tests should not be repeated more than once weekly to prevent mice from losing interest and willingness to perform the task.

### 1. Forelimb grip strength test

Use the forelimb grip strength test to measure the strength of the forelimbs. The test is based on the tendency of a mouse to instinctively grasp a grid when suspended by the tail <sup>15</sup>, and adapted from [http://www.treat-nmd.eu/downloads/file/sops/dmd/MDX/dmd\\_m.2.2\\_001.pdf](http://www.treat-nmd.eu/downloads/file/sops/dmd/MDX/dmd_m.2.2_001.pdf) ).

1.1.) Apparatus set-up: Attach a grid to a force transducer, which measures the maximum force applied by the mouse on the grid during the pull. Make sure the setting is on Peak tension node (T-PK) for pulling. The units of force can be adjusted in either ounces-of-force, grams-of-force, pounds-of-force, kilograms-of-force or Newtons.

**Note:** We prefer to work with grams as unit of values. Multiple meters are commercially available, but only axial transducers give reliable outcomes as lever-type force transducers are negatively influenced by the physical laws of the lever effect. Either a non-flexible grid or triangle can be used with bars that are 1-2 mm in diameter.

1.2) Prior to the test, assess the body weight of the mouse, to allow normalization for body weight.

1.3) Use grams as unit of values. Reset the meter at the start of each recording.

1.4) Remove the mouse from its cage by grabbing the tail and moving it horizontally towards the grid.

1.5) Check that the mouse grasps the grid tightly with both forepaws.

1.6) Pull the mouse away from the grid so that its grasp is broken; the highest force applied to the grid will be shown on the transducer's display, which can be either manually or automatically recorded.

1.7) Only take pulls into account in which the mouse shows resistance to the experimenter. Reject measures in which only one forepaw, or the hindlimbs were used and in which the mouse turned during the pull.

1.8) Let the mouse pull the bar three times in a row and then return it in the cage for a resting period of at least one minute. **Note:** Between series of pulls a resting period is necessary for the mouse to recover and avoid habit formation.

1.9) Then let the mouse perform four series of pulls, each followed by a short resting period. In this way the mouse has pulled a total of 15 times (3 pulls x 5 times = 15 pulls).

1.10) Determine the maximum grip strength and normalize for body weight by taking the average of the three highest values out of the 15 values collected.

1.11) Optional: Determine fatigue by calculating the decrement between the average of the first two and the last two series of pull  $1+2+3=A$ ,  $4+5+6=B$ ,  $10+11+12=C$  and  $13+14+15=D$ . The formula:  $(C+D)/(A+B)$  gives a value of 1 for mice which are not fatigued. This can be expressed in percentages so that a mouse without fatigue has a value of 0% and a mouse which forelimbs are completely fatigued has a value of 100%.

## 2. Hanging tests

With hanging tests, balance, coordination and muscle condition can be assessed. These tests are based on the knowledge that mice are eager to remain hanging on a wire or grid till exhaustion<sup>16</sup>. There are two distinctive hanging tests in which at the start of the test either only the two forelimbs or all four limbs are used, using a wire or grid respectively. The hanging test using the wire and the grid are the longest suspension time method adapted from [http://www.treat-nmd.eu/downloads/file/sops/dmd/MDX/DMD\\_M.2.1.004.pdf](http://www.treat-nmd.eu/downloads/file/sops/dmd/MDX/DMD_M.2.1.004.pdf) and [http://www.treat-nmd.eu/downloads/file/sops/dmd/MDX/DMD\\_M.2.1.005.pdf](http://www.treat-nmd.eu/downloads/file/sops/dmd/MDX/DMD_M.2.1.005.pdf) respectively. A fixed hanging limit is used of 600 seconds. The majority of wild type mice can hang for 600 seconds, while dystrophic mice cannot. To reduce time spend performing this test, a maximum hanging time was set in place. Mice that fall off the wire or grid before then are given up to two more tries. This is done to reinsure that mice are really unable to hang and do not fall due to clumsiness.

### 2.1) Hanging test with two limbs

2.1.1) Apparatus set-up: Tightly secure a 2-millimeter thick metal cloth hanger to a shelf with tape and maintain the hanger around 35 centimeters above a layer of bedding. **Note:**

Alternatively, a 55 centimeter wide 2-mm thick metallic wire which is tightly secured between 2 vertical stands could be used. The distance of 35 centimeters is sufficient to encourage mice to remain hanging, but also low enough to prevent mice from injuries when falling down. The wire should not vibrate or displace during the test as this could interfere with the performance of the mouse.

2.1.2) Handle the mouse via the tail and bring it near the wire.

2.1.3) Let the mouse grasp the wire with the two forepaws only, and lower the hindlimbs in such a way that the mouse only hangs with the two forepaws on the wire (Figure 2C).

2.1.4) Directly start the timer when the mouse is released. After release, strong mice try to catch the wire with all the four limbs and the tail, which is allowed (Figure 2D).

2.1.5) When a mouse shows improper behavior (like balancing on or deliberately jumping off the wire as shown in figure 2E and F), directly address this by replacing the mouse on the wire without stopping the timer.

2.1.6) When a mouse falls off the wire, stop the timer and record the hanging time.

2.1.7) When mice are able to hang for 600 seconds, take them off the wire and return them to the cage. Mice that fall before this limit are given a maximum of two more tries.

2.1.8) Record the maximum hanging time (i.e. the longest of the trials) and use this for further analysis.

## 2.2) Hanging test with four limbs

2.2.1) Apparatus set-up: Use either a hand-made square or the lid of a big cage for a rat or rabbit for this test. Position the grid 35 centimeters above soft bedding to prevent mice from harming themselves upon falling, but also to discourage mice to intentionally jump off the grid. Tightly secure the grid so that the experimenter does not have to manually hold the grid during the experiment as these movements might interfere with the mouse's performance.

2.2.1) Place the mouse on a grid so that it grasps it with its four paws.

2.2.2) Invert the grid so that the mouse is hanging and directly start the timer.

2.2.3) The test session ends for mice that are able to hang for a duration of 600 seconds. Give mice that fall off the grid earlier a maximum of two more tries.

