

Mouse Hind Limb Skeletal Muscle Functional Adaptation in a Simulated Fine Branch Arboreal Habitat

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ABSTRACT

The musculoskeletal system is remarkably plastic during growth. The purpose of this study was to examine the muscular plasticity in functional and structural properties in a model known to result in significant developmental plasticity of the postcranial skeleton. Fifteen weanling C57BL/6 mice were raised to 16 weeks of age in one of two enclosures: a climbing enclosure that simulates a fine branch arboreal habitat and is traversed by steel wires crossing at 45 degrees relative to horizontal at multiple intersections, and a control enclosure that resembles a parking deck with no wires but the same volume of habitable space. At sacrifice, *ex vivo* contractility properties of the soleus (SOL) and extensor digitorum longus (EDL) muscles were examined. Our results demonstrate that EDL muscles of climbing mice contracted with higher specific forces and were comprised of muscle fibers with slower myosin heavy chain isoforms. EDL muscles also fatigued at a higher rate in climbing mice compared to controls. SOL muscle function is not affected by the climbing environment. Likewise, mass and architecture of both EDL and SOL muscles were not different between climbing and control mice. Our data demonstrate that functional adaptation does not require concomitant architectural adaptation in order to increase contractile force.

INTRODUCTION

Over the last several years, Byron and colleagues (2009; 2011; 2015) have utilized a mouse model of climbing behavior in a fine-branch arboreal habitat to simulate musculoskeletal plasticity during growth. The motivation for Byron's research was to experimentally encourage the development of pedal grasping in an animal without innate pedal grasping morphology, in order to model (and perhaps induce) the transition from clawed, pedal grasping to a more powerful pedal grasp with a nail-bearing hallux (Byron et al., 2015), a critical putative stage in primate evolution (Gebo, 2004; Sargis et al., 2007). The results of these studies demonstrate

remarkable skeletal and central nervous system plasticity related to climbing behavior. For example, climbing mice raised in a simulated fine branch arboreal habitat exhibit increased femoral neck angles, larger patellar groove indices, more circular talar heads, increased curvature of the distal third metacarpal, relatively longer caudal vertebral transverse processes, and more robust first metatarsals (Byron et al., 2011; 2015). Additionally, these mice were characterized by thicker granular cell layers in the cerebellar lobes, areas of the brain responsible for coordination of muscle function in the tail (Byron et al., 2013).

The mechanical interactions between bone and skeletal muscle during locomotion are critical to understanding how plasticity in the musculoskeletal system is linked. Endurance exercise, as climbing in the fine branch arboreal habitat can be described, is known to affect functional properties of the muscular system in the absence of structural property adaptation. For example, voluntary wheel running in mice is known to affect metabolic properties of muscles including transformations of myosin heavy chain isoforms expressed in skeletal muscle from faster glycolytic isoforms to slower oxidative isoforms (Fitzsimons et al., 1990; Garland et al., 1995; Sullivan et al., 1995; Allen et al., 2001; Pansarasa et al., 2002; Pellegrino et al., 2005). Additionally, in some cases, these functional property adaptations are independent of structural changes in the muscles themselves (Allen et al., 2001). Therefore, the purpose of the research presented here was to investigate the functional adaptation of hind limb musculature of the growing mouse to low-impact, multi-directional mechanical loading in a simulated fine-branch arboreal habitat in order to understand the extent of plasticity of the muscular system in this model.

MATERIALS AND METHODS

Experimental design. All procedures were approved by the Indiana University School of Medicine Animal Care and Use Committee prior to beginning the study, and all animal care was performed in accordance with institutional policies. Fifteen weanling (21-day old) C57BL/6 mice

(a common inbred strain of laboratory mouse) were purchased from Envigo (Indianapolis, IN) and raised from four weeks of age to 16 weeks of age in custom housing enclosures (see below) (n=7 controls; n=8 climbers). At the end of the experiment, animals were euthanized by CO₂ inhalation.

Custom Housing Enclosures. Animals were raised in one of two custom enclosures: an experimental enclosure and a control enclosure. The experimental enclosure is a 2 ft³ Plexiglas box traversed by multiple 2.25 mm diameter steel wires that create a three-dimensional climbing substrate approximating a fine branch arboreal niche (Byron et al., 2009; 2011; 2013; 2015). Wires are oriented either horizontal to the bottom of the enclosure or at 45 degrees relative to horizontal. The purpose of the wires is to encourage the animals to develop pedal grasping (Byron et al., 2011; 2013; 2015) and to encourage balancing over the narrow substrate to induce continuous multidimensional mechanical loading in the limb skeleton. The wire diameter was chosen based on unpublished data from the Byron lab indicating maximum agility at the chosen diameter (C.D. Byron, unpublished data). The bottom of the enclosure is flooded with water to discourage animals from spending time on the floor; immediately below the top of the water level, a 0.25" caliber chicken wire "safety net" was placed to prevent drowning.

