AMERICAN JOURNAL OF PHYSIOLOGY
REGULATORY, INTEGRATIVE AND COMPARATIVE PHYSIOLOGY

Am J Physiol Regul Integr Comp Physiol 329: R537–R549, 2025.
First published September 5, 2025; doi:10.1152/ajpregu.00072.2025

RESEARCH ARTICLE

Integrated muscle protein synthesis during disuse and rehabilitation in late-midlife adults

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Abstract

The purpose of this study was to investigate the sex-specific differences in how late-midlife adults respond to short-term disuse and rehabilitation. Sixteen late-midlife adults, who were free of overt disease (8 males: 58 ± 2 yr, BMI 29.4 ± 0.8 kg·m^{-2}; 8 postmenopausal females: 56 ± 2 yr, BMI 29.1 ± 1.1 kg·m^{-2}) underwent 7 days of unilateral lower limb suspension (ULLS), followed by 7 days of rehabilitation. Vastus lateralis muscle biopsies were collected before and following ULLS [in both control (CON) and immobilized (IMM) legs] and in the IMM leg post-rehabilitation. We applied deuterium oxide to measure muscle protein synthesis (MPS), immunoblotting to assess mTORC1 signaling, and assessed changes in muscle fiber cross-sectional area (CSA) and leg strength. MPS was 17.8 ± 14.6 and 32.7 ± 10.9% lower in the IMM compared with the CON leg in males ($P = 0.32$) and females ($P < 0.05$), respectively, during immobilization. MPS was 27.5 ± 24.5 and 9.7 ± 38.9% higher in the IMM leg during the rehab compared with during the IMM phase in the males and females, respectively ($P > 0.05$). Leg extension one repetition maximum declined by 24.2 ± 2.4 and 17.1 ± 2.1% in males and females, respectively, after IMM (both $P < 0.01$), in the IMM leg with no change in the CON leg ($P > 0.05$). Our data show that late-midlife males and females experience similar reductions in MPS and muscle fiber CSA. Seven days of resistance exercise rehabilitation partially reverses the decline in muscle strength, CSA, and MPS, but longer rehabilitation periods are required for full recovery in late-midlife adults.

NEW & NOTEWORTHY This study provides novel data on the average rate of muscle protein synthesis during 7 days of disuse and 7 days of rehabilitation in late-midlife adults. Both sexes experienced a similar reduction in muscle protein synthesis, strength, and fiber cross-sectional area during disuse. Seven days of resistance exercise rehabilitation partially reverses the disuse-induced decline in muscle protein synthesis, strength, and fiber size; however, longer periods of rehabilitation are required for full recovery.

disuse-atrophy; muscle protein synthesis; rehabilitation; sex difference; skeletal muscle

INTRODUCTION

Incidences of illness or injury often require a period of short-term muscle disuse to aid recovery. Although these periods of immobilization or bed rest are essential, they result in negative health effects, including declines in muscle function (1–6), mass (3–5, 7), and insulin sensitivity (8, 9). These negative adaptations can be reversed by exercise rehabilitation (10, 11), although older people take longer to recover after a period of disuse (12). Although the physiological responses and cellular mechanisms of muscle disuse atrophy (2, 13–15) and subsequent rehabilitation (11, 12, 16) have been well studied, understanding the sex-specific differences requires further investigation.

Although the effects of short-term muscle disuse and rehabilitation on skeletal muscle mass, function (3–5, 7), and metabolism (10, 13, 17, 18) are relatively well studied. Only a very limited number of studies have directly compared how males and females respond to muscle disuse or rehabilitation. In a study of intensive care unit (ICU) patients, females exhibited a greater increase in muscle weakness compared with males after a period of bed rest (19). In contrast, in older adults with knee osteoarthritis, only males experienced a significant reduction in myosin heavy chain type 2x (MHC2x) fiber cross-sectional area

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Submitted 1 April 2025 / Revised 9 May 2025 / Accepted 25 August 2025

(CSA) (20). In healthy young subjects after 1 wk (21) and 2 wk (22) of leg immobilization, females lost greater knee extension isometric force. Furthermore, females required one extra training session to regain this deficit compared with males, although this was not significant (21). Understanding any potential differences in how males and females respond to disuse and rehabilitation could lead to better targeted interventions aimed at alleviating the negative effects of muscle disuse as well as optimizing rehabilitation.

Mechanistically, muscle mass is regulated by the balance of muscle protein synthesis (MPS) and muscle protein breakdown (MPB) (23). It is well established that short periods of muscle disuse reduce muscle protein synthesis rates (MPS), which contribute to muscle atrophy (2, 13, 17, 24, 25). This reduction in MPS can be reversed after disuse with physical rehabilitation (10) or nutritional supplementation (26), particularly in young adults. However, most of these studies have been conducted with young adult male subjects (2, 10, 13, 24, 26). In community dwelling older adults, there is some evidence that sex differences in MPS exist in older (70-yr old) (27), but not younger (37-yr old) populations (28). However, whether these differences exist during periods of muscle disuse and rehabilitation remains unknown.

The purpose of the present study was to determine the sex-specific effect of disuse and rehabilitation on muscle protein synthesis, function, and metabolism in late-midlife adults. We focused on molecular parameters, including muscle protein synthesis, and mTORC1 signaling. As well as functional parameters including leg extension strength and muscle mass. We hypothesized that females would experiences greater deficits in muscle mass and protein synthesis rates during disuse but would recover at a slower rate than males during rehabilitation.

MATERIALS AND METHODS

Participants

Sixteen late-midlife adults free of overt disease (8 males, age: 58 ± 2 yr, BMI: 29.4 ± 0.8 kg·m^{-2}; 8 postmenopausal females, age: 56 ± 1 yr, BMI: 29.1 ± 1.1 kg·m^{-2}) were included in the present study. Participants were recreationally active [i.e., spent < 8 h·day^{-1} with <100 counts/min based on accelerometry data (29)] without resistance training experience. Participant characteristics are shown in Table 1. Participants attended the clinic for a medical screening visit to assess their eligibility for participation. Inclusion criteria included: males and postmenopausal females aged 50–65 yr, BMI between 18.5 and 30 kg·m^{-2}. Postmenopausal status was confirmed by a combination of self-report (i.e., no menstruation in the previous 12 mo) and if estradiol levels were <10 pg/mL. Exclusion criteria included: endocrine or metabolic disease (e.g., hypogonadism, type 2 diabetes), cardiovascular disease, acute or chronic infection, compromised musculoskeletal function, recent injury or history of falls (within last 6 mo), anabolic steroid use (both sexes) or hormone replacement therapy use (females only) within the last 6 mo, and use of recreational drugs. During the medical screening subjects' height, body mass, and blood pressure were measured, a single blood draw was obtained and analyzed for blood count, metabolic/lipid panel, sex hormones, and for infectious diseases (e.g., Hepatitis, HIV). This study was conducted in accordance with the Declaration of Helsinki and approved by the UT Health San Antonio and University of Texas Medical Branch at Galveston institutional review boards (IRB No. 20230594H and IRB No. 1900-45). This study was registered as a clinical trial with clinicaltrials.gov (NCT04151901). Participants provided written informed consent before participating in the study.

Table 1. *Subject characteristics*

Subject Characteristics	Total	Males	Females
Age, yr	57 ± 2	58 ± 2	56 ± 2
Weight, kg	81.9 ± 4.4	90.1 ± 7.0	73.6 ± 3.3
Height, m	1.67 ± 0.03	1.75 ± 0.04	1.59 ± 0.02
Body mass index, kg·m^{-2}	29.3 ± 0.9	29.4 ± 0.8	29.1 ± 1.1

Values represent means ± SE, $n = 16$, 8 male and 8 female subjects.

Experimental Design

A graphical representation of the experimental study design is shown in Fig. 1. Following the medical screening visit, all participants attended eight study visits as part of the protocol containing 7 days of unilateral lower limb suspension (ULLS) immediately followed by four resistance exercise rehabilitation sessions over a 7-day period. Approximately 10 days before the ULLS period, participants received training from a study physical therapist on how to use the assistive devices (e.g., crutches and a walker) to ambulate safely during the disuse period. Participants were also familiarized to the exercise equipment. Participants were instructed to refrain from vigorous exercise and maintain their habitual diet for the remainder of the study. Seven days before the ULLS period participants completed one repetition (1-RM) maximum unilateral leg extension strength testing. All subsequent visits were conducted over the 14-day period of disuse and rehabilitation, and participants were asked to fast for 12 h before each visit.

