Myosin fiber type distribution and metabolic characteristics in the hindlimb muscles of sloths (Xenarthra: Pilosa)

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ABSTRACT

Sloths are canopy-dwelling inhabitants of American neotropical rainforests that exhibit suspensory locomotion and posture. These abilities involve hanging below branch from hook-like feet, and although this requires great strength and endurance, the skeletal muscle mass of sloths is reduced, thus requiring modifications to muscle architecture for large joint torque. Based on previous findings of the physiological characteristics in forelimb musculature of sloths, it is hypothesized that hindlimb muscle properties are additionally modified for sustaining force production by the predominant expression of slow myosin heavy chain (MHC) fibers that rely on anaerobic metabolism for cheap/rapid ATP synthesis. This hypothesis was tested by determining MHC fiber type (%) distribution and energy metabolism in the hindlimb muscles of three-toed (*Bradypus variegatus*, *N*=5) and two-toed (*Choloepus hoffmanni*, *N*=3) sloths using a combination of protein gel electrophoresis, immunohistochemistry, and enzyme activity assays. A primary expression of the slow MHC-1 isoform as well as moderate expression of fast MHC-2A fibers and few hybrid MHC-1/2A fibers were found in both species. Slow MHC fiber type (%) is significantly greater in the flexors of *B. variegatus*, whereas expression of fast MHC-2A fibers was significantly greater in the extensor groups of *C. hoffmannni*. MHC-1 fibers were larger in cross-sectional area (CSA) than MHC-2A fibers and comprise the greatest %CSA in each muscle sampled from both species. Enzyme assays showed elevated activity for anaerobic enzymes compared to moderately low activity for aerobic enzymes, especially in *B. variegatus*. These findings emphasize joint stabilization in the hindlimb during suspensory habits, most notably in three-toed sloths, and imply that two-toed sloths are capable of faster muscle contraction as a potential compensatory mechanism for increasing joint power. Sloth hindlimb muscles also appear to have appreciable tolerance for lactate accumulation, and primarily rely on rapid ATP re-synthesis pathways. Muscle metabolism may be indicative of dietary differences, where *C. hoffmanni* could depend on more oxidative and glycolytic energy metabolic pathways due to higher quality forage. Nevertheless, the intrinsic properties observed match well with locomotor requirements of tree sloths, and these modifications may have further evolved in unison with low metabolism and body temperature, and slow movement patterns as means to conserve energy.

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DEDICATION

I dedicate this Thesis to my family, who fully supported my decision to pursue a graduate degree. I am indebted to my father, Ryan, for his unwavering support and for helping me relocate from Utah to Ohio.

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INTRODUCTION

Sloths are arboreal folivores that spend the majority of their lives in the canopies of the Central and South American neotropical rainforests (Urbani and Bosque 2006) where they utilize below branch walking to move slowly while suspended from branches and vines (Montgomery and Sunquist 1975). Unlike primates, three-toed (Family Bradypodidae) and two-toed (Family Megalonychidae) sloths demonstrate obligatory suspensorial locomotion and their distal limbs have undergone remarkable morphological modification as a consequence of convergent evolution for their shared arboreal lifestyle (Gaudin 2004; Nyakatura 2012). Observations that support this functional morphological interpretation include: long, re-curved claws that grip support in a hook-like fashion (Britton 1941); syndactylous digits with interlocking, keel-and-groove joints that preclude lateral deviation and independent positioning of the digits (Mendel 1985, 1987); and highly mobile carpal and tarsal joints that allow lateral/medial rotations of the feet for secure purchase of the substrate during vertical climbing (Mendel 1981).

Despite their suspensory habits requiring great strength, skeletal muscle mass in tree sloths is notably reduced, accounting for only ~24% of total body mass (Grand 1978), compared to 33% reported for other arboreal mammals (Muchlinski et al. 2012). However, sloth forelimbs are strong and capable of applying nearly twice as much total joint torque as an average human (Olson et al. 2017), and their limb muscles have the intrinsic capability of sustaining torque application via economic ATP utilization (Spainhower et al. 2018), all while minimizing muscle activation during postural hanging (Gorvet et al. in preparation). These abilities are primarily due to: (1) specialized muscle architecture, which includes long moment arms for several muscles crossing the shoulder and elbow joints, (2) primary expression of slow-contracting MHC-1 fiber types that rely on creatine kinase for ATP re-synthesis, and (3) low volume of active muscle and potentially few fast-contracting motor units while performing arboreal habits. The properties of the hindlimb musculature in sloths may be expected to mirror those determined for their forelimb muscles, although potential differences could be observed relating to the differing functional roles of the limb pairs during suspensory locomotion.

