

Comprehensive Analyses of Muscle Function, Lean and Muscle Mass, and Myofiber Typing in Mice

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Abstract

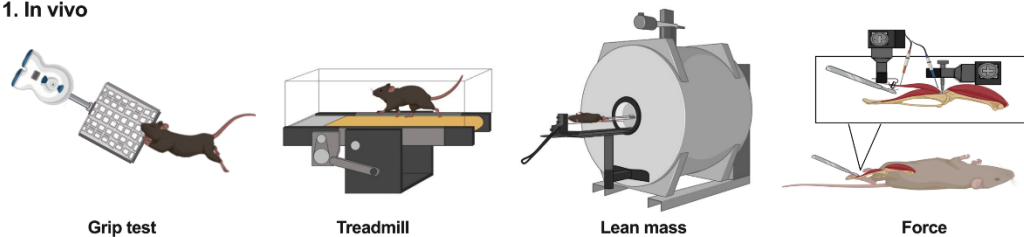
Skeletal muscle disorders commonly affect the function and integrity of muscles. Novel interventions bring new potential to rescue or alleviate the symptoms associated with these disorders. In vivo and in vitro testing in mouse models allows quantitative evaluation of the degree of muscle dysfunction, and therefore, the level of potential rescue/restoration by the target intervention. Several resources and methods are available to assess muscle function and lean and muscle mass, as well as myofiber typing as separate concepts; however, a technical resource unifying these methods is missing. Here, we provide detailed procedures for analyzing muscle function, lean and muscle mass, and myofiber typing in a comprehensive technical resource paper.

Keywords: Skeletal muscle, Myofiber type, Lean and muscle mass, Muscle cross sectional area, Muscle force, Muscle function

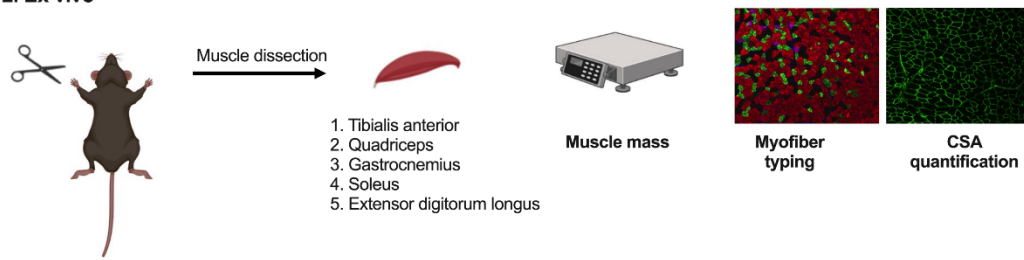
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Graphical abstract

1. In vivo



2. Ex vivo



Background

Skeletal muscle homeostasis and dysfunction depend on the adaptive changes in myofiber size, myofiber type, and force capacity. Equally important for both disease characterization and therapeutic evaluation are sensitive and precise methods for evaluating the parameters of muscle physiology in comprehensive assessments. This set of tools will enable scientists to assess not only the magnitude of the effect of a target intervention but also compare effects across different interventions.

Multiple invasive and noninvasive methods have been developed to assess muscle strength in animal models, especially rodents (Takeshita et al., 2017). Invasive methods include in situ and in vitro measurement of muscle force, whereas noninvasive methods include in vivo assessment of strength and global performance. In this protocol, we present the methods for conducting both in situ invasive measurements of muscle force and in vivo non-invasive measurements of strength and aerobic exercise tolerance in response to a pro-ergogenic glucocorticoid treatment (Quattrocchi et al., 2022).

Skeletal muscle is a metabolically active tissue made up of different types of muscle fibers, generally classified as type1, type 2A, type 2X, and type 2B according to the myosin isoform that is enriched in each of them (Talbot and Maves, 2016). Changes in muscle fiber typing are often correlated with changes in muscle strength, contractility, and/or endurance. Moreover, modifications in skeletal muscle fiber composition may be related to muscle myopathies such as muscular dystrophies, inherited myopathies preferentially affecting a specific myofiber type, and sarcopenia. Therefore, myofiber-type specification is important to provide insights for understanding the susceptibility and resistance of certain fiber types to muscle disease and to discover potential treatments based on muscle plasticity and metabolic shift. Histochemical staining for myosin ATPase or succinate dehydrogenase enzyme activity or quantification of metabolic enzymes are among the methods used to identify different myofiber types, but these cannot generally differentiate between myofibers subtypes (Reichmann and Pette, 1984). Therefore, here we report the immunofluorescence-based protocol that is routinely used in our lab for the differential staining of myosin heavy chain isoforms, which efficiently resolves the heterogeneity of muscle fibers.