2.2.4) Use the maximum hanging time (i.e. the longest of the trials) for further analysis.

## 3. Rotarod running

With the rotarod test muscle strength, coordination, balance and condition can be determined <sup>17</sup>.

3.1.) Apparatus set-up: For this test, mice have to run on a rotating tube. Ensure that the steady speed is set at 5 rotations per minute (rpm), and that the speed increases from 5 to 45 rpm in the first minute when started. After this it has to maintain its speed.

3.2) Place the mice on the tube of the rotarod when it rotates at a slow steady speed of 5 rpm. Five mice can be tested simultaneously.

3.3) Start the run once all mice are positioned. Within the first minute the speed of the tube accelerates from 5 to 45 rpm after which it maintains that speed.

3.4) Monitor the run. The running time is continuously recorded by the software. Running time stops automatically when a mouse falls off the tube as this activates the time bar positioned below the tube.

3.5) End the test session for mice that are able to run for a duration of 500 seconds. Give mice a maximum of two more tries allowing them to improve their running time, when they fall earlier.

3.6) Use the maximum running time (i.e. the longest off the trials) for further analysis.

#### **4. Treadmill exercise**

The treadmill can be used in three ways as a tool in pre-clinical research. Firstly, forced treadmill running can be used to exacerbate disease pathology as described in this protocol (see also: [http://www.treat-nmd.eu/downloads/file/sops/dmd/MDX/DMD\\_M.2.1.001.pdf](http://www.treat-nmd.eu/downloads/file/sops/dmd/MDX/DMD_M.2.1.001.pdf)). Secondly, the maximal running capability of mice and the effects of treatments on this can be assessed (See for the method to let mice run till exhaustion [http://www.treat-nmd.eu/downloads/file/sops/dmd/MDX/DMD\\_M.2.1.003.pdf](http://www.treat-nmd.eu/downloads/file/sops/dmd/MDX/DMD_M.2.1.003.pdf)). Finally, treadmill running can be used prior to another functional test to exhaust the mouse so that it performs less well in the second test<sup>13</sup>. This is done by exercising mice twice or three times weekly as described below, directly followed by either one of the functional tests described in protocol 1-3.

4.1) Apparatus set-up: There are several treadmills commercially available on which several mice can run simultaneously and for which elevation, duration and speed can be adjusted. Some treadmills are equipped with a grid to deliver low-intensity shocks to encourage mice to run. However, *mdx* mice are sensitive to stress and can easily be motivated in a friendlier manner by a gentle push with the hand in the running direction. Therefore, it is strongly encouraged to NOT use the shock grid. Generally, stimulation with the hand is only needed during the first running session.

4.2) Place the mice on the horizontal treadmill.

4.3) Start the treadmill at a running speed of 12 meter per minute. Lower speeds (8 m/min) have to be used in old mice (>15 months), where higher speeds easily lead to exhaustion.

4.4) During the first session, encourage mice to run by gently pushing them when they are near the end of the belt.



4.5) When the mice have run for a duration of 30 minutes, place them back into their cage.

4.6) Repeat this twice weekly for e.g. 12 weeks.

4.7) Allow resting periods when needed. For example, some *mdx* mice have to stop running and should be allowed to rest for a few minutes. If this happens, turn the belt off, give all mice a resting period of two minutes, turn the belt on for two minutes at 4m/min. After this, increase speed to 12m/min and allow the mice to finish the protocol. It is important that all mice complete the entire running protocol.

**Note:** In case *mdx* mice need resting periods, consider a warm-up before the 30 minutes exercise protocol. This warm-up session consists of: a two minute acclimatization period at a speed of 4m/min, immediately followed by an eight minute warm-up at 8m/min.

In our hands 4 to 16 week old female *mdx* mice are able to complete the 30 minutes exercise protocol without resting. Others have reported that in age-matched male *mdx* mice 45% of the mice do need resting periods to finish the exercise. The warm-up protocol reduces the amount of stops<sup>12</sup>

## REPRESENTATIVE RESULTS:

The forelimb grip strength of wild type and *mdx* mice increases between the age of 4 and 12 weeks and reduces again in older mice. Impairments in force can already be observed in young *mdx* mice. Representative data of 9 week old female mice are shown in figure 1A and B. Although fatigue does not differ between the strains yet at this age, *mdx* mice are weaker than wild type mice. We do not have data yet on fatigability in older *mdx* and wild type mice.

To obtain reliable and reproducible results, multiple assessments need to be done by the same experimenter. We here describe to pull 15 times per individual, however smaller numbers of pulls (as low as 5 pulls) also provide reliable data ([http://www.treat-nmd.eu/downloads/file/sops/dmd/MDX/dmd\\_m.2.2\\_001.pdf](http://www.treat-nmd.eu/downloads/file/sops/dmd/MDX/dmd_m.2.2_001.pdf)). Careful attention should be drawn to the positioning of the paws on the grid as this can largely influence outcomes. During the pull only both forepaws should be used and they have to be placed nicely next to each other (Figure 1C). When the mouse is not showing resistance to the pull, the value should not be taken into account.

For the two limb and four limb hanging tests, especially young (4-16 weeks old) wild type mice can easily reach the maximum hanging time of 600 seconds. Contrastingly, performance of young *mdx* mice is impaired (they hardly ever achieve maximum hanging time) and also deteriorates with age, even though both strains put all effort in performing these hang tests at their best abilities (Figure 2A and 3A). Larger differences in hanging times between *mdx* and wild type mice are obtained with the wire. Therefore, even small effect sizes of compounds on muscle function can be detected using this test. Hanging performance, (or any other type of performance) differs within and between individuals over time resulting in high standard deviation bars. Nonetheless, *mdx* mice consistently perform worse than age-matched wild type mice (Figure 2A). Performing multiple assessments can provide more detailed insight in functional improvements upon treatment than only endpoint measurements. It should be kept in mind that in the first session animals learn how to perform a functional test. This learning curve,

which is present in all tests, is clearly visible between 4 and 6 weeks of age. However, because mice also grow rapidly in this age period, a distinction between improvement due to learning and/or growth cannot be made. Gender differences in hanging performance for the two limb hang test have also been found. Performance of female *mdx* mice exceeds that of males by ~100 seconds, and performance of treadmill challenged female *mdx* mice is almost comparable to that of the unchallenged males (compare Figure 2A with 4A). This finding underlines the importance of using age and gender matched mice to avoid bias. We have preliminary data suggesting that differences in performance in both hanging tests between *mdx* and wild type mice increases in very old (18 months) mice.