On *day 1* of ULLS, participants reported to the clinic at 0800 and completed a dual-energy X-ray absorptiometry (DXA) scan (Lunar iDXA; GE Medical Systems, Mississauga, ON). Following this, bilateral skeletal muscle biopsies of the vastus lateralis were obtained under local anesthetic (2% lidocaine) using Bergstrom technique (30) by a licensed medical professional. Immediately following the biopsy, the muscle sample was washed with ice-cold saline and quickly assessed, and any nonmuscle tissue was discarded. Of the remaining muscle tissue, one section was flash frozen in liquid nitrogen, and a second section was oriented and mounted in Tissue Tek media (Sakura Finetek, Torrance, CA) on cork and frozen in liquid nitrogen-cooled isopentane. All samples were stored at −80°C until further analysis. A blood sample was obtained via venipuncture of the antecubital vein concomitantly with the muscle biopsies. Post-biopsies subjects ingested a bolus dose of deuterium oxide (D_2O, 150 mL, 70% Atom excess, Cambridge Isotopes, Tewksbury, MA) for measurement of integrated muscle protein synthesis (MPS) rates (18, 31). A saliva sample was collected immediately before and once every other day, following the consumption of D_2O to measure ^2H enrichment in body water. To collect the saliva samples, participants were instructed to lightly chew on a cotton sponge

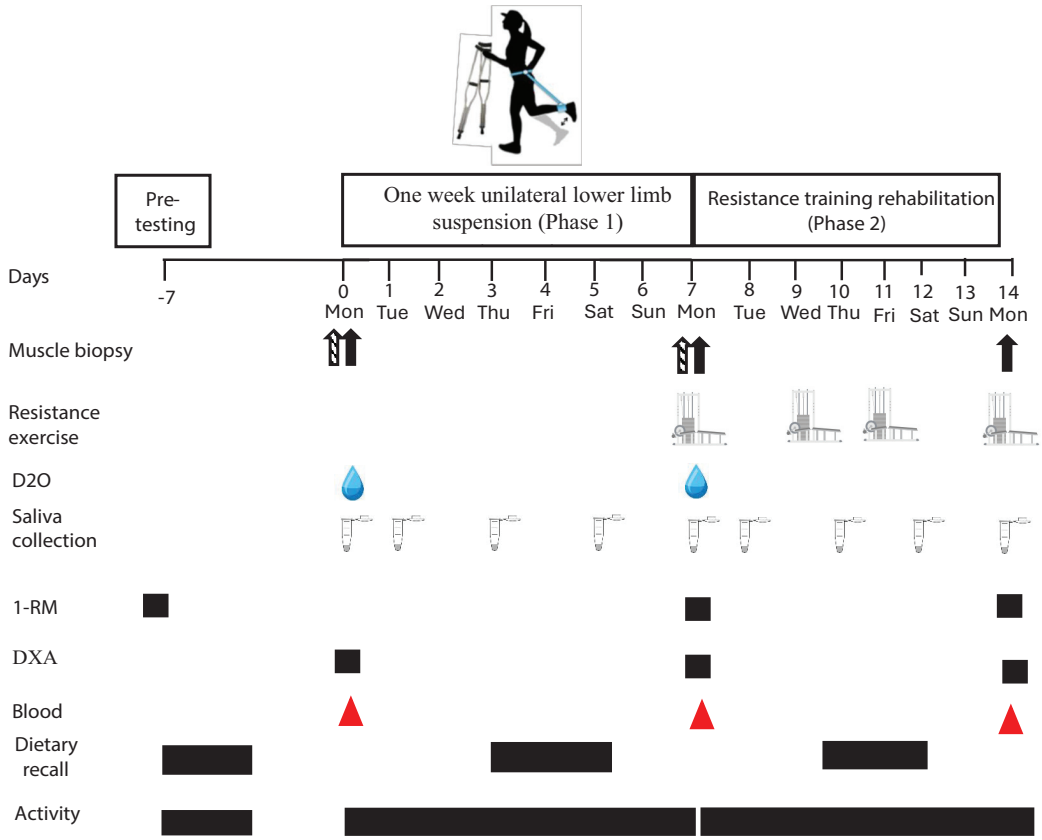

Figure 1. Study schematic. Sixteen late-midlife adults (8 males and 8 postmenopausal females) underwent 7 days of unilateral lower limb suspension followed by 7 days of rehabilitation (1 session every other day, 4 sessions total). Blood indicates venous blood sample. Arrows represent bilateral m. vastus lateralis muscle biopsies (i.e., taken from the control and immobilized legs). On *day 14* only a single m. vastus lateralis muscle biopsy was taken from the immobilized leg. D_2O, deuterated water ingestion. Activity is physical activity measured continuously by GENEactiv accelerometry. Dietary recall is habitual dietary intake recorded by self-reported, written diet diary.

(Celluron, Hartmann, Germany) until saturated. The sponge was then placed into an empty syringe, and saliva was ejected into an Eppendorf tube. Participants were fitted with a leg sling and provided with crutches and a walker to start the disuse phase and were provided with the supplies to collect their saliva samples at home.

After 4 days of ULLS, participants returned to the clinic for a monitoring visit where they completed an adherence questionnaire, the suspended leg was checked for any discomfort, and a blood sample was obtained and tested for markers of thrombosis (e.g., D-dimer). Skin temperature of the dorsal foot was measured on both legs. On *day 7* of ULLS, participants returned to the clinic with their saliva samples, and the same measurements that occurred on *day 1* were repeated (DEXA, bilateral vastus lateralis muscle biopsies, a single blood sample, and 1 RM strength testing). Participants also received a top-up dose of D_2O (50 mL), a saliva sample was collected before and once every other day throughout the rehabilitation period. Participants were provided with more saliva collection supplies for the rehabilitation phase.

Approximately 1 h following the post-ULLS biopsy, participants began the rehabilitation phase. Participants completed a 5-min treadmill-based walking warmup and were monitored to maintain a heart rate of 100 beats/min. Participants then completed a series of resistance exercise rehabilitation sessions, which included; 4 × 10 reps of unilateral leg extensions at 70% 1 RM, with a 1-min rest between legs and a 2-min rest between sets. This was followed by 4 × 10 reps of bilateral leg curls at 70% of 1 RM with a 2-min rest between sets. All exercises were done using standard gym equipment (Precor, Woodinville, MA). Rehabilitation sessions were performed 3× per week on nonconsecutive days [Monday (*day 7*), Wednesday (*day 9*), Friday (*day 11*)] and finished on Monday (*day 14*). On the final test day, participants underwent a DEXA scan, a biopsy of the immobilized leg, 1-RM strength testing, and following a 1 h break, the final rehabilitation session.

Immobilization Protocol

Unilateral lower limb suspension (ULLS) (32, 33) was utilized as the model of leg immobilization. The left leg was suspended via a strap that connected to a belt positioned around the subject's waist and a cuff around the ankle that held the left leg at 30 degrees of flexion (Fig. 1). Participants ambulated with a walker and crutches. The left leg was selected so subjects would still be able to drive an automatic transmission car. Subjects were encouraged to wear the sling for 24 h·day^{-1} (any weight bearing on the suspended leg was strictly forbidden). Participants were provided with a shower bench so they could remain seated while showering. To monitor compliance, participants were contacted daily and completed a diary detailing their daily activities.

Furthermore, participants continuously wore accelerometers (gt3x, Actigraph) around both ankles for the week of ULLS (except for when showering, Supplemental Fig. S1) and had skin temperature of the dorsal mid-foot of the ULLS and control leg measured before, during, and after the week of ULLS (Traceable Infrared thermometer, Cole Palmer, IL). A permanent marker was used to mark the location of the temperature reading to ensure the same location was used each time skin temperature was measured.

Leg Strength

Unilateral leg extension exercises were performed for both legs individually using standard gym equipment (Precor, Woodinville, MA). One repetition maximum (1-RM) strength testing was assessed using an incremental repetition procedure that was conducted separately for each leg, with the immobilized leg being tested first. Participants first completed two warm-up sets of 4 and 2 repetitions at 25% and 50% of 1-RM, respectively. Subsequently, single repetitions at 1-RM were undertaken, each separated by a 2-min rest. Following each successful attempt, the weight was increased until no extra weight could be lifted. The final 1-RM was taken as the heaviest single attempt that was successfully completed with full range of motion and correct technique.