Apart from muscle function and myofiber typing, lean mass (Kalyani et al., 2014) and muscle mass (Lovering et al., 2005) are two important determinants associated with muscle disorders or muscle changes downstream of global conditions, such as aging or type-2 diabetes. Weight curves offer an immediate proxy estimate of overall growth and mass levels but evidently cannot provide information on fat vs. lean mass or actual muscle mass. Therefore, magnetic resonance imaging (MRI)-based systems to estimate lean and fat mass can provide a better estimation of

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body composition effects of interventions, such as circadian time-specific dosing of glucocorticoids (Quattrocchi et al., 2022). Using MRI-based systems, it is possible to simultaneously quantify changes in adiposity vs. lean mass (absolute or relative) over time.

Overall, we provide a comprehensive method for the analysis of muscle function, performance, lean and muscle mass, and muscle fiber typing. These tests can be easily customized to evaluate overall muscle function, performance, and composition (especially in terms of muscle fiber types) in different situations, to study muscle physiopathology, or to assess the efficacy of therapeutic interventions.

Materials and Reagents

1. Microscope slides (Corning, catalog number: CLS294775X25)
2. Microscope slide cover glass (Globe Scientific, catalog number: 1404-10)
3. Phosphate buffered saline (PBS) (Sigma-Aldrich, catalog number: 806552)
4. Triton X-100 (Sigma-Aldrich, catalog number: T8787)
5. 10% normal goat serum (Cell Signaling, catalog number: 5425)
6. Ethanol, 200 proof (Thermo Scientific, catalog number: T038181000)
7. Isoflurane (Covetrus, catalog number: 11695-6777-2)
8. Ringer's solution (Fisher Scientific, catalog number: S25512)
9. Isopentane (Alfa Aesar, catalog number: 19387, CAS number: 78-78-4)
10. Tissue-Tek OCT compound (Sakura Finetek, catalog number: 4583)
11. PAP pen (Biotium, catalog number: 22006)
12. ProLong™ Gold Antifade Mountant with DAPI (Invitrogen, catalog number: P36931)
13. Primary antibodies (Table 1)

Table 1. Primary antibodies for muscle fiber typing

Antibody name	Company and catalog number	Dilution
Rabbit anti(α)-Laminin	Sigma, L9393	1:500
Myosin heavy chain, type IIA (SC-71)	Developmental Studies Hybridoma Bank, SC-71	1:30
Myosin heavy chain, type IIB (BF-F3)	Developmental Studies Hybridoma Bank, 10F5	1:10
Myosin heavy chain (slow) (BA-F8)	Developmental Studies Hybridoma Bank, A4.840	1:10

14. Fluorescently tagged secondary antibodies (Table 2)

Table 2. Secondary antibodies for muscle fiber typing

Antibody Name	Company and Cat No.	Dilution
Goat anti-rabbit IgG (H+L) Alexa Fluor™ 488	Invitrogen, A-11034	1:1,000
Goat anti-mouse IgG2b Alexa Fluor™ 647	Invitrogen, A-21242	1:1,000
Goat anti-mouse IgG1, Alexa Fluor™ 488	Invitrogen, A21121	1:1,000
Goat anti-mouse IgM, Alexa Fluor™ 594	Invitrogen, A-21044	1:1,000

15. Goat serum (Invitrogen, catalog number: 31872)
16. Dimethyl sulfoxide (DMSO) (Fisher Scientific, catalog number: D1319)
17. Prednisone powder (Sigma-Aldrich, catalog number: P6254)

18. 1 mL insulin syringe with needle (BD, catalog number: 329424)
19. Permeabilization reagent: 1% (v/v) Triton X-100 in PBS
20. Blocking buffer: 10% (v/v) goat serum in PBS
21. Prednisone stock (5 mg/mL): 5 mg prednisone powder in 1 mL DMSO
22. Physiological solution: sterile 0.9% saline solution (Cytiva Z1379)
23. Vehicle stock: 1 mL DMSO.

Equipment

A. Grip and treadmill assays

1. Grip strength meter with single sensor for mice (max 1 kg capacity) with standard pull bars (Chatillon Instruments, Digital Force Gauge, catalog number: DFIS-2)
2. Open treadmill with stimulus assembly, ~10% uphill incline, individual mouse lanes, drive motor to adjust speed (3–100 m/min range), and exercising belt with grip-prone texture (Columbus Instruments, catalog number: Exer3/6)