The control enclosure is a 2 ft³ Plexiglas box that is vertically stratified similar to the climbing enclosure. Instead of wires, however, the enclosure consists of four levels with two 45 degree inclined ramps connecting levels like a parking deck. The vertical stratification of the enclosure with Plexiglas levels and no wires obviates the need for mice to balance over narrow substrates but still provides them with ample three-dimensional space to explore.

Feeding boxes, water sources, and nesting sites were initially located in close proximity to one another. After one week, the location of feeding boxes and water sources were randomized to encourage mice to explore all corners of the enclosures. Food and water were available *ad libitum*.

Body composition. Prior to sacrifice at 16 weeks of age, mice were anesthetized by inhalation of 1.7% isoflurane mixed with oxygen (1.5 L/min) and were scanned with a PIXImus II dual x-ray absorptiometer (GE Medical Systems, Lunar Division, Madison, WI) to record total body mass, lean mass, and fat mass.

Ex vivo muscle contractility. Mice were euthanized at 16 weeks of age and the soleus (SOL) and extensor digitorum longus (EDL) muscles were removed for *ex vivo* contractility measurements. These muscles are standard targets for *ex vivo* contractility assays for several reasons: they have different functions (SOL, plantarflexion; EDL, dorsiflexion), they are comprised of different proportions of slow and fast myosin heavy chain isoforms (SOL, slow MHC-1 fibers; EDL, faster MHC-2 fibers), and they are easy to attach to the testing apparatus because they have small tendons on both proximal and distal ends of the muscle belly. Using our previously described approach (Sato et al., 2017), muscles were excised from the hind limbs while immersed in a physiologic salt bath (Ringer's solution) consisting of 136.9 mM NaCl, 2.68 mM KCl, 1.84 mM CaCl₂ dihydrate, 1.03mM MgCl₂ hexahydrate, 5.55 mM dextrose, 11.91 mM NaHCO₃, and 0.44 mM NaH₂PO₄ anhydrous (pH 7.4). Once dissected, stainless steel hooks were fastened to the muscle tendons using 4-0 silk sutures, and muscles were fastened at one end to a force transducer (Aurora Scientific, Inc., Aurora, Ontario) and at the other to an adjustable hook in a fixed position. Muscles were then submerged between two platinum electrodes in a stimulation chamber filled with additional Ringer's solution and bubbled with a 95%/5% concentration of oxygen/carbon dioxide. Electrodes were used to stimulate muscles to contract and the resulting contractility data were recorded and analyzed with the Dynamic Muscle Control (v5.410) and Dynamic Muscle Analysis (v5.111) software packages (Aurora Scientific, Inc., Aurora, Ontario), respectively. Muscle optimal length – the length at which maximum twitch force is achieved – was established, and force-frequency and fatigue-

stimulation regimens were initiated. The force-frequency regimen consisted of supramaximal contractions (1A voltage, 0.5 ms pulse width, 350 ms stimulation duration) triggered at incremental stimulation frequencies (1 to 150 Hz). Between stimulations, muscles were allowed to rest: 1 minute for frequencies between 1 and 70 Hz; 3 minutes for frequencies between 100 and 150 Hz). All muscles reached maximum contractile force at about 100 Hz. Therefore, specific force (size-corrected maximum contractile force) was calculated at 100 Hz by dividing absolute force by the whole muscle physiologic cross-sectional area (PCSA), calculated as follows (Sato et al., 2017): $PCSA (mm^2) = \text{mass (mg)} / [L_o (mm) \times (L/L_o) (1.06 \text{ mg/mm}^3)]$, where L_o is the optimal length of the muscle, L/L_o is the fiber-to-muscle length ratio, and 1.06 mg/mm^3 is the density of skeletal muscle (Mendez and Keyes, 1960; Moorwood et al., 2013). Fiber-to-muscle length ratios of 0.51 for the EDL and 0.72 for the SOL were taken from the literature (Burkholder et al., 1994) and used to calculate PCSA. Half relaxation time – a proxy for calcium sequestration by the sarcoplasmic reticulum prior to contraction – was also measured at 100 Hz. Finally, muscles were stimulated at 70 Hz to fatigue with 100 repeated contractions – one contraction every 0.7 seconds.