Leg Lean Mass

Leg lean mass (LLM) was determined by dual-energy X-ray absorptiometry (DXA) on *days 0*, *7*, and *14* (Lunar iDXA; GE Medical Systems). To standardize and minimize the effects of fluid shifts, subjects were required to lie supine for 10 min before scanning.

Diet and Physical Activity Monitoring

For 3 days before the unilateral lower limb suspension (ULLS) period as well as throughout ULLS and rehabilitation, participants physical activity was measured using an accelerometer (GENEactiv) that was worn around the waist and each ankle. Participants were asked to maintain their habitual activity level in the habitual period before crutches, as well as during ULLS (to the best of their ability despite using crutches/a walker to avoid whole body sedentariness) and rehabilitation. Participants were asked to wear the accelerometer continuously, and data were recorded at 60 Hz sampling frequency. Physical activity data from the GENEactiv accelerometers that were worn around the waist were converted into 60 s epochs and used to estimate time spent performing total physical activity (all intensities) using standard cut-off points (29) for the pre-ULLS, ULLS, and rehabilitation periods, respectively. For the GENEactiv accelerometers that were worn around the ankle of the ULLS and control legs during the ULLS period. Vector magnitude counts were calculated for both legs for each day of the ULLS period to assess total movement in the ULLS leg and control leg, respectively, as a measure of compliance (Supplemental Fig. S1). Vector magnitude counts were calculated from the counts in each axis [x, y, and z, which is the raw acceleration data that was converted into counts using Actilife software (Actigraph, Pensacola, FL)], which were combined using the following formula:

$$\mathrm{VM} = \sqrt{X^2 + Y^2 + Z^2}$$

where X, Y, and Z are counts in the x, y, and z axes.

Participants diet was recorded for 3 days before ULLS, during ULLS, and during rehabilitation (1 weekend day and 2 weekdays), respectively, by a self-reported written diet diary. Subjects were asked to refrain from alcohol intake and maintain a similar diet during and throughout the study. Dietary analysis was completed using ASA24 (National Cancer Institute, Bethesda, MA).

Immunohistochemistry

Muscle samples from both the immobilized (IMM) and control (CON) legs at both pre-ULLS (PRE) and post-ULLS (POST), and the IMM leg post-rehab (REHAB) were preserved for immunohistochemistry. Samples were embedded in Tissue Tek compound (Sakura Finetek, Torrance CA) on cork and frozen in liquid nitrogen-cooled isopentane. Subsequently, seven-micron-thick sections were cut in a cryostat (Cryostar NX70, Epredia, Portsmouth, NH) and were air-dried for 1 h. For immunofluorescence staining and muscle fiber detection, unfixed slides were immersed in acetone for 10 min at −20°C, then rinsed for 1 × 5 min in 1 × phosphate-buffered saline (PBS), 7.5 pH. Subsequently, slides were incubated overnight at 4°C in primary antibodies (MyHC-1, BA-D5c, 1:100, MyHC-2a, SC-71c, 1:100, MyHC-2x, 6H1-s 1:10; Developmental Studies Hybridoma Bank, IA, laminin, 1:200, Cat. No. L9393, Sigma Aldrich, St. Louis, MO). The next day, after washing the slides in PBS (3 × 5 min), slides were incubated for 1 h at room temperature with secondary antibodies [MyHC-1, Alexa Fluor (AF) 647, 1:250, MyHC-2a, AF488, Cat. No. A21121, 1:500, MyHC-2x, AF555, Cat. No. A21426, 1:500, Invitrogen, Carlsbad, CA; laminin 1:500 dilution of AF568, Invitrogen, Cat. No. 11011]. Subsequently, slides were rinsed three times with PBS and post fixed in methanol before being mounted with fluorescent mounting media (Vectashield, Cat. No. H-1000; Vector Laboratories, Burlingame, CA) and a coverslip applied.

Image Acquisition and Analysis

All muscle sections were imaged at ×10 magnification using the tile and stitching functions on a Nikon Eclipse Ti2-U inverted microscope equipped with Nikon Elements Ar software package (Nikon Americas Inc, Melville, NY). Image analysis was completed in a blinded manner and performed using MyoVision version 2 software (34) to calculate cross-sectional area of type 1, type 2a, and type 2x muscle fibers. A pixel conversion ratio value of 0.654 µm/pixel was used to account for the size of the images. A detection range from 500 to 12,000 µm^2 was used to ensure artifacts were removed (e.g., structures between laminin stains, which were likely small blood vessels or large fibers that may have not been in cross section).

Myofibrillar Bound ^{2}H Alanine Enrichments and Body Water ^{2}H Enrichments

The enrichments of [2^H]alanine in the myofibrillar fraction of skeletal muscle tissue samples were determined, as described previously (35, 36). Briefly, 50 mg of whole frozen muscle was mechanically homogenized in 7.5 volumes of

ice-cold homogenization buffer [50 mM Tris-HCL (pH 7.4), 1 mM EDTA, 10 mM β-glycerophosphate, 1 mM EGTA, 50 mM NaF, 0.5 mM activated sodium orthovanadate, and 1 complete mini protease inhibitor cocktail tablet per 50 mL of buffer (Roche Holding AG, Basel, Switzerland)]. Homogenized samples were centrifuged (10 min, 2,200 *g* at 4°C), and the pellet was washed in 500 μL of ice-cold homogenization buffer, centrifuged (10 min, 700 *g* at 4°C), and solubilized (750 mL of 0.3 M sodium hydroxide at 50°C for 30 min). Following centrifugation (10,000 *g* at 4°C for 10 min), myofibrillar proteins were precipitated from the supernatant by adding 500 μL of 1 M perchloric acid and vortexing for 40 s and pelleted by centrifugation (10 min, 700 *g* at 4°C). The pellet was washed twice in 70% ethanol, and amino acids were hydrolyzed in 2 mL of 6 M hydrochloric acid at 110°C for 24 h. The samples were subsequently dried under a vacuum (Savant SpeedVac, Thermo Fisher Scientific), reconstituted in 3 mL of acetic acid (25%), passed over cation exchange resin columns (100–200 mesh; H^+ form; Dowex 50WX8; Sigma Aldrich Company Ltd., St Louis, MO), and eluted with 6 M NH_4OH, before being dried again under vacuum. Samples were resuspended in 1 mL of 0.1% formic acid in acetonitrile and 1 mL distilled water, centrifuged (3 min, 10,000 *g* at 4°C), and the supernatant was aliquoted, dried under a vacuum, and stored at −20°C. Amino acids were derivatized by adding 50 μL *N*-tert-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) + 1% tert-butyl-dimethylchlorosilane and 50 μL acetonitrile, and vortexed and heated for 40 min at 95°C. The samples were then transferred to a gas chromatography vial. Alanine enrichment was analyzed using a Thermo Fisher Delta V Advantage IRMS (Bremen, Germany) fitted with a Trace 1310 gas chromatograph with an online high-temperature thermal conversion oven (HTC) at 1,420°C. The sample (1 μL) was injected in splitless mode at an injection port temperature of 250°C. The peaks were resolved on a 30 m 0.25 mm ID × 0.25 μm film Agilent Technologies DB-5 capillary column (temperature program: 110°C for 1 min; $10°C \cdot min^{-1}$ ramp to 180°C; $5°C \cdot min^{-1}$ ramp to 220°C; $20°C \cdot min^{-1}$ ramp to 300°C; hold for 2 min) before pyrolysis. Helium was used as the carrier gas with a constant flow of $1 mL \cdot min^{-1}$. Any amino acid eluting from the gas chromatograph was converted to H_2 before entry into the IRMS via pyrolysis reaction performed by the HTC oven. Deuterium enrichment of myofibrillar protein-bound alanine was determined by monitoring ion masses 2 and 3, thereby establishing the 2H/1H ratio of the amino acid. A series of known standards was applied to assess the linearity of the mass spectrometer.