B. Force-frequency analysis in tibialis anterior muscles

1. Surgical scissors (Fine Science Tools, catalog number: 14060-10)
2. Mouse handling forceps (Fine Science Tools, catalog number: 11036-20)
3. Weighing scale (Ohaus, catalog number: 80000022)
4. In-situ apparatus setup from 3-in-1 whole animal system for mice (Aurora Scientific, catalog number: 1300A)
5. Ohaus Pioneer precision balance (Fisher Scientific, catalog number: 01-922-178)
6. Digital caliper (Thermo Fisher, catalog number: 14-648-17)
7. Isoflurane vaporizer (SurgiVet Classic T3, catalog number VCT302) and induction chamber (Patterson Scientific, catalog number: 07-8917760)
8. Surgical suture (Ethicon, catalog number: K871)

C. Myofiber typing

1. Leica cryostat microtome (Leica Biosystems, catalog number: CM1510S)
2. Humidified container (Thomas Scientific, catalog number: 1219D68)
3. Nikon Eclipse Ti inverted microscope (Nikon Instruments, catalog number: Ti-E)

D. MRI scans to determine lean mass ratios

1. EchoMRI whole-body composition analyzer (EchoMRI, catalog number: 100H)
2. Sample tubes dedicated to mice comprised between 20 and 40 g of body mass (EchoMRI, Houston, TX)
3. Standard internal calibrator tube (canola oil) (EchoMRI, Houston, TX)

Software

1. Built-in software EchoMRI version 140320. Data were analyzed when hydration ratio was >85%
2. ImageJ (Fiji, <https://imagej.nih.gov/ij/>)
3. ASI 611A Dynamic Muscle Analysis v5.300
4. ASI 610A Dynamic Muscle Control v5.500

Procedure

The protocols and example data presented here are from wildtype (WT) male mice from the C57BL/6 background (JAX strain #000664). Number of animals used per cohort should be decided based on an appropriate power analysis tailored to the scope of the study. See notes at the end for considerations regarding age and sex of test mice.

A. Quantitation of grip strength and treadmill performance

1. Grip strength
 - a. Record the body weight of each mouse prior to the grip assay.
 - b. To record the force for a mouse, hold its tail, let the mouse grip to the bars of the grip strength meter with its forelimbs, and then pull the animal away from the metal grid. Ensure to pull with a constant, gentle movement, keeping the mouse parallel to the work surface (**Figure 1**).
 - c. Repeat the grip force measurements three times per mouse. Let the mouse rest for a minute between the pulls and record the highest number.
 - d. Normalize grip strength to body weight.

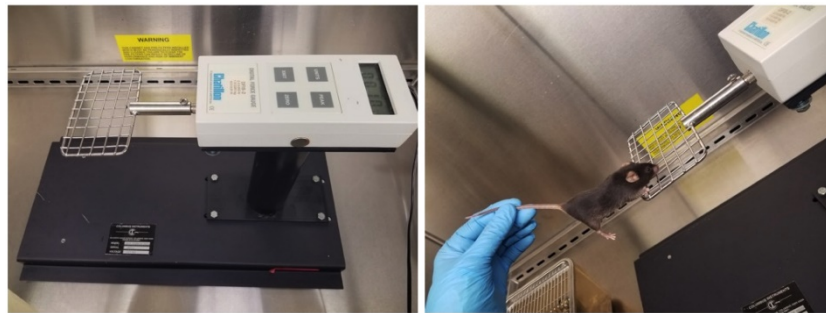


Figure 1. In vivo assessment of muscle strength in mice using grip strength meter

Notes:

- Examine the toes of the mice to make sure there are no visible wounds on them before the test.
 - Make sure that the mouse is gripping nicely to the bars before pulling it; otherwise, this can result in a false low value.
 - Ensure that the mouse is only holding bars with the forearms and the body is parallel to the worksurface.
2. Treadmill assay
 - a. Place each mouse in individual treadmill lanes. Each lane is equipped with a shock grid that delivers a foot shock. The shock current in the grid is usually set at the lowest level 1 (**Figure 2**).
 - b. Start the conveyor belt at 3 m/min and gradually increase the speed by 1 m/min every minute (1 m/min² acceleration). If analyses of weight-normalized work or power are required, regulate the treadmill belt on an upward incline (generally 10°).
 - c. Monitor each mouse to ensure they are running (**Figure 2**). Mice that stop running are pushed to the shock grid through the moving treadmill belt. Signs of exhaustion (e.g., 30 s on the shock grid without successful efforts to start running again) are defined as treadmill assay endpoints.
 - d. Record the distance (m) and the total time (s) until exhaustion.
 - e. Analyze the treadmill assay performance as either time-to-exhaustion or distance-to-exhaustion. If an incline was used, weight-normalized work and/or power can be calculated using previously reported formulas (Castro and Kuang, 2017):

$$\text{Work (J)} = \text{body mass (kg)} \times \text{gravity (9.81 m/s}^2\text{)} \times \text{vertical speed (m/s} \times \text{angle expressed in radians)} \times \text{time (s)}$$

$$\text{Power (W)} = \text{work (J)/time (s)}$$

Calculations should be adjusted to take into account acceleration or varying speed, if used.