Some mice display inappropriate behavior to avoid hanging on the wire like; balancing on the wire, jumping off the wire deliberately etc. (Figure 2D-E), although the majority of mice comply with the test and hang with either two or four limbs (Figure 2B-C). Occasionally, strong mice jump off the wire intentionally. They hang prior to jumping with only the two hindlimbs and the tail on the wire and look down to estimate the distance to the ground. Inappropriate behavior that is occasionally seen on the grid during the four limb hanging test consists of deliberately jumping off the grid or climbing on the grid. All inappropriate forms of behavior can be easily distinguished and should not be allowed. Mice that avoid hanging in one of these ways should be directly placed back on the wire or grid without stopping the timer.

On the rotarod, *mdx* mice hardly ever run for the maximum running time of 500 seconds, while a larger proportion of wild type mice do (Figure 3B). With age, running performance of both strains decreases. Some mice are able to clamp tightly on to the rotating tube and avoid running by ‘cartwheeling’ around. This cannot be corrected for and is a severe limitation of the test when multiple mice start doing this for prolonged periods, thereby increasing variation within the experimental groups. Especially for some mice which partly run and partly cartwheel, and during the transition from cartwheeling into running fall.

Forced treadmill running is an easy and effective exercise to exacerbate disease pathology in non-treated *mdx* mice, while wild type mice undergoing the same protocol are not affected. Generally, mice become familiar with the treadmill after an initial training session and are willing to run, especially when multiple mice are running simultaneously. Old *mdx* mice (over 15 months of age) have difficulties in running and cannot cope with the same running speed of 12m/min for 30 minutes used for young mice. Therefore a slower running speed of 8m/min for 30 minutes is recommended enabling all mice to finish the entire protocol. *Mdx* mice are especially vulnerable to eccentric contractions, therefore downhill running can only be used for a short duration.

Alternatively, other functional tests like the two limb hanging wire test can be performed directly after running (Figure 4A). Using this study design, differences between strains or treatment arms are likely to increase as treadmill challenged untreated *mdx* mice are less capable of performing these tests than sedentary *mdx* mice<sup>13</sup>.

As mentioned earlier, when studying muscle function in *mdx* mice, the C57BL/10ScSnJ wild type strain needs to be used which is of the corresponding genetic background. We advise this as even between inbred wild type strains treadmill running performance differs<sup>18, 19</sup>. But also in

non-invasive functional tests, functional performance is influenced by genetic backgrounds. Figure 4 illustrates this in three representative graphs where performance of *mdx* mice on a BL/10 background and on a mixed background consisting of BL/10, BL/6J, DBA2 and 129OLA are compared. As can be appreciated the mixed background mice perform better in the hanging wire tests and worse on the rotarod.

### Figure Legends:

**Figure 1: Forelimb grip strength, representative results and correct positioning of the paws.** **A.** Forelimb grip strength normalized for bodyweight of 9 weeks old female *mdx* (n=5) and wild type (n=4) mice. Grip strength is already impaired in young *mdx* mice. Asterisks indicate  $P<0.05$  and data are presented as mean $\pm$ st.dev. **B.** Fatigue of the same individuals as shown in A, was on average less than 10% and did not vary between strains. **C.** To obtain reliable data, attention should be to the positioning of the paws during forelimb grip strength analysis. Correct positioning of the mouse; two forepaws are next to each other, hindlimbs are not touching the grid and the mouse is pulling in a straight line. **D.** Incorrect positioning of the forepaws; the mouse is not pulling in a straight line. When this happens, or when only one forepaw or also the hindlimbs are used, the mouse turns around during pulling or lacks to show resistance, data should be discarded.

**Figure 2: Two limb hanging tests, representative results and appropriate and inappropriate hanging behavior.** **A.** A representative example of the two limb hanging test performed once weekly in *mdx* (n=18 4-10 weeks, n=13 11&12 weeks, n=10 13 weeks) and age and gender-matched wild type mice (n=6). A learning curve is visible for both strains in the first few weeks of testing. Performance of *mdx* mice was worse compared to that of wild type mice. Data presented as mean $\pm$ st.dev. Maximum hanging time allowed is indicated by the dotted line. **B.** The correct starting position of this test is with the two forepaws. **C.** Depending on the functional ability of the mouse it can also use the hindlimbs and tail. **D and E.** A small subset of mice, especially strong wild type mice, can occasionally avoid hanging by climbing on the side bars or balancing on the wire. Some mice intentionally jump off the wire.

**Figure 3: Four limb hanging and rotarod running test.** **A.** Four limb hanging performance assessed once weekly in male *mdx* (n=18 4-10 weeks, n=13 11&12 weeks, n=8 13 weeks) and wild type (n=6) mice. Over time, *mdx* mice hang less long than wild type mice. **B.** Rotarod running times did not differ between young male *mdx* (n=18 4-10 weeks, n=13 11&12 weeks, n=10 13 weeks) and wild type mice (n=6).

**Figure 4: The effect of forced treadmill running exercise protocol on functional performance and skeletal muscle pathology in 16 week old female mice.** Muscle pathology was deliberately exacerbated by letting mice run on a horizontal treadmill three times a week at 12m/min for 30 minutes for a duration of 12 weeks. Directly after running, mice had to participate in the two limb hanging test. While all wild type mice (n=5) remain hanging till the maximum allowed, all *mdx* mice (n=6) fall off the wire earlier ( $P<0.001$ , data presented as mean $\pm$ st.dev.). **B.** The presence of membrane damage was determined by assessing serum Creatine Kinase (CK) levels that leak out of muscle fibers through tears in the membrane. CK levels were elevated in *mdx* mice compared to wild type mice before exercise. Treadmill exercise

immediately increased levels ( $P < 0.01$  indicated by asterisk, data presented as mean $\pm$ st.dev.) in *mdx* mice, while they remained low in wild type mice. **C-D.** Muscles of *mdx* mice are very vulnerable to treadmill exercise, worsening disease pathology extensively after a few weeks of running. These Haematoxylin and Eosin stainings of the quadriceps of a 16 week old non-exercised (C) and treadmill exercised (D) *mdx* mouse show that extensive fibrosis and necrosis are developed. **E.** Muscles of wild type mice undergoing the same running protocol are not affected.