In contrast to terrestrial locomotion in upright mammals, the hindlimbs of sloths are the braking appendages and the forelimbs are propulsive elements. Specifically, sloth

hindlimbs play a greater role in stabilizing the body to avoid aberrant pendular swinging (Nyakatura and Andrada 2013) during below-branch walking and arboreal maneuvering (e.g., bridging tree branches), whereas the forelimbs perform mechanical work to pull the body forward (Granatosky and Schmidt 2017). Moreover, in a previous study on sloth forelimb muscle fiber types (Spainhower et al. in press), muscle performance was concluded to be tuned for suspension by the predominant expression of slow and fast fiber types that are equally capable of keeping pace with contractile demand by utilizing rapid, non-oxidative ATP re-synthesis. While these characteristics partially explain how the forelimbs are employed for propulsion, the braking/stabilizing function of the hindlimbs that may be the main weight-bearing appendages may require a different suite of intrinsic properties. The opportunity to integrate muscle fiber types with system-level function remains to be evaluated in the hindlimbs of tree sloths, thus myosin heavy chain (MHC) expression and energy metabolism of the hindlimb musculature, and how both may be related to prolonged suspension are the focus of this study.

Four MHC isoforms can be expressed in mammalian limb muscles, and they are in order from slowest-to-fastest-contracting: MHC-1, MHC-2A, MHC-2X, and MHC-2B (Bottinelli 2001; Toniolo et al. 2004). Each isoform corresponds to a generalized 'fiber type' of similar nomenclature (e.g., Type I and Type 2A fibers). MHC isoform expression is the main determinant of intrinsic fiber contractile properties (Schiaffino and Regianni 2011), namely ATP turnover rate (myosin ATPase activity: Bottinelli et al. 1994), filament sliding velocity (V_f: Butcher et al. 2010), fiber shortening velocity (V_0: Bottinelli et al. 1991), and fiber power output (W: Bottinelli et al. 1999). Fast MHC isoforms and their corresponding fiber types MHC-2A, -2X, and -2B, are more powerful than MHC-1 isoform fibers, but because their myosin isoforms bind and hydrolyze ATP more rapidly, fast MHC fiber types are expected to be fatigable. On the other hand, slow-contracting MHC-1 fibers are expected to be fatigue resistant, and thus are predicted to be better suited for behaviors requiring prolonged and/or repetitive contractions (Rupert et al. 2014; Thomas et al. 2017). Accordingly, MHC fiber type distribution influences the intrinsic contractile properties of whole muscles, and by convention, the energy metabolism of muscle fibers is related to their MHC isoform content. For example, fast MHC fiber types are believed to rely more on glycolytic pathways, whereas slow MHC-1

fibers are often reported to exclusively utilize oxidative cellular metabolism (Lieber 2009; Schiaffino and Reggiani 2011, and references therein). However, a series of recent studies (e.g., Kohn et al. 2011a, b; Curry et al. 2012), including a previous report on muscle enzyme activities in sloth forelimbs (Spainhower et al. in press), indicate little correlation between muscle energy metabolism and MHC isoform content. Fiber type distribution may be largely constrained by phylogeny (Westerblad et al. 2002; Rupert et al. 2014), while the metabolic characteristics of skeletal muscle fibers can be modified to a greater extent via muscle use to match functional demands (Kohn 2014; Thomas et al. 2017; Spainhower et al. 2018). Therefore, sloth muscle fibers can coexpress slow-contracting MHC and anaerobic metabolism, and this atypical combination of intrinsic properties may have co-evolved with their adaptive slow movement patterns (Engelmann 1985, McDonald and De Iuliis 2008).

This analysis of MHC fiber type distribution and enzyme activity in the hindlimb muscles of the pale-throated three-toed sloth (*Bradypus variegatus*) and Hoffmann's two-toed sloth (*Choloepus hoffmanni*) aims to further validate our understanding of how mammalian skeletal muscle can become adapted for strong, sustained contractions necessary for suspension. It is hypothesized that their muscles have evolved contractile properties and energy metabolism modifications that prevent fatigue for maintaining moderate-to-high levels of torque application. In particular, a primary expression of slow MHC-1 fibers that rely on anaerobic metabolism for cheap/rapid ATP synthesis is expected to be observed in the hindlimb musculature of sloths. Moreover, these intrinsic properties may be emphasized in the large proximal limb muscles to stabilize the hip and knee joints by relatively greater MHC-1 expression than forelimb muscles, whereas the digital flexor muscles of sloths may show additional modifications of heterogeneity of MHC fiber type and energy metabolism that is specialized for grip force and/or prolonged grip on the substrate.

MATERIALS AND METHODS

Animals and Muscle Sampling

A total of eight sloths were used in this study (*N* = 5, *B. variegatus*; *N* = 3, *C. hoffmanni*). Morphometric details for each individual and muscle sampling strategy are presented in Table 1. The Sloth Sanctuary of Costa Rica provided access to frozen sloth cadavers. Specimens were thawed for 24–30 h prior to dissection. Tissue was harvested from the following muscles of the right hindlimb: m. sartorius (SRT), m. gluteus medius (GLM), m. adductor magnus (ADDM), m. semimembranosus (SM), m. semitendinosus (ST), m. biceps femoris vertebral and femoral heads (BF), m. vastus lateralis (VL), m. tibialis cranialis (TCN), m. extensor digitorum longus (EDLO), m. gastrocnemius medial and lateral heads (GAS), m. soleus (SOL), and m. flexor digitorum profundus all heads (FDP). This suite of muscles was selected for their proximal-to-distal distribution along the hindlimb, in addition to several being standard comparators for muscle fiber properties across mammals (e.g., GLM, VL, GAS/SOL), and others having a primary role in hip, knee, tarsal, or digital flexion.