Figure 2. In vivo evaluation of mouse muscle endurance and fatigue by treadmill

Notes:

- This test is a form of forced exercise and requires aversive stimuli to keep the animal running. We use electric shock as an aversive stimulus and closely ensure that animals do not get excessive electric shocks, as it can affect the downstream assays.
- If assaying grip strength and treadmill on the same day, perform grip assay before the treadmill assay to avoid possible effects of fatigue. Allow mice to walk freely in the cage for 30 min between grip and treadmill assays.
- Also ensure that the experiments are conducted in a blinded fashion.
- Measure grip strength and treadmill performance at baseline (before injection) and post treatment (24 h after last prednisone dose).
- For both assays, keep operator-dependent variables (e.g., operator, instruments, room conditions, time of day) constant across cohorts and time points.
- Avoid collecting tissues immediately after a treadmill assay.
- It is recommended to collect tissues at least 24 h after the last treadmill assay.

B. For the quantitation of muscle force

1. Anesthetize mouse with 1.5% isoflurane inside the anesthesia induction chamber for approximately 2–5 min. The anesthesia system consists of an induction chamber, a transparent plastic box to confine one mouse at a time in a closed space connected to the oxygen flow, set to a maximum of 0.5 L per min, and to isoflurane gas, set to 1.5% (**Figure 3**).

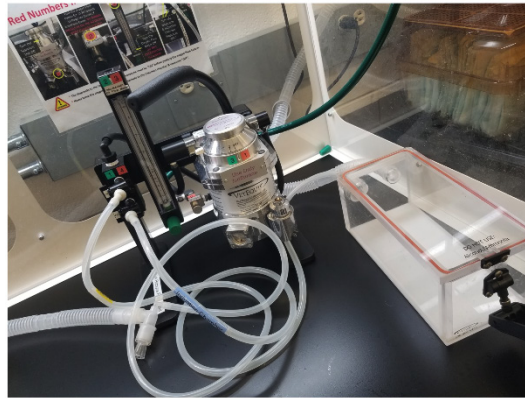


Figure 3. Anesthesia induction chamber

2. Lay the mouse supine on the assay platform of the in-situ apparatus setup (Aurora). During the whole procedure, maintenance of anesthesia is supplied to animals through a nose cone delivering 1.5% isoflurane.
3. Spray 70% ethanol to the specific hind limb (right or left) that you intend to perform the experiment on.
4. Make a small incision near the ankle and remove the hindlimb skin to uncover the tibialis anterior (TA) muscle, ensuring tendons and muscles are not damaged.
5. Detach the TA muscle from the tibia by sliding the forceps between the muscle and the bone.
6. Using a surgical suture, secure the distal tendon to the force probe.
7. Gently sever the tendon and separate the muscle from the tibia. The TA muscle should be linked to the force probe on the distal side and to the knee region on the proximal side.
8. While keeping the mouse on the platform, block the knee with the dedicated clamp.
9. Adjust the platform to ensure that the probe is in line with the knee.
10. Place two electrical probes into the leg, one at the distal end of the TA and the other under the kneecap near the sciatic nerve (**Figure 4**).