**Figure 5: Effect of a mixed background on functional performance in *mdx* mice.** Differences in genetic background influence functional performance. To illustrate this, performance of male *mdx* (BL/10 background, n=18 4-10 weeks, n=13 11&12 weeks, n=10 13 weeks) and *mdx* (mixed BL/10, BL/6J, DBA2 and 129OLA background, n=5) mice was compared over time. **A.** Two limb hanging test performance significantly differed between the two strains. **B.** Four limb hanging test results were slightly higher in the mixed background *mdx* mice. **C.** Rotarod running times also slightly differed between the strains. Data presented as mean $\pm$ st.dev.

## DISCUSSION:

The described functional tests presented here are reproducible, easy to perform and applicable to wild type and dystrophic mice independent of their age. The tests provide useful tools to pre-clinically assess muscle function, strength, condition and coordination. When testing the effects of a compound on the natural history of the disease, the non-invasive tests described here (forelimb grip strength, both hanging tests and the rotarod test) can be nicely combined in a functional test regime where these tests are performed on consecutive days. These protocols are not detrimental to *mdx* mice and can be used in a longitudinal manner<sup>10</sup>. It should be kept in mind that outcomes of each of these tests are generated by different or partly overlapping muscle groups instead of an individual muscle. Therefore, using a combination of multiple tests is recommended to obtain a more complete picture and thereby better insight in the functionality of the experimental groups. Alternatively, functional improvements of a sole muscle can be assessed using muscle physiology measurements<sup>20</sup>.

Like behavioural test, also functional tests can show extensive variation between different mice, or within a mouse between different assessments. To reduce variation, all tests should be performed by the same experimenter who is familiar with the mice. External variables like smells and sounds in the room, time of the day and the day of the week on which the test is performed should be kept as constant as possible. Mice should be gender and age matched. When using treadmill running to exacerbate disease progression, it is essential to use a standardized protocol in which all running parameters (running time, speed and slope) are kept constant over time for all experimental groups, so that all mice are equally treated. Although the majority of mice are keen to participate in the functional tests and most animals show high levels of willingness, some mice (primarily strong wild type mice) occasionally avoid performing the test and show avoidance behavior. When this behaviour is not corrected for, false conclusions could be drawn<sup>21</sup>. Fortunately, these types of behaviour are only observed occasionally and can be corrected for by placing the mouse back on the wire, grid or rotarod, or pulling another time on the grip strength meter.

Improvements in one functional test (e.g. hanging test assessing muscle function) does not necessarily have to co-occur with improvements in another test (e.g. forelimb grip strength assessing sole muscle strength). In *mdx* mice, improvements in muscle function can be distinguished earlier than in muscle strength. This is also seen in DMD patients participating in clinical trials where clinically meaningful improvements in the 6-minute walk test do not co-occur with improvements in muscle strength<sup>6, 7</sup>. However, this may in part depend on the working mechanism of the compound tested and it is possible that other compounds improve strength and not function. Therefore the results of the tests should be interpreted with the mechanism of action of the compound in mind.

## ACKNOWLEDGMENTS:

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## DISCLOSURES:

The authors have nothing to disclose.