Muscle fiber typing. Contralateral SOL and EDL muscles to those that were tested for *ex vivo* contractility were harvested for immunohistochemical examination of muscle myosin heavy chain isoforms (fiber types). The middle of the muscle bellies (~ 1 cm³) were excised, mounted to cork blocks using tissue freezing medium (TBS Durhman, NC), flash frozen using isopentane cooled in liquid nitrogen, and then stored at -80°C until sectioning. Muscle blocks were cut into transverse serial cross-sections (10 µm) and reacted with primary monoclonal antibodies (DSHB University of Iowa) specific to slow myosin heavy chain 1 (MHC-1 (S58)), fast MHC-2a (SC-71), and MHC-1, 2A, 2B (BF35) to determine fiber type proportions. Monoclonal antibody concentrates were diluted 1:200 in phosphate buffered saline (pH 7.4). Serial cross-sections were blocked using 5% goat serum, incubated with primary monoclonal antibodies overnight at

4°C, and reacted with a biotinylated (anti-mouse) secondary antibody labeled with a streptavidin horseradish peroxidase enzyme conjugate. Immunohistochemical reactions were facilitated using the Histostain Plus kit (Invitrogen). Positive reactions were visualized with DAB chromagen and hematoxylin counter stain to observe fiber and nucleus morphology. Muscle fiber-type proportions were determined by dividing the total number of positive reacted fibers to specific monoclonal antibodies in the entire muscle cross-section by the total number of fibers in the cross-section (Organ et al., 2016).

Statistical analysis. Data in figures are presented as mean \pm standard error. All statistics were performed in SPSS v.24 (IBM SPSS Statistics). The overall hypothesis tested was that the EDL and SOL muscles of climbing mice would have enhanced morphological and functional properties. Thus, one-tailed independent samples Student's t-tests were used to evaluate group differences. To test for potential differences in muscle fatigability, *ex vivo* fatigue data were evaluated using ordinary least squares regression on the group mean data for each stimulation number. For both groups (climbers and controls), two regressions were calculated: the first regression was fit to the first 10% of the stimulations and the second regression was fit to the last 90% of the stimulations (Sato et al., 2017). Regressions coefficients (i.e., slopes) were compared using one-tailed independent samples t-tests (Plotnick, 1989; Organ and Ward, 2006). A priori α -levels for all statistical tests were set at 0.05.

RESULTS

Whole body mass was similar between climbing mice and control mice (Fig. 1), even while the composition of whole body mass differed: climbing mice had higher whole-body lean mass and lower whole-body fat mass than control mice (Fig. 1). Individual masses and physiologic cross-sectional area (PCSA) of the soleus (SOL) and extensor digitorum longus (EDL) muscles also were similar between climbing and control mice (Figs. 2a,b). In fact, the

only morphological differences in muscles between climbing and control mice were found in the fiber type proportions of the EDL muscles: climbing mice had a higher proportion of MHC 2a/x fibers and a lower proportion of MHC 2b fibers than control mice (Fig 3b); SOL muscles had similar fiber type proportions between groups (Fig. 3a).

Ex vivo contractility tests revealed that climbing mice EDL muscles contracted with higher specific forces than those of control mice (Fig. 4a), but half relaxation times were not different between groups (Fig. 4b); SOL muscles functioned similarly in climbing and control mice, with no statistical differences in specific forces or half relaxation time (Figs. 4a,b). Finally, fatigue tests showed that SOL muscles fatigued at the same rate in climbing and control mice for both regions of the curve (Fig. 5a), whereas EDL muscles in the climbing mice fatigued at a higher rate than those of control mice in both regions of the curve (Fig. 5b).

DISCUSSION

This experiment aimed to examine the effects of low-impact exercise on the growing mouse hind limb musculature by raising mice in a simulated fine branch arboreal habitat. Previous work in a similar model showed extensive structural adaptations of the hind limb skeleton, including development of a rudimentary grasping hallux, altered foot and ankle skeletal linear dimensions, and structural adaptations of the hallucal metatarsal (Byron et al., 2009; 2011; 2015). Because bone and muscle are both highly plastic tissues (e.g., Lieber, 2002; Dudley-Javoroski and Shields, 2008; Burr and Allen, 2013), and because we know bone structure is altered in this model, we expected to find significant changes in hind limb muscle structure and function in climbing mice compared to control mice.