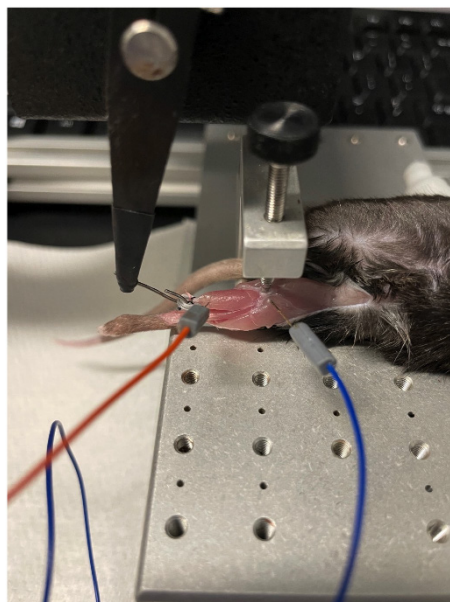


Figure 4. In vivo quantitation of muscle force

11. Run test pulses to adjust muscle tension to the equilibrium point. The equilibrium point is the muscle tension at the initial resting point to which the muscle returns to after test pulses of electricity are administered. Record the muscle length in millimeters at equilibrium with a digital caliper (L_0).
12. Run the force-frequency test through tetanus stimulations at increasing frequency (i.e., from 25 to 200 Hz with intervals of 25 Hz). Pause 5 min between stimulation bouts. Record force (in N) for each isometric contraction (P_0).
13. Repeat procedure (B3–B12) and test on contralateral TA muscle.
14. Proceed to euthanasia and collect and record each TA muscle with a precision scale.
15. Calculate specific force (N/mm^2) for each tetanus frequency as $(P_0 \text{ N})/[(\text{muscle mass mg}/1.06 \text{ mg}/\text{mm}^3)/L_f \text{ mm}]$. $1.06 \text{ mg}/\text{mm}^3$ is the mammalian muscle density. $L_f = L_0 \times 0.6$, where 0.6 is the muscle to fiber length ratio in TA muscle (Burkholder et al., 1994). The specific force is often converted to and reported as N/cm^2 units.

Notes:

- Avoid delays while performing this test.
- Keep muscle hydrated by adding Ringer's solution.
- Ensure that the electrodes are secured properly during the assessment.

C. For the quantitation of muscle mass

1. Tibialis anterior (TA)
 - a. Immediately following the force experiment, perform the cervical dislocation on the mice.
 - b. Position the mouse in supine position and pin all the limbs in the dissection pad.
 - c. Remove the surgical suture from the distal tendon of the TA muscle that is used in the force experiment.
 - d. Hold the distal tendon with the forceps. Measure the length (the muscle length will be used for the calculation of the force) and then resect the TA muscle at the knee region in the proximal side (**Figure 5**). Make sure to resect only the TA and not the extensor digitorum longus muscle, which is adjacent to the TA.
 - e. Weigh the tibialis to the nearest 0.1 mg.

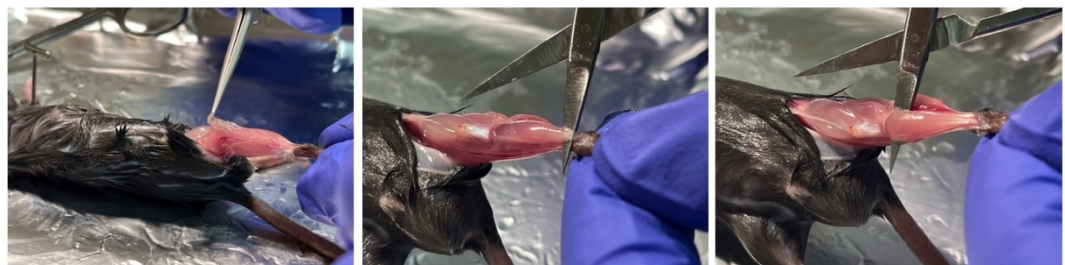


Figure 5. Isolation of murine tibialis muscle

2. Gastrocnemius (GA) and soleus
 - a. Identify the GA and gently remove the fascia covering it.
 - b. Cut the distal GA tendon and insert the forceps (**Figure 6**).
 - c. Rub back and forth from the distal to proximal end with the forceps to open a gap.
 - d. Once there is an opening, cut the tendon as close as possible to the foot.
 - e. Lift the tendon with the forceps and cut the tendon as close as possible to the knee.
 - f. Identify the soleus muscle (beneath the GA in darker pink color) and then use the fine forceps to hold one of its ends and cut it with the scissors.
 - g. Hold the free end with forceps and then cut another end.
 - h. Weigh the resected GA and soleus to the nearest 0.1 mg.



Figure 6. Harvesting of GA muscle

3. Quadriceps (QD)
 - a. Identify the QD and remove the fascia.
 - b. Cut closest to the knee. Hold the free end with forceps and cut towards the proximal end pointing the scissors down and forward (**Figure 7**).
 - c. After reaching proximal end, resect the muscle.
 - d. Weigh the QD to the nearest 0.1 mg.



Figure 7. Isolation of murine QD muscle

4. Calculation of muscle mass
Calculate muscle mass as muscle weight of individual muscles over body weight and/or tibia length.

D. For myofiber typing

1. Sample preparation
 - a. The myofiber typing can be performed in any muscle tissue. After weighing muscle for the muscle mass, it can be prepared for myofiber typing.
 - b. Carefully, place the tissue on a syringe plunger and cover with OCT compound.
 - c. Immediately snap-freeze the tissue by immersing in a metal cup filled with isopentane and cooled in liquid nitrogen for 30–40 s.
 - d. Store the frozen tissue at -80 °C for future use.
 - e. To section the frozen tissue, cut 10 µm transverse sections using the cryostat microtome.
 - f. Use a brush to pick up the sections and place them on a microscope slide.
 - g. Store the slides with appropriate sections at -80 °C for future use.
2. Immunostaining
 - a. Thaw the slides to room temperature for 30 min.
 - b. Using a PAP pen, draw a perimeter around the tissue to create a hydrophobic barrier.