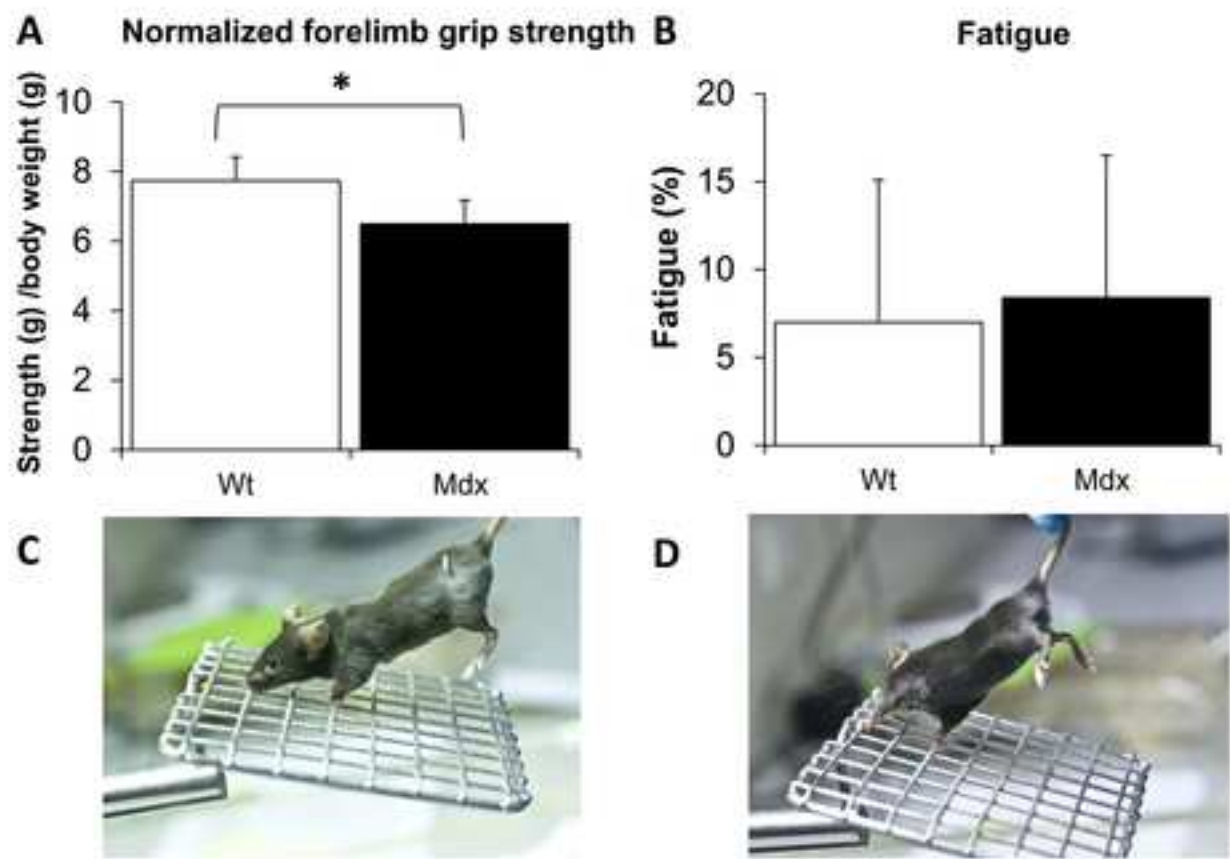
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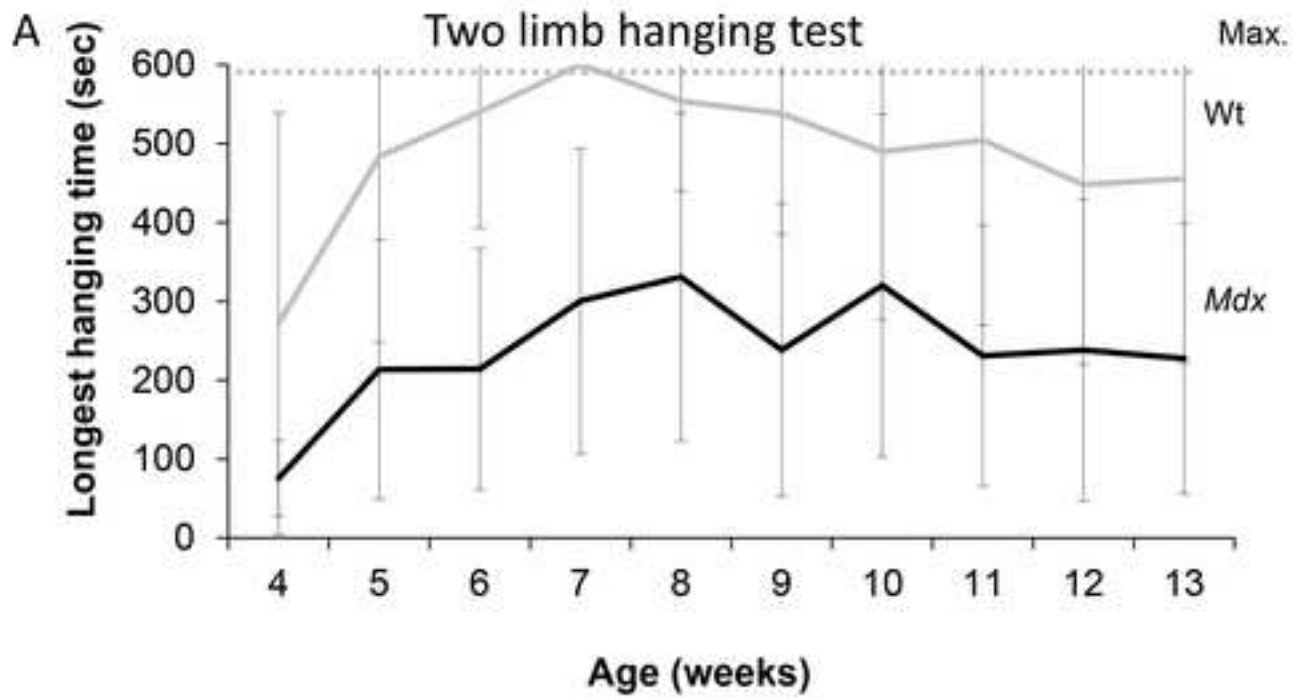
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Figure 1