Data collected with dual x-ray absorptiometry indicated that whole body mass did not differ between climbing mice and control mice at 16 weeks of age. However, the composition of that mass was significantly different between groups: climbing mice had a higher whole body lean mass and lower whole-body fat mass than control mice. Higher whole body lean masses

would suggest that individual muscle masses are higher in the climbing mice. But, this relationship is not reflected in the masses of individual muscles: soleus (SOL) and extensor digitorum longus (EDL) muscle masses were not different between groups. Moreover, the physiologic cross-sectional areas (PCSA) of SOL and EDL did not differ between groups, suggesting that living in the climbing enclosure did not enhance muscle architectural properties like mass and fiber (fascicle) length. Yet, the *ex vivo* contractile data empirically demonstrated enhanced specific force of the EDL muscles in climbing mice compared to control mice.

On the one hand, we have data that suggest no changes in skeletal muscle structure (and estimated function, i.e., PCSA) resulting from low-impact climbing behavior during growth. On the other hand, we have directly measured functional parameters of the EDL muscles that indicate enhanced specific force in climbing mice. On the surface, these appear to be conflicting results. But we explain these data by recalling the shifted fiber type data seen in the EDL, but not in the SOL muscle where there were no differences in functional properties between climbing and control mice. Climbing mice EDLs were characterized by higher proportions of myosin heavy chain (MHC) types 2a and 2x (and lower proportions of type 2b) than control mice. Based on our data, we suggest that climbing mice EDL MHC-2b fibers likely transformed to MHC-2x fibers. Those MHC-2x fibers may have further transformed to MHC-2a fibers. However, it should be noted that we could not directly identify the MHC-2x fibers because our assay lacked a monoclonal antibody specific solely to MHC-2x. The transformation from MHC-2b to MHC-2x (and perhaps MHC-2a) is important to consider because there is a well-established pattern of variation in contraction velocities of fibers expressing different MHC isoforms: MHC-2b fibers are the fastest contracting, followed by MHC-2x fibers, MHC-2a fibers, and finally MHC-1 fibers (Schiaffino and Reggiani, 1996, 2011). The transformation of EDL MHC-2b fibers to MHC-2x fibers is consistent with studies showing that endurance exercise leads to fiber type transformations from faster MHC isoforms to slower MHC isoforms,

especially MHC-2b to MHC-2x (Fitzsimons et al., 1990; Garland et al., 1995; Sullivan et al., 1995; Allen et al., 2001; Pansarasa et al., 2002; Pellegrino et al., 2005).

Furthermore, we suspect that the transformation of MHC-2b to MHC-2a/x fibers in the EDL of climbing mice explains two other aspects of our data. Typically, the cross-sectional area (CSA) of skeletal muscle fibers expressing different MHC isoforms varies in a predictable pattern: MHC-2b fibers have the largest CSA, MHC-1 fibers have the smallest CSA, and MHC-2a and MHC-2x fibers are intermediate in size (Schiaffino and Reggiani, 2011). As contractile force is proportional to muscle fiber CSA (i.e., fibers with larger CSA produce larger forces), the transformation from MHC-2b fibers to MHC-2x in the EDL maintains fibers with considerably large CSA while increasing the ability of the muscle to generate ATP via beta oxidation, as MHC-2x and moreover MHC-2a fibers have increased beta oxidation potential versus MHC-2b fibers (Schiaffino and Reggiani, 2011). These muscle fiber properties in the EDL of climbing mice allow for more forceful contractions to be sustained over longer contraction periods – contractile properties which would be beneficial for stability during fine branch arboreal locomotion. This significant increase in MHC-2x and MHC-2a fiber proportions enables the EDL to contract with higher specific force because of the comparatively large CSA of the individual MHC-2x and MHC-2a fibers. Additionally, the EDL muscles of climbing mice fatigued at a higher rate than the EDLs of control mice. Fiber type transformations from MHC-2b to MHC-2x fibers, but not from MHC-2x to MHC-2a fibers would explain why climbing mice EDLs were more fatigable: MHC-2b and MHC-2x fibers are fast glycolytic fibers (or fast fatigable) when characterized histochemically based on ATPase enzyme activity; MHC-2a fibers are less fatigable than MHC-2b and MHC-2x fibers because they are fast oxidative-glycolytic (or fast fatigue resistant) fibers. So, if the overall proportion of MHC-2x fibers is increased while MHC-2a fibers is constant, the overall EDL composition in climbing mice would be characterized by a higher number of fast fatigable fibers (MHC-2b and MHC-2x), which would make the whole muscle fatigue at a higher rate than controls. Given more time to adapt, we suspect that fiber

types would transform further to MHC-2a and even perhaps increasing populations of MHC-1 fibers in the EDL, creating EDLs in climbing mice that are even more fatigue resistant. This would be consistent with long term studies on endurance training in elite athletes who have higher proportions of slow MHC isoforms in their muscles than controls which gives them greater stamina for muscle function over long distances or time periods (Serrano et al., 2000; Shoepe et al., 2003; Westerblad et al., 2010).