- c. Place the slides in a humidified chamber and add enough permeabilization reagent to cover the tissue (usually 200 μ L per section).
- d. Incubate at 37 °C for 30 min followed by 10 min at room temperature.
- e. Gently wash the slides once with 1 $\times$ PBS.
- f. Add freshly prepared blocking buffer to cover the section. Incubate for 1 h at room temperature.
- g. Wash the slides once in 1 $\times$ PBS.
- h. Prepare the appropriate dilutions for the primary antibodies (see Table 1) in blocking buffer: BA-F8, SC-71, and BF-F3 and overlay onto the sections. SC-71, BF-F3, and BA-F8 antibodies are used for staining type IIA, type IIB, and type I myofibers, respectively.
- i. Incubate in the refrigerator (4 °C) overnight.
- j. Gently wash the sections once with 1 $\times$ PBS to remove unbound primary antibody.
- k. Overlay the sections in secondary antibodies (see Table 2) diluted in blocking buffer: goat anti-mouse IgG2b Alexa Fluor™ 647, goat anti-mouse IgG1, Alexa Fluor™ 488, and goat anti-mouse IgM, Alexa Fluor™ 594.
- l. Incubate for 1 h at room temperature in the dark.
- m. Gently wash the sections three times in 1 $\times$ PBS.
- n. Mount slides with ProLong™ Gold Antifade Mountant with DAPI.
- o. If needed, store slides at 4 °C protected from light for future imaging. Slides can be saved for approximately one month for imaging.
- p. Quantitate myofiber types (**Figure 8**) by taking images with Nikon microscope in at least five serial sections and quantitate as the percentage of total counted myofibers. Conduct all analyses blinded to treatment.

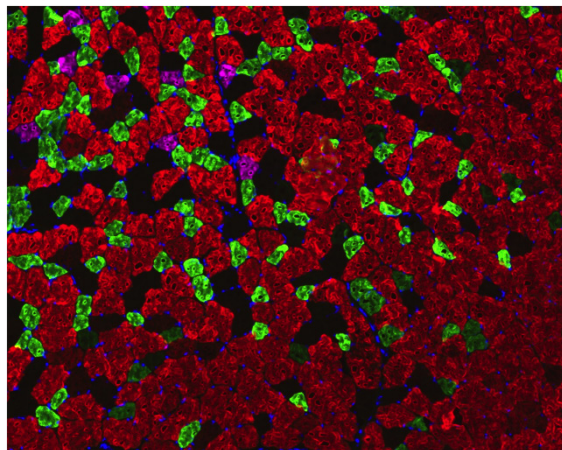


Figure 8. Representative image of immunohistochemical staining of TA section. Type 1 fibers stained in magenta, type 2A stained in green, type 2X showed no staining, and type 2B stained red. Nuclear staining in blue (original magnification, 20 $\times$).

E. Myofiber cross-sectional area (CSA)

The sample preparation for the myofiber CSA and myofiber typing is the same (see section D1 for sample preparation).

1. Immunofluorescence
 - a. Thaw the slides to room temperature for 30 min.
 - b. Using a PAP pen, draw a perimeter around the tissue to create a hydrophobic barrier.
 - c. Place the slides in a humidified chamber and add enough permeabilization reagent to cover the tissue (usually 200 μ L per section).
 - d. Incubate at 37 °C for 30 min followed by 10 min at room temperature.
 - e. Gently wash the slides once with 1 $\times$ PBS.
 - f. Add freshly prepared blocking buffer to cover the section. Incubate for 1 h at room temperature.
 - g. Wash the slides once in 1 $\times$ PBS.
 - h. Prepare anti(α)-Laminin antibody (see Table 1) in blocking buffer and overlay onto sections. Incubate in the refrigerator (4 °C) overnight.
 - i. Gently wash the sections once with 1 $\times$ PBS to remove unbound primary antibody.
 - j. Dilute goat anti-rabbit IgG (H+L) Alexa Fluor™ 488 (see Table 2) in blocking buffer and incubate for 1 h at room temperature in the dark.
 - k. Gently wash the sections three times in 1 $\times$ PBS.
 - l. Mount slides with ProLong™ Gold Antifade Mountant with DAPI.
 - m. If needed, store slides at 4 °C protected from light for future imaging. Slides can be saved for approximately one month for imaging.
2. CSA quantitation
 - a. Perform imaging with a Nikon Eclipse Ti inverted microscope using 10 $\times$ and 20 $\times$ (short-range) objectives (**Figure 9**).
 - b. Conduct CSA quantitation on >400 myofibers per tissue per mouse using ImageJ software.
 - c. For linear calibration, use the line selection tool to draw a line between the two points of known distance (such as the scale bar).
 - d. Transform the line length from pixels into units of measure (μ m) using command Analyze $\rightarrow$ Set scale.
 - e. Manually outline the myofibers perimeter by means of an area selection tool (polygonal) (**Figure 9**). Myofibers at the edge of the image or with deformed/altered shapes (*) (**Figure 9**) are excluded from counting to remove the presence of artifacts.
 - f. Display area measurements in a data window using the command Analyze $\rightarrow$ Measure.