**Figure 2**



**B**



**C**



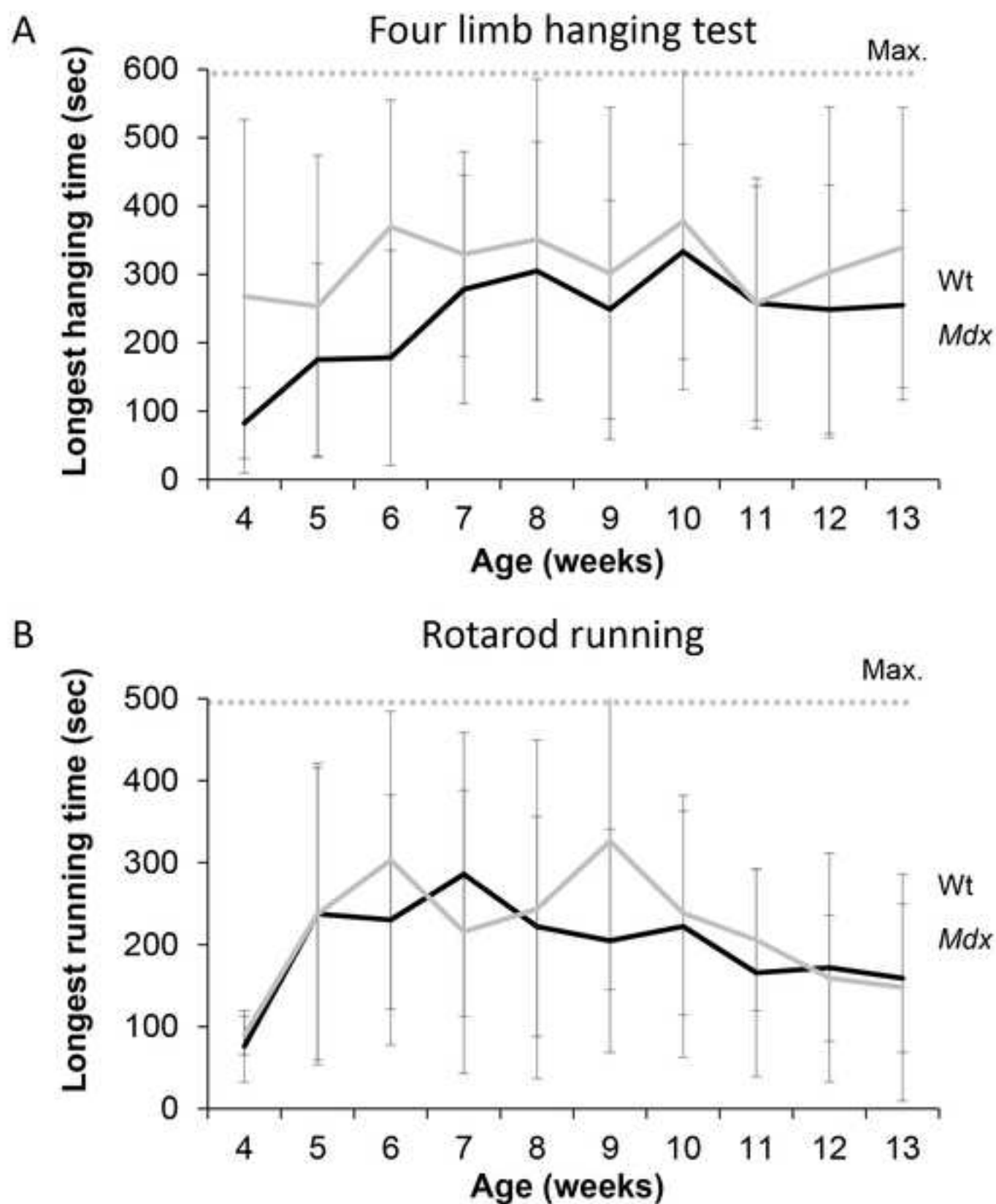
**D**



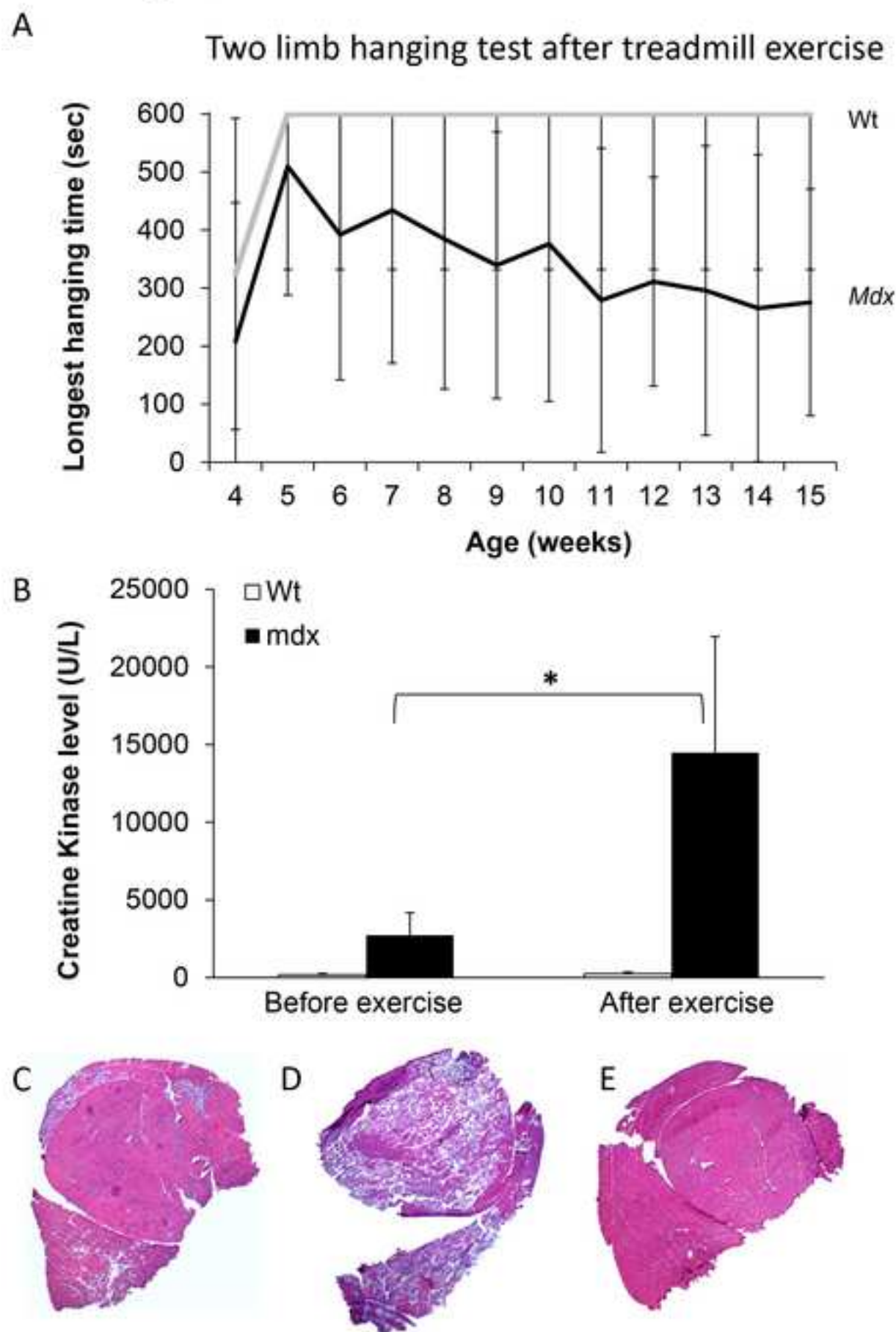
**E**



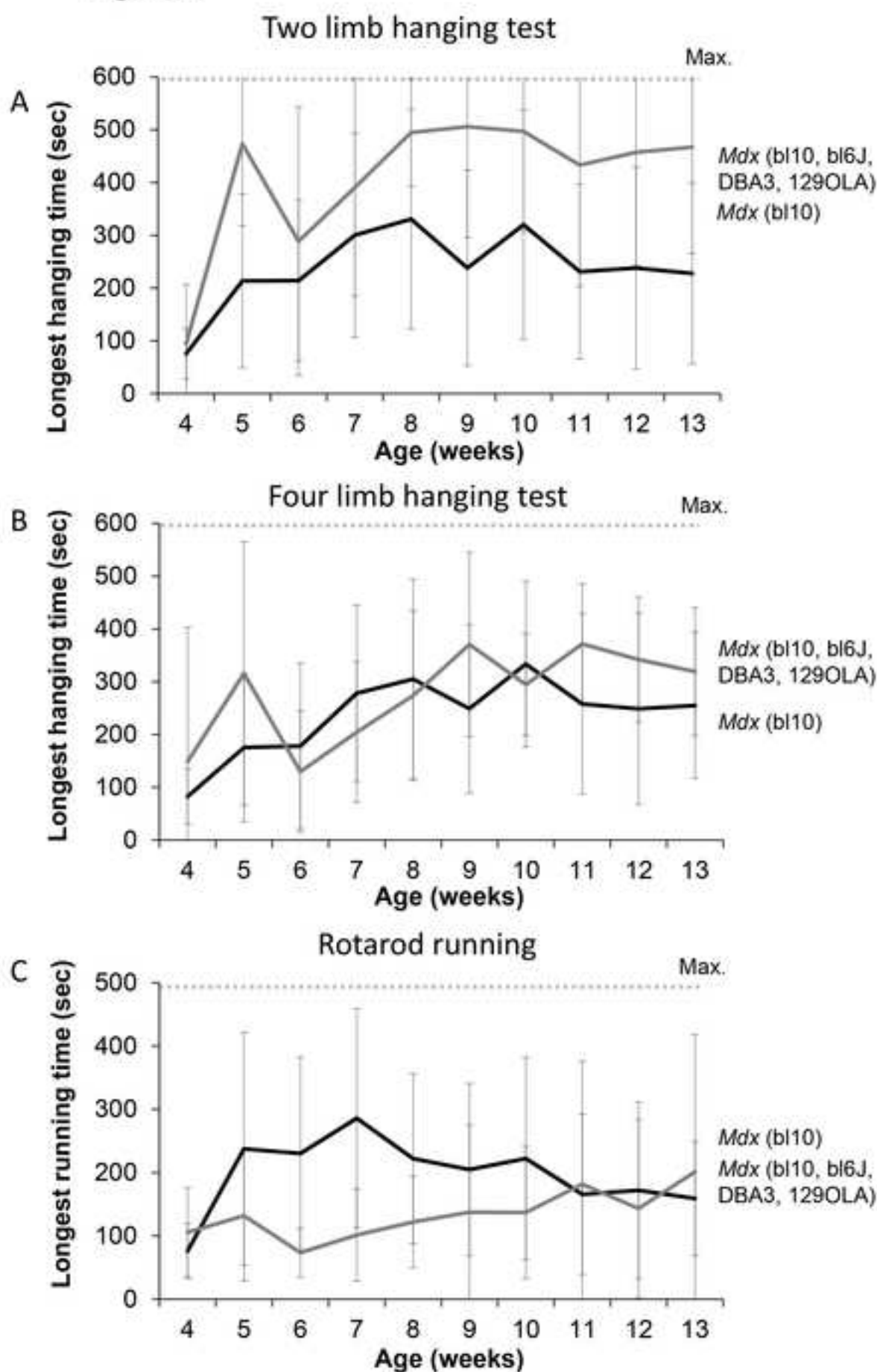
**Figure 3**



**Figure 4**



**Figure 5**



\*Excel Spreadsheet- Table of Materials/Equipment  
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Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
Mouse grip strength meter	Chatillon DFE (re-sold by Columbus Instruments)	# 80529	
Hanging wire 2 limbs device	Cloth hanger or custom made device		
Hanging wire 4 limbs device	Lid of rat cage or custom made device		
Rotarod	Ugo Basil	# 47600	
Treadmill for mice Exer 3/6	Columbus Instruments	# 1055SRM	



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MS # (internal use):



### **Editorial comments:**

\*The reference Chapillon et al., 1998 (under step 3) is missing in the reference list.

*We have now added this reference to the reference list.*

### **Reviewers' comments:**

#### **Reviewer #1:**

##### *Manuscript Summary:*

It is excellent to have such formal comparisons of functional tests.

What is really useful to the field is the provision of the reference data for each of the procedures described, to show the expected values for mdx and control mice. It is especially useful where data are compared over a period of a few weeks/months as shown in Fig 2B. This also indicates the degree of variation to be expected. It would be ideal if time course data were provided for ALL of the tests. Such information is not readily available.

*We thank the reviewer for the suggestion and have now incorporated longitudinal data of the functional tests whenever available (Figure 3, 4 and 5).*