The motivation for this study centered on understanding how non-skeletal tissues adapt to habitual mechanical loading compared to skeletal tissues. Previous work by Byron and colleagues using a similar mouse model of fine-branch arboreal climbing demonstrated considerable skeletal adaptation over the same time course (Byron et al., 2009; 2011; 2015). Additionally, the Byron laboratory demonstrated significant central nervous system plasticity in their climbing mice, as the granular cell layers of cerebellar lobules responsible for coordinating muscle function of the tail are thicker, suggesting more muscle coordination (Byron et al., 2013). We expected to see adaptation of the hind limb skeletal musculature in the present study. We observed functional adaptation in the form of greater specific force of the EDL in climbing mice compared to controls, but without structural adaptation in the form of enhanced mass or architecture. Because most bones of the limb are loaded in bending as a result of the combined forces of gravity and muscle contraction (Currey, 1984, 2002), differences in muscle strength should influence skeletal form. Data from our lab not reported here indicate that mechanical properties of the femur and tibia are enhanced in climbing mice compared to controls (Joll et al., 2015). Remarkably, results presented here suggest that fiber type morphology has the potential to alter skeletal structure and function even when muscle mass and architecture are not different.

Results from our study should be interpreted in the context of the following limitations. Although our sample size was sufficient to demonstrate significant differences in muscle contractile properties, it is certainly possible that a larger sample size would reveal more

differences than we have shown here. Further, examination of SOL and EDL muscle *ex vivo* contractility is standard in the literature because they are amenable to easy testing and represent generally fast (EDL) vs. slow (SOL) skeletal muscle. They are not, however, specifically involved in pedal grasping. Flexor digitorum longus would be a better muscle to directly examine pedal grasping development, but this muscle is deeper than SOL, it is more difficult to excise intact, and we have not had success measuring contraction in this muscle. Therefore, we are left with the gold standard of EDL and SOL to examine both contractile properties as well as structural and metabolic properties. But, because SOL and EDL muscles are predominately comprised of MHC fibers at one end of the contraction velocity spectrum (i.e., SOL muscles are predominately MHC-1 and MHC-2a/x fibers whereas EDL muscles are predominately MHC-2a/x and MHC-2b fibers), fiber type transformations are more difficult to observe than if fibers were of a more mixed variety like in the tibialis anterior muscle or gastrocnemius muscle. Future experiments will examine these mixed muscles for fiber type transformations. Finally, we were unable to measure individual fiber cross-sectional areas from our serial sections. These measurements would have allowed us to evaluate whether muscle fibers experienced any hypertrophy and would have helped us better evaluate our contractility data.

In summary, our data demonstration that muscle function – especially specific force – is enhanced in the EDL of climbing mice compared to controls, but there is no functional adaptation in the SOL muscles. We attribute the changes in muscle contractile properties of the EDL to fiber type transformation from faster MHC isoforms to slower ones. Future work will incorporate a larger range of histomorphometric data to validate our functional data.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to report. The results of this research have not been published previously in whole or in part, except in abstract format.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: JMO. Performed the experiments: JER, JEJ, WYE. Analyzed the data: JMO, JER. Contributed to the writing of the manuscript: JMO, JER, JEJ, WYE.

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FIGURE LEGENDS

Figure 1. Body composition data derived from dual x-ray absorptiometry. *significantly different from controls (p<0.05).

Figure 2. Mass and architecture of soleus (SOL) and extensor digitorum longus (EDL) muscles.

Figure 3. Fiber type proportions of soleus (SOL) and extensor digitorum longus (EDL) muscles. MHC, myosin heavy chain. *significantly different from controls (p<0.05).

Figure 4. *Ex vivo* contractile properties of soleus (SOL) and extensor digitorum longus (EDL) muscles. *significantly different from controls ($p < 0.05$)

Figure 5. Fatigability of soleus (SOL) and extensor digitorum longus (EDL) muscles. *significantly different from controls ($p < 0.05$).