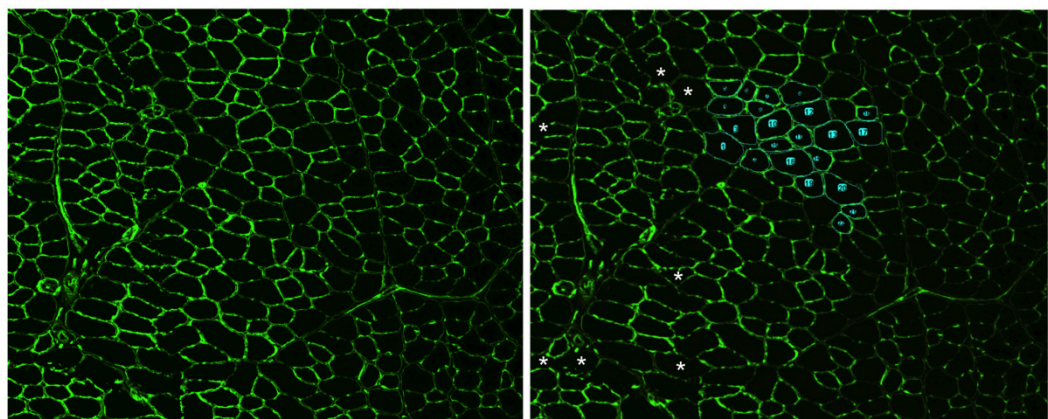


Figure 9. Representative immunofluorescence images of TA sections. Anti(α)-Laminin (green) staining (original magnification, 20 $\times$); (*) indicates representative myofibers with deformed shape.

Note: The protocol for myofiber CSA quantitation can also be adopted for hematoxylin and eosin-stained sections.

F. Quantitation of lean mass

1. Open the EchoMRI program on the desktop.
2. Create a folder to save the raw data automatically once the experiment is completed.
3. Place the solid calibration holder (for mouse) into MRI and preform a system test by inserting the calibration holder. The process takes approximately 4 min to complete. The system is calibrated using the standard internal calibrator tube (canola oil).
4. Remove the calibration holder from the machine.
5. Holding the mice with its tail, place it in an appropriately sized holder and slide the adjustable barrier to minimize area of movement. Some degree of mobility is fine and will not compromise measurement (Figure 10).



Figure 10. Body composition analysis using EchoMRI

6. Record the reading and repeat the scan with an interval of 20 s.
7. Remove the rodent from the tube holder and wipe out any urine/feces or gross debris with a brush/paper towels before proceeding to the next animal.
8. When hydration ratio was >85%, analyze the data using the built-in software EchoMRI version 140320.

Notes:

- If metal ear tags are used for mouse identification, use non-magnetic ones that will not affect the animal, machine, or measurements by EchoMRI.
- Once all measurements have been performed, wash tube holders and sanitize all parts. Sanitization washing must occur between different strains, species, and principal investigators' animals to prevent potential cross contamination of rodent pathogens.

G. Quantification of muscle function, lean and muscle mass, and myofiber typing in regimen-specific glucocorticoid treatment

The data presented below (**Figure 11**) shows examples of implementation of our protocol on muscle function, lean and muscle mass, and myofiber typing. Here, we show the effects of dosage time of prednisone (a commonly used glucocorticoid); data presented are adapted from Quattrocchi et al. (2022).

1. Glucocorticoid regimens

- Prior to injection, record the weight of each mouse.
- Prepare 50 μL (final volume) of physiological solution with the appropriate volume of prednisone stock to a final dose of 1 mg/kg [e.g., 4 μL of a 5 $\mu\text{g}/\mu\text{L}$ stock (total of 20 μg) for a 20 g mouse].
- For the vehicle, mix corresponding volumes of vehicle stock and sterile physiological solution.
- With a single-use insulin syringe per dose, load the appropriate solutions. Make sure no air is introduced.
- Expose the mice abdomen and perform an intraperitoneal injection.
- Frequency of intermittent dosing: **once-weekly regimen** consists of 1 $\times$ prednisone dose (day 1) followed by 6 $\times$ vehicle doses (day 2–6) per week. It is controlled by once-weekly vehicle injection.
- Time of dosing: Injections were conducted either at the beginning of the light phase (ZT0; lights-on) or at the beginning of the dark phase (ZT14; lights-off). Experiments were performed 24 h after the single pulse or the last injection in chronic treatment.
- Follow the regimen for a total of 12 weeks for chronic treatment. All in vivo, ex vivo, and postmortem analyses are conducted blinded to the treatment group.