##### *Major Concerns:*

Since many of these techniques for mdx mice have been described carefully as Standard Operating Procedures on the TreatNMD website (<http://www.treat-nmd.eu/research/preclinical/dmd-sops/>), it would seem essential to make some reference to these established/approved protocols. These include Treadmill running (DMD\_M.2.1.003), 2 versions of the hanging wire test (DMD\_M.2.1.004 and DMD\_M.2.1.005) and Grip Strength (DMD\_M.2.2.001). The descriptions of these are very comprehensive, with data also being presented in some cases. Comment should be made regarding WHY these additional descriptions are actually required for these well-described protocols? It would also be appropriate to specifically comment on differences or improvements for the protocols described here, compared with the widely available and well-used protocols mentioned above,

*We thank the reviewer for this suggestion. In addition to providing the link to the TREAT-NMD SOP website, we have now also added cross references for each protocol with the available TREAT-NMD SOPs throughout the manuscript. The Journal of Visualized Experiments provides an ideal platform where videos of the described tests will be provided. Especially for these types of assessments, these movies will be a very useful addition to the TREAT-NMD SOPs. JoVE requires an accompanying paper for the videos, which is the current manuscript. Only very minor differences exist between the grip strength protocol provided here and the TREAT-NMD SOPs, and for clarification we have now outlined these in the corresponding representative results section. For both*



*hanging tests, we perform experiments exactly as described for the longest suspension version in the SOPs, while the treadmill protocol is also as described in the SOP.*

*Minor Concerns:*

Treadmill exercise. The mdx mice are very prone to stress and therefore the electric shock stimulus to make them run should NOT be used. This point should be clarified in the text.