Figure 1

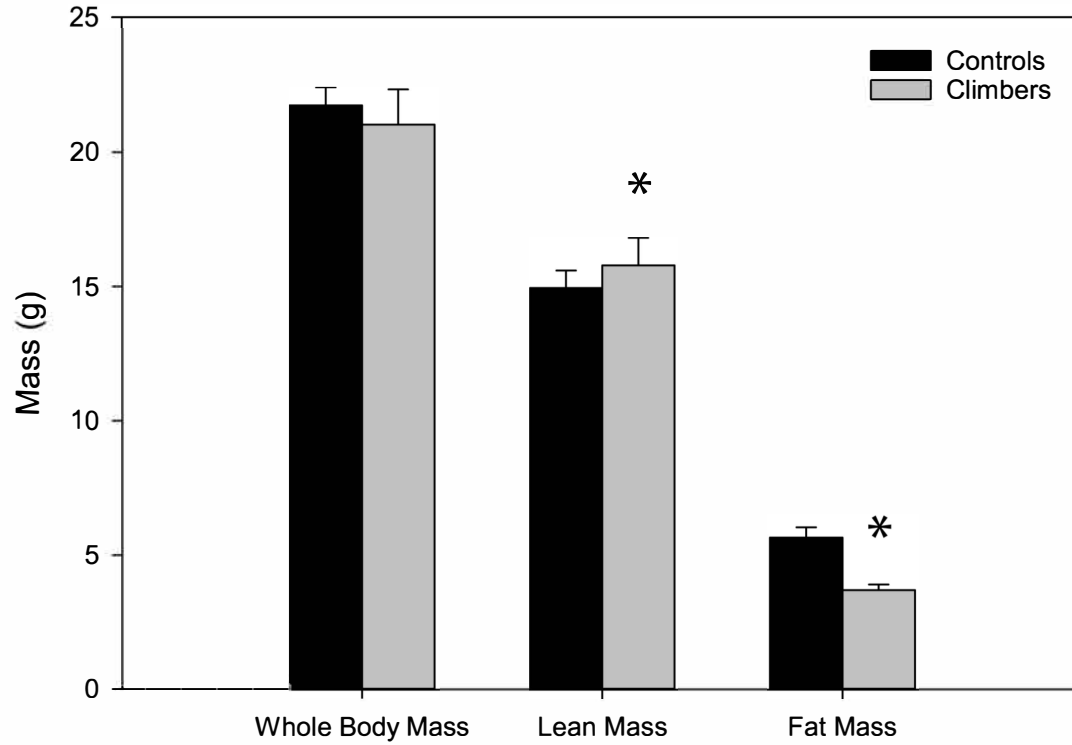


Figure 2

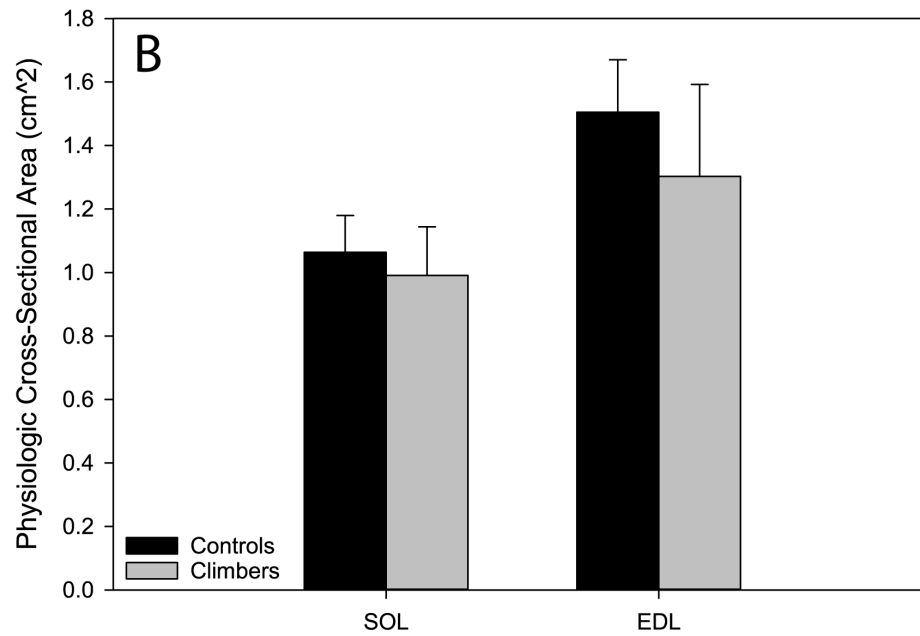
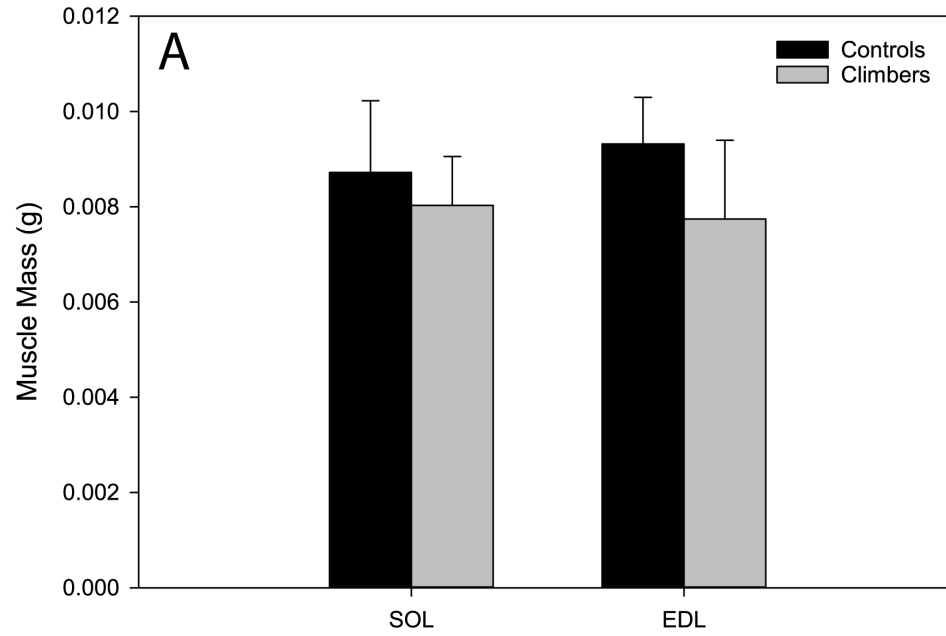