Body water enrichment was measured from collected saliva samples using an inline gas preparation system (GasBench II, Thermo Scientific), connected to the above isotope ratio mass spectrometer, as described previously (37). In brief, 5 mg of activated charcoal and 200 mg of copper powder were added to an exetainer (Labco Ltd., UK), along with 200 μL of the saliva sample. Afterward, the sample was flushed with 2% H_2 in helium for 7 min and allowed to equilibrate at room temperature (4–6 h) before analysis.

Calculation of Fractional Synthesis Rate

Integrated myofibrillar fractional synthesis rates (FSRs) were calculated based on the incorporation of the mean body water ^2H enrichment over the ULLS and rehabilitation phases as a precursor pool into myofibrillar-bound proteins. Previous work has shown that the body water ^2H pool is a valid precursor pool for the calculation of myofibrillar protein synthesis rates (corrected by a factor of 3 × 7 based on ^2H labeling of alanine during de novo synthesis), which shows excellent agreement with plasma alanine as an alternative precursor pool (24, 38). FSR was calculated as follows:

$$FSR = -Ln\left(\frac{1 - \left[\frac{(APE_{Ala})}{APE_P}\right]}{t}\right),$$

where APE_{Ala} is the deuterium enrichment of protein-bound alanine, APE_P is the mean precursor enrichment over the time period, and t is the time between biopsies (39).

Immunoblotting

Muscle tissue (20 mg) was homogenized using glass on glass homogenization, in precooled tubes in a buffer containing; 50 mM Tris·HCl, 250 mM mannitol, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol tetraacetic acid, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, and 5 g/mL soybean trypsin inhibitor. The homogenates were then centrifuged, and supernatants were reserved, subsequently, total protein concentration was determined by bicinchoninic acid (BCA) assay. Protein concentration was normalized, and samples were mixed with Laemmli sample buffer and heated at 70°C for 10 min. Proteins were resolved by Laemmli SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked in Tris-buffered saline with 0.1% Tween-20 (TBS-T) plus 5% bovine serum albumin (BSA) and probed with the primary antibody overnight at 4°C. Primary antibodies included phosphorylated $rpS6^{Ser235/236}$ [Cell Signaling Technology (CST), Cat. No. 4858] and $4E\text{-}BP1^{Thr37}$ (CST, Cat. No. 2855), with β-tubulin (CST, Cat. No. 2128) used as a housekeeping protein. Membranes were then washed with TBS-T and probed with fluorescent IRDye 800CW Donkey anti-rabbit secondary antibody (LI-COR Biosciences, Lincoln, NE, Cat. No. 925-32213) for 1 h at room temperature. Proteins were visualized using a LI-COR Odessey CLx Imaging System (LI-COR Biosciences, Lincoln, NE). Subsequently, membranes were stripped (Restore Fluorescent Western blot striping buffer, Thermo Scientific, Waltham, MA) and reblocked with TBS-T plus 5% BSA, and primary antibodies for total-rps6 (CST, Cat. No. 9452S) and total-4E-BP1 (CST, Cat. No. 9452) were applied overnight at 4°C. Membranes were again washed with TBS-T and probed with fluorescent IRDye 800CW Donkey anti-rabbit secondary antibody (LI-COR Biosciences, Lincoln, NE, Cat. No. 925-32213) for 1 h at room temperature. Proteins were again visualized using a LI-COR Odessey CLx Imaging System (LI-COR Biosciences, Lincoln, NE).

Mitochondrial Respiration

Small bundles of skeletal muscle fibers (2–5 mg) were blotted, weighed, and gently teased apart using fine-tipped forceps

to partially separate fibers without removing them from the fiber bundle. Each fiber bundle was permeabilized for 15 min on ice in BIOPS buffer, containing 50 µg/mL saponin to selectively permeabilize cell membranes. Fiber bundles were then rinsed for 15 min in ice-cold MiR05 buffer (110 mM sucrose, 60 mM potassium lactobionate, 2 mM $MgCl_2$, 20 mM taurine, 10 mM KH_2PO_4, 0.5 mM EGTA, 20 mM HEPES, and 1 g/L bovine serum albumin). Following the rinse step, bundles were placed inside the Oroboros Oxygraph-2k (O2k) (Oroboros Instruments, Innsbruck, Austria) with MiR05 buffer, set at 37°C with the stir bars spinning at 750 revolutions/min. Supplemental oxygen was added to each chamber to maintain O_2 concentrations between 400 and 200 µM throughout the experiment. Mitochondrial respiration rate was measured via the sequential titration of substrates (5 mM pyruvate, 2 mM malate, and 10 mM glutamate), ADP (5 mM), succinate (10 mM), cytochrome c (10 µM), and finally, the ionophore CCCP (5 µM). Background oxygen consumption was measured following the addition of antimycin A (2.5 µM).

Data Analysis

All data are presented as means ± SE, and all statistical analyses were conducted in GraphPad Prism version 10.0 (GraphPad Software, San Diego, CA). A three-way mixed model ANOVA with sex (male vs. female, between subject's), leg (immobilized vs. control), and time (pre-ULLS, post-ULLS, and postrehabilitation) as within subject's factors was used to compare differences in leg extension strength. A two-way mixed model ANOVA was used to assess changes in integrated muscle protein synthesis rates during the disuse period with sex (male vs. female, between subjects), leg (immobilized vs. control, within subjects) as factors. A two-way mixed model ANOVA was used to assess changes in integrated muscle protein synthesis rates in the immobilized leg between the disuse and rehabilitation periods with time (disuse vs. rehabilitation, within subjects) and sex (males vs. females, between subjects) as factors. For muscle fiber CSA in the immobilized leg, a two-way mixed model ANOVA with sex (males vs. females, between subjects) and time (disuse vs. rehabilitation, within subjects) as factors was used. A one-way repeated measures ANOVA was used to assess for changes in protein content/phosphorylation in the immobilized leg. For all ANOVAs, when a significant interaction was found, Bonferroni post hoc tests were applied to locate individual differences. Statistical significance was set at $P < 0.05$.

RESULTS

Compliance with Unilateral Lower Limb Suspension

To check for compliance with the unilateral lower limb suspension (ULLS) protocol, we used both lower limb skin temperature and accelerometry data. Total activity counts in the suspended leg were lower compared with the control leg (mean ULLS leg = 154,634 ± 8,192 vector magnitude counts/day; mean control leg = 321,967 ± 23,108 vector magnitude counts/day, $P < 0.001$) throughout every day of the ULLS period (Supplemental Fig. S1). Dorsal foot skin temperature in the ULLS leg decreased over time throughout the disuse period [pre-ULLS = 95.0 ± 0.5°F vs. during ULLS (*day 4*) = 93.5 ± 1.6°F] and was significantly reduced after 7 days (post-ULLS = 92.9 ± 0.5°F, $P < 0.001$). The right (weight bearing) leg dorsal foot skin temperature did not change over time [pre-ULLS = 94.6 ± 0.6°F, during ULLS (*day 4*) = 96.1 ± 0.4°F, post-ULLS = 94.9 ± 0.5°F]. Together these data indicate that all subjects were very compliant with the ULLS protocol.

Leg Strength

Maximum unilateral leg extension one repetition maximum (1-RM) decreased by—24.1 ± 2.1% in males (pre-disuse 1-RM = 65.1 ± 6.8 kg, post-disuse 1-RM = 49.4 ± 4.6 kg) and to the same extent—17.0 ± 2.3% in females (pre-disuse 1-RM = 32.2 ± 1.6 kg, post-disuse 1-RM = 26.8 ± 1.0 kg) (leg × time × sex interaction, $P > 0.05$, $\eta_P^2 = 0.08$, 95% CI [0.00, 0.102], but time × leg interaction was significant ($P < 0.05$, $\eta_P^2 = 0.30$, 95% CI [0.00, 0.34]) (Fig. 2). The main effect of sex was significant ($P < 0.001$, $\eta_P^2 = 0.53$, 95% CI [0.20, 1.00]) with males showing greater overall strength than females. Males were significantly stronger than females for both IMM and CON leg at pre-ULLS ($P < 0.05$). After the exercise rehabilitation period, males leg extension 1-RM was 59.2 ± 6.1 kg (increased 19.8 ± 5.0% vs. post-ULLS) and females was 31.0 ± 2.4 kg (increased 16.0 ± 8.6% vs. post-ULLS). There was no difference in 1-RM for the IMM leg between post-ULLS and post-rehab in either the males or females. Furthermore, there was no difference in 1-RM between the IMM leg and CON leg post-rehab in either the males or females. 1-RM did not change in males or females after ULLS or after rehabilitation in the CON leg.