Some labs have reported that mdx mice need a warm up time BEFORE they are able to cope with a speed of 12m/min (e.g Radley-Crabb H et al, 2012, Neuromusc. Disorders. Feb;22(2):170-82). This helps to reduce the amount of stopping time and thus reduces the variability between mdx mice for the formal exercise bout. Was this an issue in the present study? Perhaps the need for this additional step could be discussed

*We agree with the reviewer that the shock stimulus should not be used and have now rephrased the sentence to clarify this.*

*Our lab has experience with exercising young (<17 weeks of age) and old (>15 months) female mdx mice. In our hands all young females are able to finish the exercise protocol (30 min at 12m/min) without resting. Old female mdx mice are able to run without resting for 30 min at 8m/min. We have no experience with male mdx mice. The discrepancy between our study and that of Radley et al. and De Luca et al. might be gender related. These labs have tested male mice and both encountered mice that needed rests. We have now incorporated this and the suggested additional warm-up step in the protocol and discussion section of the manuscript.*

**Reviewer #2:**

*Minor Concerns:*

I suggest rewriting of some paragraphs that sound confused probably because of several rephrasings occurred during the first draft. I suggest a more clear separation of the two ways of using treadmill for mdx mice: as regular, forced exercise to worsen pathology, and as outcome measure to estimate muscle function.

*We have implemented all the reviewer's suggestions, except where outlined below.*

Lines 78-91: I think it is not clear which tests you consider "disease affecting" and therefore not appropriate for measurements, and which not. I think this paragraph needs to be more structured. Line 82: please distinguish between treadmill as exercise and treadmill as outcome. The use of treadmill as exercise should be described in a separate sentence to avoid confusion.

Line 152: four pulls, if one series of three was already done and a total of five series is planned

Line 153: This sentence is confusing here. It was already clear that the mouse pulls three times in a row, then rests for one minute, then pulls three times again until five series of three pulls each are performed. One can re-summarize here that in this way the mouse has pulled a total of 15 times, but I would not say again "repeat the measurements".

Line 198, 215 and 238: It is not clear to me why these mice are given two more tries, and if the maximum hanging time is the longest of the three trials or the sum of them.

*Mice that were unable to reach the maximum time limit of the test were given up to two more tries to ensure that mice really cannot hang any longer and to rule out that they fell off due to clumsiness. We have now added this for clarification.*

Line 221: Quotations are in numbers in the text

Line 243-247: The protocol described here is the one to exacerbate pathology (exercise protocol). The abstract says that behavioral outcome measures will be described in this article. Therefore, I think it would be appropriate to report here also the deviations for the exhaustion protocol. Alternatively, report from the beginning the rationale not to use treadmill as outcome and to use treadmill only as forced exercise to worsen pathology.

Line 246: With "alternatively", it sounds like there is a third way to use the method.

*This is indeed a third method as in this method the treadmill is used as a tool to exhaust mdx mice so that they perform less well in the functional test that is performed directly after treadmill running. This allows for a better separation between treatment arms. We have now rephrased this for clarification.*

Line 255-256: I think that for the exercise protocol the settings should be standardized, including slope.

Line 269: Maybe it would be useful to have an idea of how many breaks an mdx mouse could need per run (is it normally one or five breaks?). Is the timer stopped during the breaks, to ensure that all mice did run 30 min?

*In our hands, female mdx mice aged <17 weeks old running at 12m/min do run without stopping. So we cannot provide that input. Others have reported that age-matched male mdx mice do need rests. We have incorporated these references in the manuscript.*

Line 277-278: Is there an age where fatigue differs between mdx and wt? It may be useful to know at which age the assessment of fatigue as outcome measure makes sense. *This is an interesting question, to which we have as yet no answer.*

Line 283: only C shows the forepaws in the right position.

Lines 301-311: "two" refers to wire and grid tests. But then only wrong behaviors on wire are described. Only later and after a conclusion sentence (All these inappropriate...), the grid test is mentioned for the jumping issue. I think this paragraph needs a clearer structure. Line 301: tests. Line 304: I would remove "these". The sentence refers only to the described inappropriate behavior (2E-F) and not to the appropriate ones (2C-D). Also, I would place this sentence after having listed all inappropriate behaviors (including deliberately falling and climbing up the grid) and conclude with the sentence about placing mice back without stopping the time: at present, this sentence is repeated twice.

Line 310: placed

Line 324: difficulties in

Line 332: This sentence refers to the hanging tests and should be incorporated two paragraphs above

Line 334: neither figure 2A nor 3A show data on female mice for this comparison. You probably meant 4A.

Line 352: the picture shows an average value that is below 10%. I suggest "was on average less than 10%"

Line 361: weeks

Line 367: What is the minimum number of mdx and wt mice required to be able to show significant differences despite the natural variability? These numbers would be useful for each of the tests described.

Line 374: Is there an age at which differences are significant? Does this figure mean that the four limb hanging test and the rotarod test can be used only at younger ages? Or is it not significant at all ages? In this case, I think a comment is needed.

*We have experience with the functional tests described using mice ranging from 1 to 19 months of age. The difference in 4 limb hanging performance is already apparent in young mice – we have preliminary data suggesting that the difference increases in very old (18 months old) mice. We have now added this. Rotarod does not differ, even at an older age. This might be due to the carting observed in both mdx and wild type mice. We have now made a comment regarding this in the discussion section.*

Line 378: forced treadmill running. It is important to keep the distinction of the two treadmill protocols throughout the paper (the exercise protocol and treadmill as muscle function measurement). If treadmill is used as outcome measure, it should not influence pathology. I suggest to use the words "exercise protocol", or similar, to make the distinction

Line 391: A comparison with H&E in sedentary mdx would help estimating the exercise damage next to CK values

Line 394: influence

Line 407: again, here is the discrepancy about the treadmill tests, that was listed as outcome measure in the abstract and is no more listed in the discussion because only the exercise protocol was described.

Line 408: these protocols

Line 422: adapt slope accordingly to corrections eventually done in line 255-256.

Line 425: I wonder if "devotion" is appropriate here?

Line 427: "correct" appears three times in three lines. I suggest here "biased (or false) conclusions could be drawn" - without observations.

Lines 493-494: this citation is reported twice

Last page: hanging