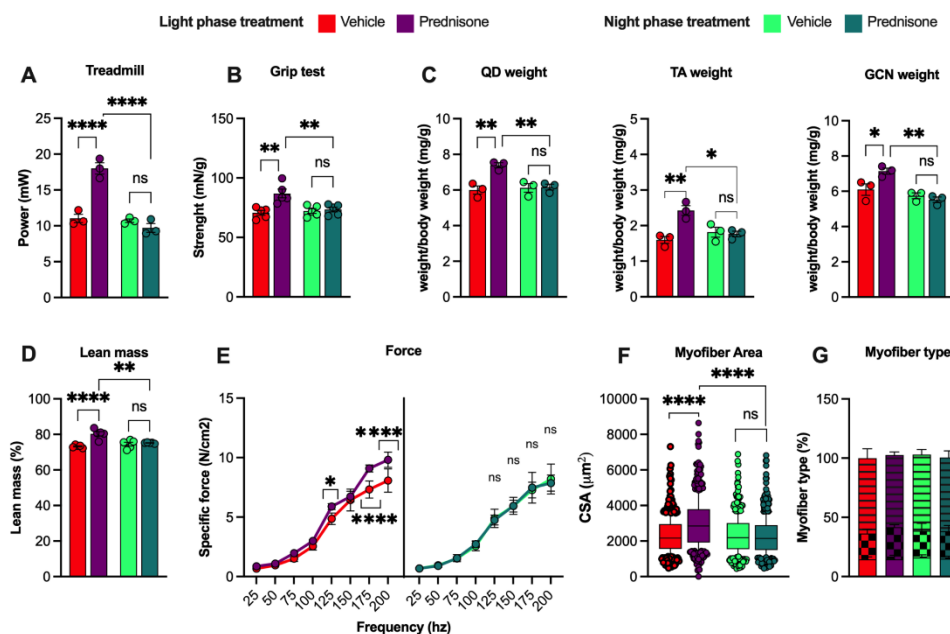


Figure 11. Effects of light-phase vs. dark-phase intermittent prednisone treatment on muscle bioenergetics. Results are shown after a 12-week-long treatment with intermittent once-weekly prednisone with dosing restricted to ZT0 (light phase) vs. ZT14 (dark phase). In wildtype (WT) mice, compared to isochronic vehicle controls, ZT0, but not ZT14, prednisone improved (A) treadmill performance, (B) grip test, (C) muscle weight, (D) lean mass, (E) muscle force, and (F) myofiber area. However, no changes were reported in (G) the distribution of myofiber types in tibialis anterior muscles. Lean mass, grip strength, myofiber cross-sectional area (CSA), and myofiber typing data were adapted from Quattrocchi et al. (2022). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$; 1-way ANOVA + Sidak (A-D, F-G), 2-way ANOVA (curves in E).

Notes

1. Here, we used 1 mg/kg prednisone dose to successfully discriminate the effects of diverging the once-weekly day dosing from the once-weekly night dosing in WT mice lines. Other glucocorticoids, dosages, and intermittence intervals should be tested to evaluate differences/similarities with analyses and trends discussed here.
2. A 12-week-long treatment enables for stabilization of the divergent regimen-specific changes, with the additional consideration of ages at start and endpoint.
3. Here, we report methods and data related to glucocorticoid treatments in 4-months-old mice at start. This age-at-start is convenient for 12-week-long regimens. Indeed, treated mice will be 4 months old at start and approximately 7 months old at endpoint. Analyses at different ages will yield different results and therefore age-at-analysis should be considered when performing these tests.
4. Regarding sex as biological variable, biological sex impacts almost every virtual parameter of muscle physiology and its response to drug treatments, including glucocorticoid intermittence (Salamone et al., 2022). Therefore, analyses in sufficiently powered cohorts of both male and female mice are recommended, as well as data reporting as aggregated and disaggregated by sex.

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Competing interests

MQ is listed as co-inventor on a patent application related to intermittent glucocorticoid use filed by Northwestern University (PCT/US2019/068618). All other authors declare they have no competing interests.

Ethics

Mice were housed in a pathogen-free facility in accordance with the American Veterinary Medical Association and under protocols fully approved by the Institutional Animal Care and Use Committee at Cincinnati Children's Hospital Medical Center (#2020-0008).

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