Leg Lean Mass

There was no difference in leg lean mass pre-ULLS in females (IMM leg = 6,622.5 ± 16.0 g; CON leg = 6,779.8 ± 16.2 g, $P > 0.05$) or males (IMM leg = 9,300.4 ± 35.9 g; CON leg = 9,427.5 ± 41.3 g, $P > 0.05$). The main effect of time and leg were nonsignificant, but the main effect of sex was significant

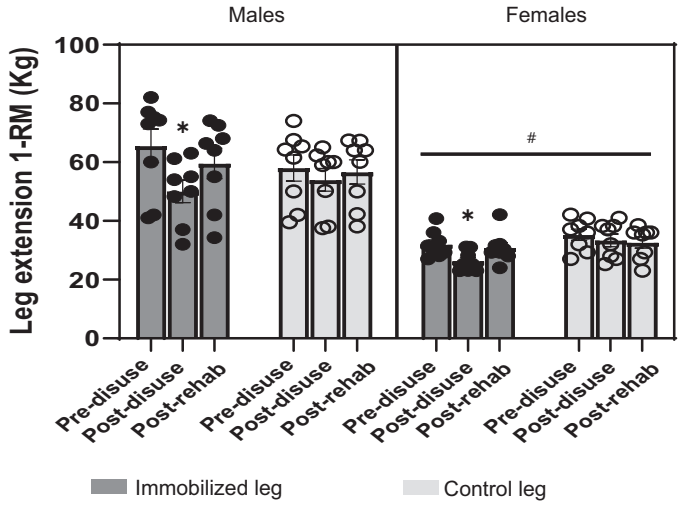

Figure 2. Leg extension strength in males and females, in both the control and immobilized legs, pre-disuse, post-disuse, and post-rehabilitation. A three-way ANOVA was used to see if male and female leg strength was altered by 7 days of unilateral lower limb suspension and 7 days of resistance exercise rehabilitation (4 sessions, 1 session every other day). The main effect of time and sex were significant ($P < 0.001$). The time × leg and time × sex interaction effects were significant, but the leg × sex and leg × time × sex interaction effects were nonsignificant $P > 0.05$. The leg × sex and leg × time × sex interaction effects were nonsignificant ($P > 0.05$). *Significant difference from pre-disuse, #significant main effect of sex. $n = 16$, 8 male and 8 female subjects. Data are means ± SE.

($P < 0.001$), such that the females had lower leg lean mass than males at all timepoints. The time × leg, time × sex, and time × leg × sex interactions were nonsignificant, such that leg lean mass did not change after disuse or after rehabilitation in either the males or females in the IMM and CON legs.

Muscle Fiber Cross-Sectional Area

Analysis of mean fiber cross-sectional area (CSA) showed no change in myosin heavy chain (MyHC) type 1 and type 2x fibers in the immobilized leg after the disuse period or after the rehabilitation period (time × sex interaction and main effect of time both, $P > 0.05$) (Fig. 3, *A* and *C*). Although the main effect of sex was significant with males having greater CSA compared with females at all time points for all fiber types MyHC type 1, type 2a, and type 2x ($P < 0.05$). In females, for MyHC type 1 fibers approached a trend to decrease from pre- (3,501.1 ± 703.2 μm^{-2}) to post-disuse (3,157.2 ± 647.5 μm^{-2}) ($P = 0.12$). MyHC type 2a fibers experienced a reduction in mean CSA from pre- to post-disuse to the same extent in both males (pre-disuse CSA = 4,042.2 ± 299.6, post-disuse CSA = 3,652.9 ± 280.0 μm^{-2}, −9.6 ± 1.0%, $P < 0.05$) and females (pre-disuse CSA = 3,252.9 ± 722.1 μm^{-2}, post-disuse CSA = 2,919.5 ± 646.4 μm^{-2}, −10.2 ± 7.0%, main effect of sex $P < 0.05$, $\eta_P^2 = 0.35$, 95% CI [0.006, 0.431]) and time ($P < 0.001$, $\eta_P^2 = 0.88$, 95% CI [0.758, 0.914]), time × sex interaction ($P > 0.05$, $\eta_P^2 = 0.17$, 95% CI [0.00, 0.364]) (Fig. 3*B*). This reduction in MyHC type 2a CSA after disuse was partially recovered by rehabilitation in males (post-rehabilitation CSA = 3,898.8 ± 244.1 μm^{-2}, $P > 0.05$ from pre-ULLS) and females (post-rehabilitation CSA = 3,040.4 ± 660.0 μm^{-2}, $P > 0.05$ from pre-ULLS).

Body Water Enrichment and Daily Myofibrillar Protein Synthesis Rates

Saliva ^2H enrichment over the week of ULLS and the week of rehabilitation can be observed in Fig. 4, *A* and *B*. Twenty-four hours after the D_2O bolus dose, body water enrichment increased to 0.54 ± 0.05% and decayed over the week of ULLS. Twenty-four hours after the top-up dose of D_2O on the first day of rehabilitation, body water enrichment increased to 0.52 ± 0.04% and decayed over the week of rehabilitation.

When assessing how integrated muscle protein synthesis (MPS) rates changed during disuse in the IMM and CON leg of males and females, the main effect of leg was significant ($P < 0.05$, $\eta_P^2 = 0.25$, [95% CI: 0.00, 0.36]), but sex was nonsignificant ($P > 0.05$, $\eta_P^2 = 0.006$ [95% CI: 0.00, 0.12]) and the leg × sex interaction was nonsignificant ($P > 0.05$, $\eta_P^2 = 0.02$ [95% CI: 0.00, 0.19]) (Fig. 4). Seven days of ULLS reduced

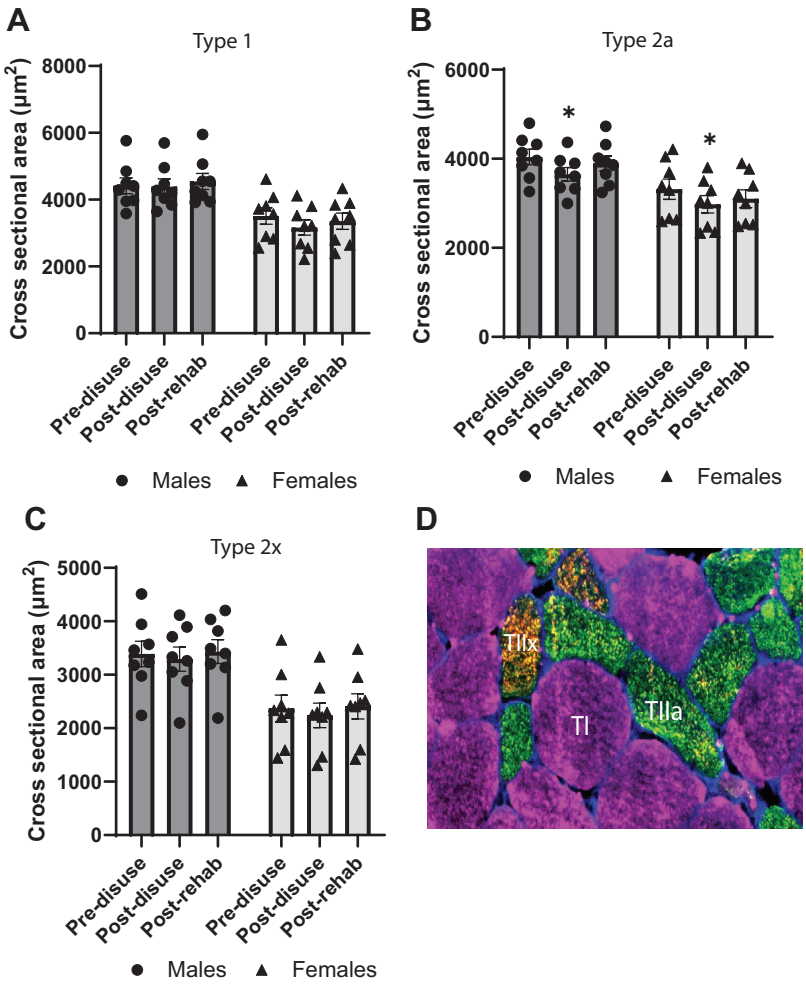

Figure 3. Vastus lateralis muscle fiber cross-sectional area in the immobilized leg pre-disuse, post-disuse, and post-rehabilitation in males and females, in MyHC type 1 (*A*), type 2a (*B*), and type 2x (*C*) fibers. Data presented are means ± SE. *D*: representative image. Two-way repeated measures ANOVA were used to compare each fiber type over time. *Significant difference from predisuse $P < 0.05$. $n = 16$, 8 male and 8 female subjects. Data are means ± SE.