Figure 3

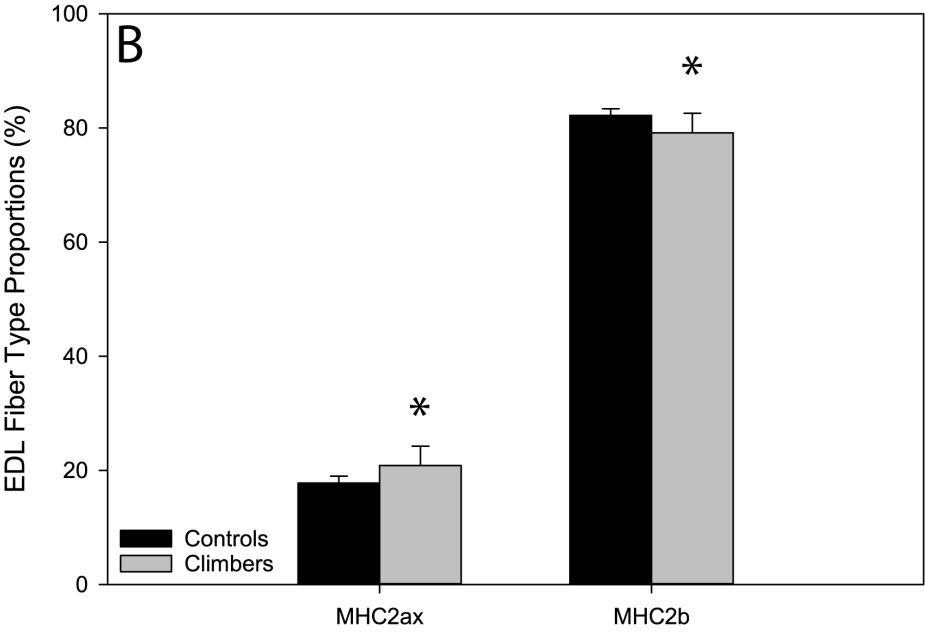
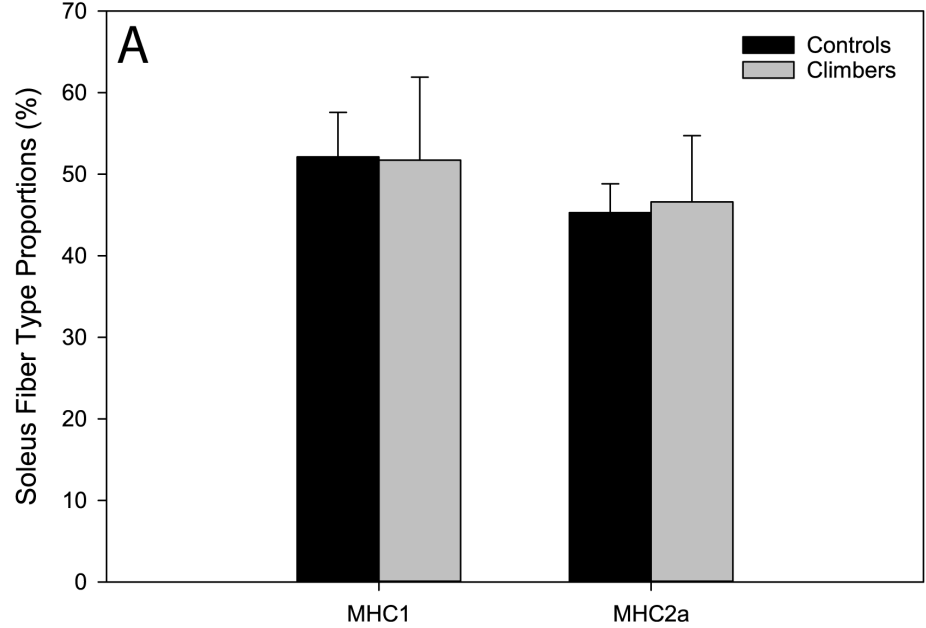


