1. Materials and Methods

1.1 Experimental animals

Male Fischer Brown Norway hybrid rats (F344 X BN F1) were obtained from the National Institutes of Aging colony. Rats, 3 months old, were singly housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International - accredited animal quarters. Food and water were provided ad libitum. Temperature and light:dark cycle (dark cycle – 7:00 am to 7:00 pm) were held constant. The animals were acclimated to housing conditions for one week and randomized to the SSC trained group or nontrained group. For the trained group, 3-month-old rats were exposed to SSC training for one month, thereupon reaching 4 months of age (Figure 1). At 7 months of age, performance testing was completed for both groups of rats – rats that underwent the training and rats which were never trained. After performance testing and while still anesthetized (isoflurane gas 2-3% by volume), all animals were euthanized by pentobarbital (100-300 mg/kg body weight) intraperitoneal injection followed by exsanguination. All animal procedures were done in accordance with the Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press) and approved by the Animal Care and Use Committee at the National Institute for Occupational Safety and Health in Morgantown, WV.

1.2 SSC training

The SSC training was based on a previously described procedure which resembles high-intensity resistance training and has repeatedly been demonstrated to induce gains in performance and muscle mass for 3 month old rats (Cutlip et al. 2006; Naimo et al. 2019; Rader

and Baker 2017; Rader et al. 2016a; Rader et al. 2016b). Each rat was anesthetized with isoflurane gas (2-3% by volume), placed supine on a heated table, the left knee was secured in 90º flexion, and the left foot was secured to a fixture containing a load cell. Platinum electrodes were placed subcutaneously at the region of the common peroneal nerve for activation of dorsiflexor muscles at 4-V magnitude, 0.2-ms pulse duration, and 120-Hz frequency, optimal settings for maximal contraction (Geronilla et al. 2003).

Exposure to SSCs consisted of 8 sets with 2-minute intervals between sets and 10 SSCs per set with 2-second intervals between SSCs to be comparable to the timing experienced during resistance training (Vaczi et al. 2011). For each SSC, the dorsiflexor muscles were maximally activated, while the ankle was set to 90º for 100 ms (i.e. isometric phase), then rotated to 140º at 60º/s (i.e. stretch phase), returned to 90º at the same velocity (i.e. shorten phase), and lastly, deactivated 300 ms later. The velocity of 60° per second was chosen to promote muscle adaptation rather than overt muscle degeneration and injury – a feature observed at higher velocities (e.g. 500° per second) (Baker et al. 2010; Baker et al. 2008; Cutlip et al. 2006). Each rat was exposed to this protocol 3 times per week (i.e. Monday, Wednesday, and Friday) for 4 weeks. Performance measures (i.e. torque values for the isometric and stretch phases and work values for the stretch and shorten phases) for the first SSC of each of the sessions during the first and last week of training were averaged to determine initial and final training values, respectively. At 3 months following the cessation of training (i.e. 7 months of age), muscles were exposed to a SSC to assess performance and compared with that of muscles from age-matched (i.e. 7 month old) nontrained rats. Immediately following this assessment, both right and left tibialis anterior (TA) muscles were surgically removed, weighed, and the tibia lengths recorded.

1.3 Total RNA and mRNA analysis

A ~65 mg portion of frozen TA muscle tissue was homogenized with a Mini-BeadBeater 8 (Biospec) while in a vial of 1 ml of TRIzol with 1.0 mm zirconia beads (BioSpec, Cat#11079110zx). The RNAqueous phenol-free total RNA Isolation Kit (Ambion, Cat# AM1912) was used to isolate RNA and total RNA concentration was quantified (NanoDrop 2000c, Thermo Fisher Scientific, Waltham, MA). The cDNA was synthesized utilizing 0.5 µg of RNA and the RT^2 First Strand Kit (Qiagen, Cat# 330401). The expression of genes relevant to growth and energy sensing was investigated using the RT^2 Profiler™ PCR Array for mTOR signaling (Qiagen, Cat# PARN-098Z) per manufacturer's instructions. Gene expression was considered significantly differentially regulated when fold regulation exceeded 1.3-fold regulation (below 0.769 fold change or above 1.3 fold change) and $P < 0.05$(Rader and Baker 2020; Rader et al. 2017).

1.4 Immunofluorescence

The mid-belly of each TA muscle was covered with tissue freezing media and immersed in isopentane (-160 °C). This tissue was then cryosectioned at 12 µm thickness. Sections were fixed in HistoChoice (Sigma-Aldrich; H2904) for 45 minutes, washed (3 x 5 minutes in PBS), washed (3 x 5 minutes in PBS), and then blocked with 5% goat serum in 0.4% Triton X-100 in PBS for 1 hour. A primary polyclonal antibody for laminin (Sigma-Aldrich; L9393; 1:50) was applied for 1 hour. Sections were washed (3 x 5 minutes in PBS) and secondary antibody (donkey anti-rabbit IgG Cy3 at 1:100 in PBS with 0.4% Triton X-100) was applied for 30 minutes. After 3 x 5 minute washes in PBS, sections were mounted with Prolong™ Gold