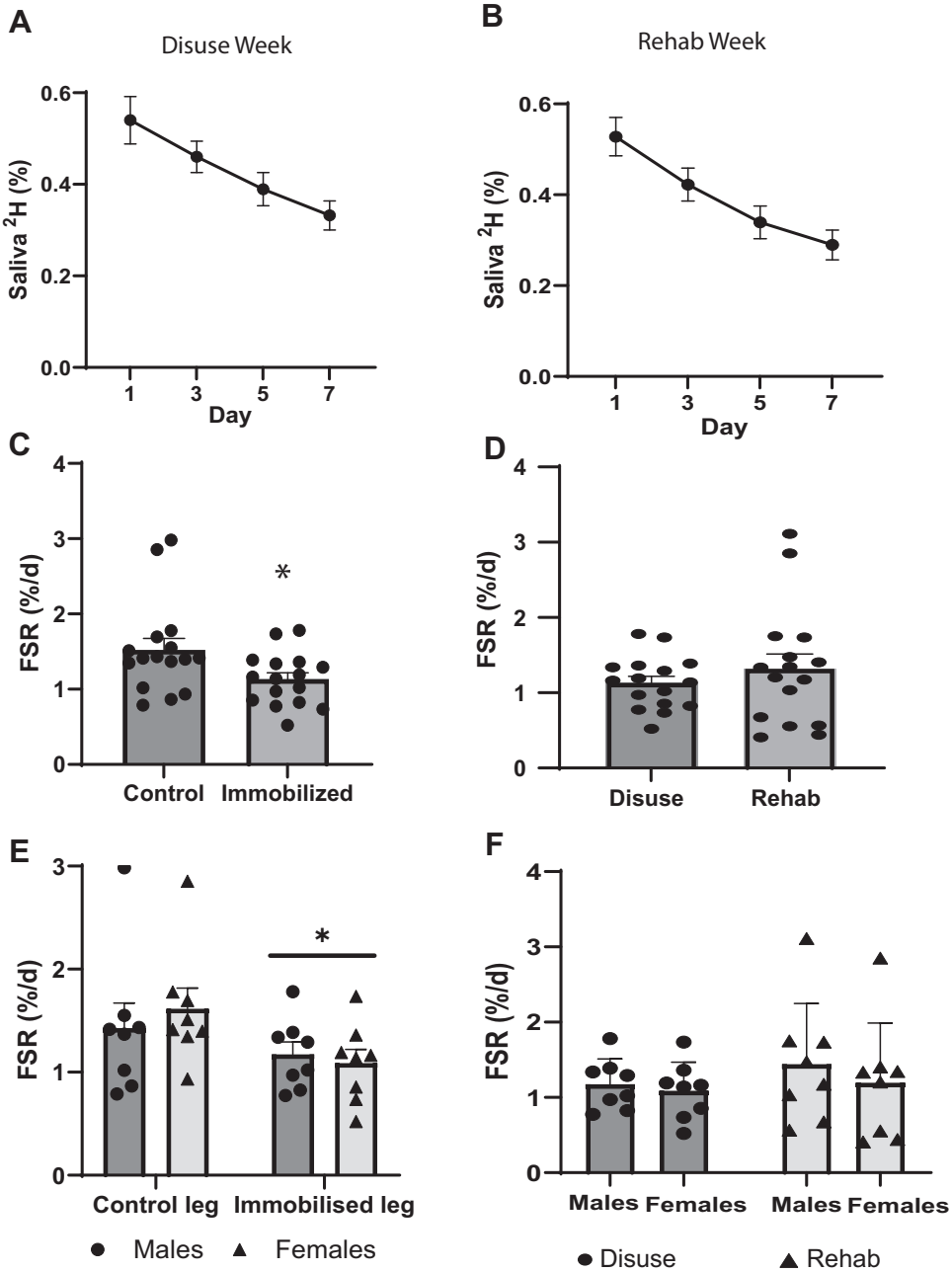

Figure 4. Myofibrillar protein FSR (%·day^{-1}) calculated from the saliva precursor pool. Previous research (38) has shown that when using deuterium oxide (^2H_2O) as a stable isotope tracer saliva ^{2}H enrichment can be used as a precursor pool to calculate FSR instead of the traditionally used plasma precursor pool ([^{2}H]-alanine) as they correlate strongly when they are used to calculate FSR. *A* and *B*: saliva ^{2}H enrichment (%) during the week of ULLS and rehab week, respectively. *C* and *D*: myofibrillar FSR (%·day^{-1}) during the week of ULLS and the week of rehab, respectively, males and females combined. *E* and *F*: myofibrillar FSR (%·day^{-1}) during the week of ULLS and rehab with males and females presented separately. *Significant difference from control leg ($P < 0.05$). $n = 16$, 8 male and 8 female subjects. Data presented are means ± SE. FSR, fractional synthesis rate; ULLS, unilateral lower limb suspension.

integrated MPS rates in the disused leg compared with the control leg ($P < 0.01$) and to a similar extent in males (CON leg FSR = 1.43 ± 0.24%·day^{-1}, IMM leg FSR = 1.17 ± 0.10%·day^{-1}) and females (CON leg FSR = 1.62 ± 0.20%·day^{-1}, IMM leg FSR = 1.08 ± 0.14%·day^{-1}) (Fig. 4). Although not significant the interaction differences [(IMM − CON) males—(IMM − CON) females] was −0.28, which suggests a small nonsignificant difference toward a greater reduction in MPS in females compared with males.

When assessing how 1 wk of physical rehabilitation affected integrated MPS rates in the IMM leg between the disuse and rehabilitation phases. Although numerically higher (IMM leg MPS rates were 22.8 ± 24.7% and 9.6 ± 39.9% higher during the rehabilitation compared with the disuse in males and females, respectively) the main effect of time ($P > 0.05$, $\eta_p^2 = 0.04$ [95% CI: 0.00, 0.20]) and sex ($P > 0.05$, $\eta_p^2 = 0.02$ [95% CI: 0.00, 0.19]), as well as the time × sex ($P > 0.05$, $\eta_p^2 = 0.005$ [95% CI: 0.00, 0.14]) interaction were all nonsignificant.

Immunoblotting for mTOR Signaling

There was no change in the phosphorylation of rpS6$^{Ser235/236}$ or 4E-BP1^{Thr37} between pre- and post-ULLS and post-ULLS and postrehabilitation time points in the IMM leg (both, $P > 0.05$, Fig. 5). No change was observed in total-rpS6, total-4E-BP1, or the housekeeping protein β-tubulin at any point during the study in the IMM leg.

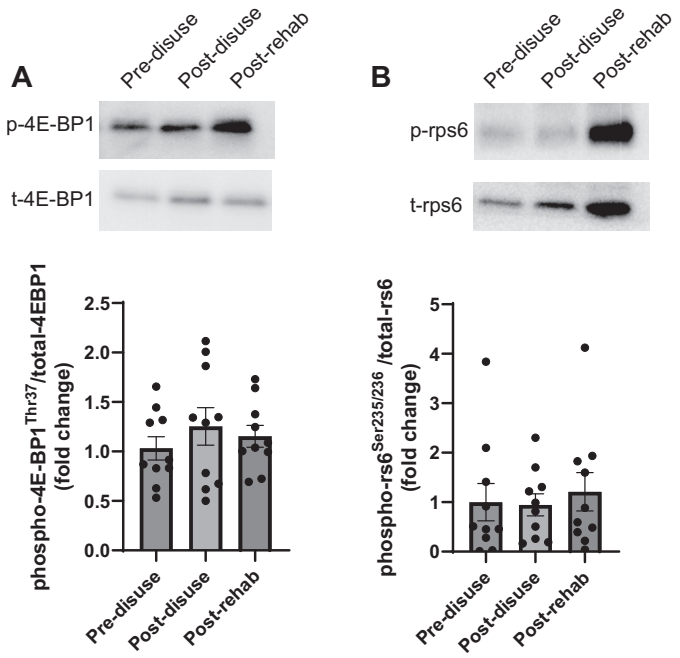

Figure 5. Changes in protein expression of 4E-BP1^{Thr37} (*A*) and rpS6$^{Ser235/236}$ (*B*) pre-disuse, post-disuse, and post-rehabilitation in the immobilized leg. Protein phosphorylation for all targets is presented relative to respective total protein expression, with baseline normalized to 1. Values are means ± SE. $n = 10$ male and female subjects combined. Significance was set at $P < 0.05$. No significant time effect was found for 4E-BP1^{Thr37} or rpS6$^{Ser235/236}$. Representative Western blot lanes appear above each graph for the pre-disuse, post-disuse, and post-rehabilitation time points.