Figure 4

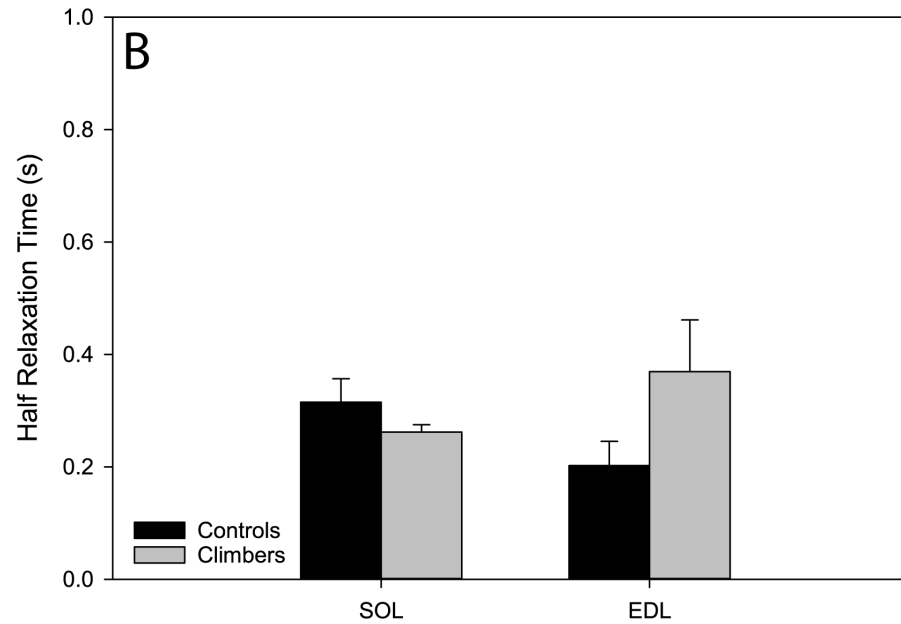
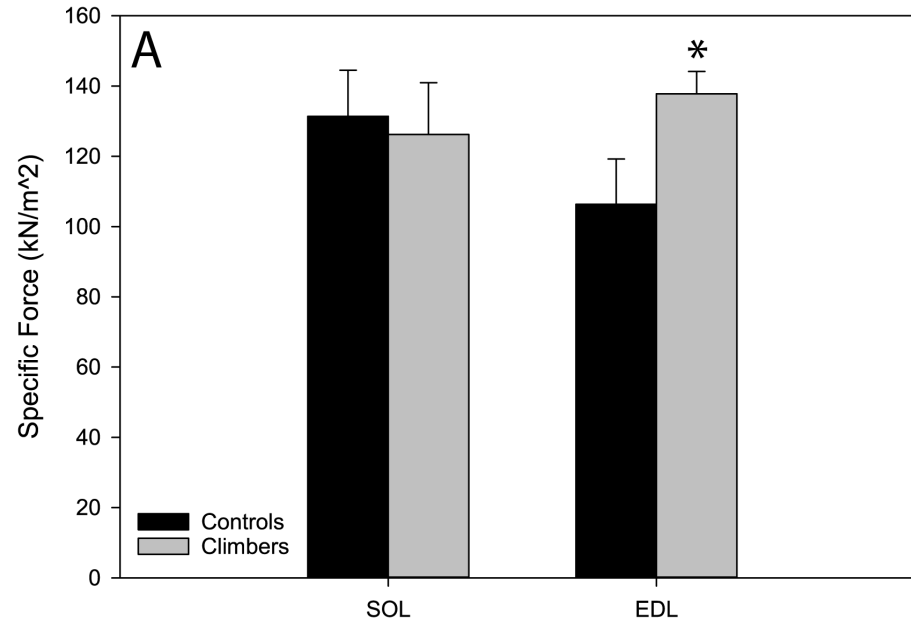


Figure 5