Antifade Reagent (Thermo Fisher Scientific; P36931) with 4′, 6-diamidino-2-phenylindole (DAPI). With the investigator blinded to sample identification, each muscle section was imaged by a standardized method (Baker et al. 2006; Rader and Baker 2020; Rader et al. 2018; Rader et al. 2016b). At 2 mm offset from either side of the midpoint, 5 equally spaced fields (at 20X magnification) were imaged for a total of 10 images. Image analysis utilized ImageJ (version 1.46, National Institutes of Health, USA). Each muscle fiber (118 ± 2 fibers per section) was traced to determine muscle fiber size. Number of nuclei within each muscle fiber was counted to assess number of nuclei per muscle fiber. Nuclei not in contact with the laminin boundary were designated as central nuclei. Muscle fiber area per nucleus was determined for each muscle fiber by dividing the muscle fiber area by the number of nuclei per fiber. ImageJ functions of watershed and particle analysis were utilized for each DAPI image to yield nuclei size and circularity of all the nuclei (2626 ± 70 nuclei per section) in the muscle tissue for the regions imaged (Eidet et al. 2014; Rader and Baker 2020). Small nuclear fragments (< 5 μm^2) were omitted from the measurements by modifying particle area range (Eidet et al. 2014). The index of circularity was determined by the equation 4π (area/perimeter^2) with a perfect circle as a value of 1 and increasing elongation as the value decreases.

References

Baker, B.A., Hollander, M.S., Kashon, M.L., and Cutlip, R.G. 2010. Effects of glutathione depletion and age on skeletal muscle performance and morphology following chronic stretch-shortening contraction exposure. European journal of applied physiology **108**(3): 619-630. doi:10.1007/s00421-009-1258-4.

Baker, B.A., Hollander, M.S., Mercer, R.R., Kashon, M.L., and Cutlip, R.G. 2008. Adaptive stretch-shortening contractions: diminished regenerative capacity with aging. Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme **33**(6): 1181-1191. doi:10.1139/H08-110.

Baker, B.A., Mercer, R.R., Geronilla, K.B., Kashon, M.L., Miller, G.R., and Cutlip, R.G. 2006. Stereological analysis of muscle morphology following exposure to repetitive stretch-shortening cycles in a rat model. Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme **31**(2): 167-179. doi:10.1139/h05-009.

Cutlip, R.G., Baker, B.A., Geronilla, K.B., Mercer, R.R., Kashon, M.L., Miller, G.R., et al. 2006. Chronic exposure to stretch-shortening contractions results in skeletal muscle adaptation in young rats and maladaptation in old rats. Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme **31**(5): 573-587. doi:10.1139/h06-033.

Eidet, J.R., Pasovic, L., Maria, R., Jackson, C.J., and Utheim, T.P. 2014. Objective assessment of changes in nuclear morphology and cell distribution following induction of apoptosis. Diagn Pathol **9**: 92. doi:10.1186/1746-1596-9-92.

Geronilla, K.B., Miller, G.R., Mowrey, K.F., Wu, J.Z., Kashon, M.L., Brumbaugh, K., et al. 2003. Dynamic force responses of skeletal muscle during stretch-shortening cycles. European journal of applied physiology **90**(1-2): 144-153. doi:10.1007/s00421-003-0849-8.

Naimo, M.A., Rader, E.P., Ensey, J., Kashon, M.L., and Baker, B.A. 2019. Reduced Frequency of Resistance-Type Exercise Training Promotes Adaptation of the Aged Skeletal Muscle Microenvironment. Journal of applied physiology. doi:10.1152/japplphysiol.00582.2018.

Rader, E.P., and Baker, B.A. 2017. Inflammaging and the Age-Specific Responsiveness to Stretch-Shortening Contractions. Exercise and sport sciences reviews **45**(4): 195-200. doi:10.1249/JES.0000000000000123.

Rader, E.P., and Baker, B.A. 2020. Age-dependent stress response DNA demethylation and gene upregulation accompany nuclear and skeletal muscle remodeling following acute resistance-type exercise in rats. Facets (Ott) **5**(1): 455-473. doi:10.1139/facets-2019-0060.

Rader, E.P., Naimo, M.A., Ensey, J., and Baker, B.A. 2017. Agonist muscle adaptation accompanied by antagonist muscle atrophy in the hindlimb of mice following stretch-shortening contraction training. BMC Musculoskelet Disord **18**(1): 60. doi:10.1186/s12891-017-1397-4.

Rader, E.P., Naimo, M.A., Ensey, J., and Baker, B.A. 2018. High-intensity stretch-shortening contraction training modifies responsivity of skeletal muscle in old male rats. Experimental gerontology **104**: 118-126. doi:10.1016/j.exger.2018.02.009.

Rader, E.P., Layner, K., Triscuit, A.M., Chetlin, R.D., Ensey, J., and Baker, B.A. 2016a. Age-dependent Muscle Adaptation after Chronic Stretch-shortening Contractions in Rats. Aging Dis **7**(1): 1-13. doi:10.14336/AD.2015.0920.

Rader, E.P., Naimo, M.A., Layner, K.N., Triscuit, A.M., Chetlin, R.D., Ensey, J., et al. 2016b. Enhancement of skeletal muscle in aged rats following high-intensity stretch-shortening contraction training. Rejuvenation Res **2**(2): 93-102. doi:10.1089/rej.2016.1816.

Vaczi, M., Tihanyi, J., Hortobagyi, T., Racz, L., Csende, Z., Costa, A., et al. 2011. Mechanical, biochemical, and electromyographic responses to short-term eccentric-concentric knee extensor training in humans. Journal of strength and conditioning research / National Strength & Conditioning Association **25**(4): 922-932. doi:10.1519/JSC.0b013e3181c1fa47.