Mitochondrial Respiration

Leak (state 2) respiration was unaffected by unilateral lower limb suspension in both females and males in both the IMM and CON leg (time × leg × sex, $P > 0.05$). Coupled respiration after the addition of succinate (state $3_{+\text{succinate}}$) remained unchanged by unilateral lower limb suspension in males and females and in both legs (time × leg × sex, $P > 0.05$). Finally, electron transfer system capacity (maximal uncoupled respiration) was also unaltered in males and females across the week of ULLS in both the IMM and CON legs (time × leg × sex, $P > 0.05$; Fig. 6).

Skeletal muscle mitochondrial respiratory function was also unaltered during rehabilitation. Leak (state 2) respiration, coupled respiration after the addition of succinate (state $3_{+\text{succinate}}$) and electron transfer system capacity (maximal uncoupled respiration) were unaltered in IMM leg after disuse compared with after rehabilitation for both males and females (time × sex, $P > 0.05$; Fig. 6).

Dietary Analysis

Table 2 shows participants habitual dietary intake for 3 days preceding unilateral lower limb suspension, 3 days during unilateral lower limb suspension, and for 3 days during rehabilitation. No differences in macronutrient intake (protein, carbohydrate, or fat) or energy intake were observed between males and females over the course of the study (i.e., pre-ULLS, ULLS phase, and rehab phase) (sex × time, $P > 0.05$).

DISCUSSION

In the current study, we applied a deuterated water approach to assess integrated myofibrillar protein synthesis (MPS) rates throughout 1 wk of unilateral lower limb suspension and 7 days of physical rehabilitation in healthy late-midlife males and postmenopausal females. We report that late-midlife males and postmenopausal females experienced the same reduction in integrated MPS over 7 days of unilateral lower limb suspension. This was accompanied by a reduction in the cross-sectional area (CSA) of type 2a muscle fibers as well as a reduction in leg extension strength, both of these reductions were of a similar magnitude in males and females. Although females did approach a trend for a decline in type 1 fiber CSA. Seven days of physical rehabilitation partially recovered MPS, leg extension strength, and muscle fiber cross-sectional area in males and females.

During the week of unilateral lower limb suspension (ULLS) integrated MPS reduced by ~25.7% (3.7%·day^{-1}) in all subjects. Although there was non significant time × sex interaction, there was a small effect size ($\eta_P^2 = 0.02$) as females experienced a 32.7% (4.7%·day^{-1}) reduction in integrated MPS in the IMM compared with CON leg, whereas males experienced a 17.8% (2.5%·day^{-1}) reduction. However, the change in integrated MPS during disuse was not accompanied by a change in basal anabolic signaling. Most of the previous work that has measured integrated MPS using deuterated water during short-term unilateral leg immobilization has been conducted on younger adults. Previous research in males, who underwent short-term leg immobilization (i.e., ≤ 1 wk), showed that integrated MPS declined by −17% (4%·day^{-1}) (18) and −36% (5.1%·day^{-1}) (24). In contrast, in young females, the reduction in integrated MPS was slightly lower at −14% (0.67%·day^{-1}), although this was over 2 wk (25). In the present study, it should be noted that although non significant the magnitude of reduction in integrated MPS in females was almost twice that of males (i.e., −32.7% vs. −17.8%). This would support previous research that has shown that females experience greater muscle weakness after a period of bed rest during an intensive care unit (ICU) stay (19). One reason for this could be that short-term disuse induces anabolic resistance to protein ingestion (13) and older females have a diminished response to protein ingestion compared with age-matched males in free living conditions (27). In the present study, there was no difference in protein intake between the sexes during the disuse period (Table 2). Thus, it may be the case that the reduced anabolic response to protein ingestion accumulated over the disuse period and resulted in a numerically (but not significantly) lower MPS in females compared with males during disuse. Interestingly, we detected minimal changes in basal mTORC1 signaling following disuse and rehabilitation. This is somewhat to be expected as we did not provide a protein anabolic challenge to our participants as we did in our bed rest study in older adults (17).

Seven days (1 session every other day, 4 sessions total) of resistance exercise rehabilitation partially recovered integrated MPS in the immobilized leg by 16.2% (2.3%·day^{-1}) in all participants. Although there was no significant difference in integrated MPS between males (22.8, 3.3%·day^{-1}) and females (9.6%, 1.4%·day^{-1}) during rehab, there was a small interaction effect size ($\eta_P^2 = 0.005$), which may suggest that

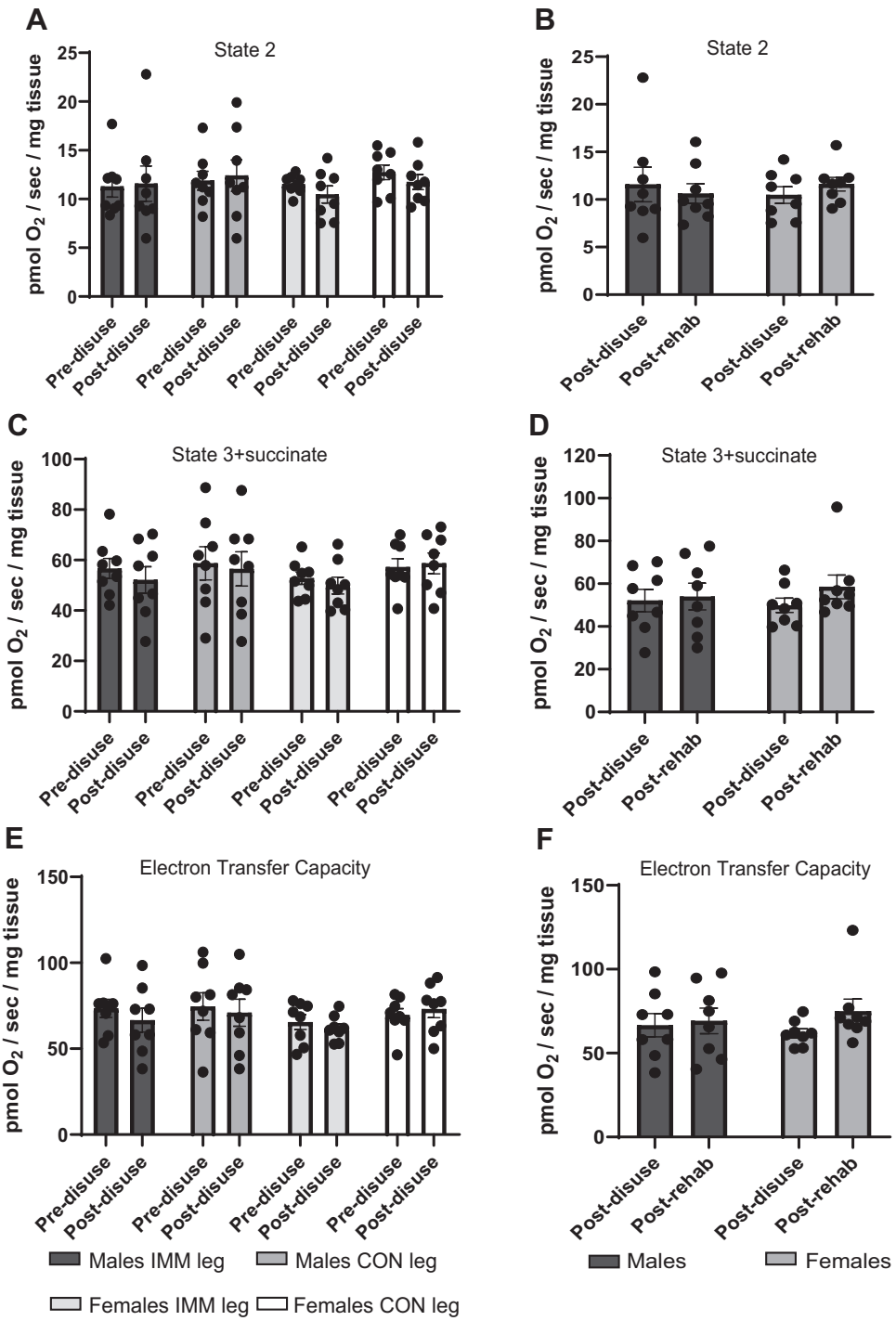

Figure 6. Skeletal muscle mitochondrial respiration measurements. *A*, *C*, and *E* display mitochondrial respiration in males and females in both the immobilized (IMM) and control (CON) legs pre- and post-disuse. *B*, *D*, and *F*, display mitochondrial respiration measurements in the IMM leg in males and females at post-ULLS and post-rehab timepoints. Data are means ± SE, $n = 16$; 8 male and 8 female subjects. ULLS, unilateral lower limb suspension.

late-midlife males may recover integrated MPS to a greater extent than females during short-term rehabilitation after short-term muscle disuse. This may be due to older postmenopausal women having a reduced anabolic response to resistance exercise compared with age-matched males (40–42), potentially due to reduced estrogen levels (43). Alongside integrated MPS not fully recovering during rehabilitation in females, muscle fiber cross-sectional area also failed to fully recover compared with pre-ULLS levels in males and to a similar extent in females. This agrees with previous work that states that older individuals require longer periods of rehabilitation to fully recover from a period of disuse (11) and with regards to leg strength, young adult females took one extra session to regain leg strength compared with males after 1 wk of unilateral leg immobilization (21).

In addition to measuring skeletal muscle size and integrated MPS, we examined changes in unilateral leg extension strength. In both males and females, leg extension strength was significantly reduced post-ULLS. There was a small

Table 2. *Dietary intake and physical activity levels measured during; predisuse, disuse, and rehabilitation*

	Predisuse		Disuse		Rehabilitation	
	Males	Females	Males	Females	Males	Females
Energy intake, Kcal·day^{-1}	2,461 ± 354	2,031 ± 175	2,258 ± 341	1,803 ± 140	2,264 ± 256	2,294 ± 169
Protein intake, g·day^{-1}	114 ± 24	75 ± 7	120 ± 20	77 ± 5	114 ± 20	91 ± 8
Protein intake, g·kg·bm·day^{-1}	1.2 ± 0.2	1.1 ± 0.1	1.3 ± 0.2	1.1 ± 0.1	1.3 ± 0.2	1.4 ± 0.2
Fat intake, g·day^{-1}	107 ± 15	86 ± 7	93 ± 13	77 ± 12	99 ± 9	106 ± 10
CHO intake, g·day^{-1}	238 ± 46	244 ± 13	224 ± 40	172 ± 15	229 ± 39	236 ± 19
Light physical activity, h·day^{-1}	1.0 ± 0.1	0.9 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	1.3 ± 0.1	1.1 ± 0.1
Moderate physical activity, h·day^{-1}	2.1 ± 0.2	1.8 ± 0.2	2.2 ± 0.3	1.7 ± 0.2	3.0 ± 0.5	1.9 ± 0.2
Vigorous physical activity, h·day^{-1}	0.3 ± 0.1	0.1 ± 0.2	0.3 ± 0.2	0.1 ± 0.02	0.3 ± 0.04	0.1 ± 0.1
Total physical activity, h·day^{-1}	3.4 ± 0.3	2.7 ± 0.2	3.7 ± 0.4	2.8 ± 0.2	4.6 ± 0.6	3.1 ± 0.4

Values represent means ± SE, $n = 16$, 8 male and 8 female subjects.

interaction effect size ($\eta_P^2 = 0.008$) for males to decline to a greater extent compared with females (−24% vs. −17%). Previous research has suggested that young adult females lose a greater amount of leg strength compared with age-matched males after 7 (21) and 14 days of unilateral leg immobilization (22). This difference is unlikely to be due to the reduction in muscle size after disuse, as males and females experienced the same reduction in type 2a fiber cross-sectional area. Furthermore, in young adults, females also seem to experience a greater loss of neuromuscular function compared with males (44), however, further investigation is required to assess if this is the case in older adults.

Most of the previous research that has assessed the effectiveness of interventions aimed at alleviating the negative effects of short-term muscle disuse as well as promoting recovery during rehabilitation have focused on males (4, 6, 45–48). Given that integrated MPS rates declined by a similar amount in late-midlife males and postmenopausal females after short-term ULLS, it is likely that interventions aimed at reducing muscle atrophy during disuse i.e., electrical stimulation will work in females, as has already been demonstrated in males (49, 50). For other interventions that have shown mixed results at alleviating reductions in muscle atrophy during short-term disuse, namely, protein or essential amino acid supplementation, which has sometimes been successful (4, 6, 45) and other times not (46, 48, 51). It is plausible that due to females experiencing a numerically (but not significantly) greater reduction in integrated MPS compared with males, these interventions will have a greater chance at working in late-midlife postmenopausal females. Conversely, due to the numerically greater inhibition in integrated MPS in late-midlife females compared with males during disuse and the numerically blunted increase in integrated MPS in females during rehabilitation. Late-midlife females may benefit more from interventions that sensitize the muscle to anabolic stimuli as has been demonstrated by omega 3 supplementation (25). As basal phosphorylation of mechanistic target of rapamycin complex 1 (mTORC1) is elevated in skeletal muscle of older adults (52). Future work should investigate interventions that aim to reduce this hyperphosphorylation of mTORC1, before and during disuse, as well as during rehabilitation to assess if this improves the efficacy of interventions that aim to increase integrated MPS during disuse and rehabilitation (i.e., protein supplementation).

The present study has some limitations. Firstly, previous research has shown that when using the ULLS model the control (weight bearing) leg experiences overuse (53, 54), which must be acknowledged especially when making comparisons to the suspended leg. It should also be noted that participants may vary widely in this compensatory activity. Secondly, to reduce participant burden, participants completed 1 wk of rehabilitation. However, rehabilitation from an injury or surgery (e.g., knee arthroplasty) would require longer periods of rehabilitation (55), which may limit the applicability of this study to a real-world situation. Third, due to the small sample size of the study some of the results presented are exploratory and should be followed up in future research studies.

In conclusion, our data show that late-midlife males and postmenopausal females experience a reduction in integrated MPS and muscle fiber cross-sectional area that is similar between males and females. Both males and females experienced reductions in leg strength, although males experienced the numerically greater decline. Seven days of resistance exercise rehabilitation partially reverses the decline in muscle strength, cross-sectional area and integrated MPS but longer rehabilitation periods are required for full recovery in late-midlife adults.

DATA AVAILABILITY

Data will be made available upon reasonable request.

SUPPLEMENTAL MATERIAL

Supplemental Fig. S1: https://doi.org/10.6084/m9.figshare.29640884.v1.

ACKNOWLEDGMENTS

We thank the research participants for time and effort completing this study. In addition, we acknowledge to the nursing staff at the Institute for Translational Sciences Clinical Research Center.

GRANTS

This work was supported by NIH/NIA Grant Nos. R01 AG064683, P30 AG044271, and P30 AGO24832.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

S.P.K., E.J.A.-L., A.J.M., E.V., D.P.-J., and B.B.R. conceived and designed research; S.P.K., Z.D.V.R., H.K., V.R., and D.R.A. performed experiments; S.P.K., Z.D.V.R., H.K., V.B., D.R.A., and B.B.R. analyzed data; S.P.K., E.J.A.-L., A.J.M., D.R.A., E.V., and B.B.R. interpreted results of experiments; S.P.K., H.K., and B.B.R. prepared figures; S.P.K. and B.B.R. drafted manuscript; S.P.K., Z.D.V.R., H.K., V.B., E.J.A.-L., A.J.M., V.G.R., D.R.A., E.V., and B.B.R. edited and revised manuscript; S.P.K., Z.D.V.R., H.K., V.B., E.J.A.-L., A.J.M., V.G.R., D.R.A., E.V., and B.B.R. approved final version of manuscript.

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