Whole muscles or tissue blocks were placed in conical tubes, frozen, and shipped (on dry ice) to Youngstown State University (YSU) for cold storage (–80ºC) until analysis. This work was conducted between 2015 and 2017. All dissection procedures complied with protocols approved by the Costa Rica Ministerio de Ambiente, Energia, y Telecomunicaciones (MINAE: R-033-2015 to R. Cliffe; R-008-2017 to M. Butcher); tissue harvesting for biochemical analyses approved by the Comision Nacional para la Gestion de la Biodiversidad (CONAGEBIO: R-033-2015 to R. Cliffe; R-027-2017-OT to M. Butcher); permissions to export biological tissue approved by Sistema Nacional de Areas de Conservacion (SINAC-ACLAC-PIME-R-033-2015); and procedures involving analysis of frozen tissue approved by YSU IACUC (Protocols: 01-12 and 04-12).

Protein Sample Preparations

Muscle tissue homogenates were prepared for electrophoresis following established previous methods (Talmadge and Roy 1993; Mizunoya et al. 2008; Rupert et al. 2015; Thomas et al. 2017). Protein samples (diluted in gel sample buffer: ~0.125 mg/mL) were heated (95ºC) for 5 min before being loaded on gels. Muscle tissue from the heart and limbs of additional laboratory mammals (e.g., rat and rabbit) was prepared by the same

methods to serve as band standards for MHC-1β [rabbit and sloth heart (left ventricle)] and MHC-2X and 2B [rat gastrocnemius]. Separate homogenates for muscle enzyme activities were prepared using the methods of Kohn et al. (2011a). Briefly, small portions of tissue were weighed (to the nearest mg) and added to a 100 mM potassium phosphate buffer (pH: 7.30) to a final ratio of 1:19. The tissue was homogenized with a Tissue Tearor 985-370 sonicator (Biospec Products, Bartlesville, OK, USA) for two 30 sec cycles and cold centrifuged at 13,000 rpm for 7 min. The supernatant was decanted into microcentrifuge tubes and either immediately used for enzyme kinetics assays or stored frozen at –80°C until experimentation.

Protein Analyses: SDS-PAGE and Peptide Analysis

MHC isoforms were separated using an SDS-PAGE protocol as previously described in detail (Hazimihalis et al. 2013; Rupert et al. 2015). The total acrylamide percentage equaled 8% and 4% in the separating gel and stacking gel, respectively. The electrode buffer was 50 mM Tris (pH 8.3), 75 mM glycine, and 0.5% SDS; upper buffer was 6X the concentration of the lower buffer and also contained 0.12% β-mercaptoethanol (Mizunoya et al. 2008). Electrophoresis was run on a mini-PROTEAN Tetra system (Bio-Rad, Hercules, CA, USA) at 140 V for 22–24 h at 4°C (Talmadge and Roy 1993; Mizunoya et al. 2008). Gels were stained with silver for visualization of the MHC isoforms and imaged using a Pharos FX Plus system (Quantity One software: Bio-Rad).

MHC bands resolved by SDS-PAGE were also sectioned from the select gels, placed in sterile microcentrifuge tubes containing a 5% acetic acid buffer, and sent to The Ohio State University for mass spectrometry peptide analysis of the isoforms. Isoform identity was confirmed by matching the peptide sequences with published MHC sequences for Mammalia accessed by MASCOT identification software (Thomas et al. 2017). The identity of each MHC isoform coincided with the largest number of overlapping peptide segments in a known sequence.

Immunohistochemistry (IHC)

Muscle blocks were mounted to cork using OCT tissue embedding medium (Sakura Finetek USA, Inc., Torrance, CA) and flash frozen in isopentane cooled in a liquid nitrogen bath (Butcher et al. 2010). Subsequently, transverse serial sections (10 μm) were

cut frozen on a Leica 1850 cryostat (Leica Microsystems, Buffalo Grove, IL, USA) at –20ºC and mounted on charged Superfrost slides (Thermo Fisher Scientific, Waltham, MA USA). Serial sections of each muscle were reacted against a panel of five monoclonal antibodies (mAbs) specific to slow and fast MHC isoforms used to identify MHC isoform fiber type. Antibodies with known immunospecificity against MHC isoforms were purchased (as supernatants/concentrates) from the Developmental Studies Hybridoma Bank (DSHB: University of Iowa, IA, USA) and Sigma-Aldrich. BA-D5 and S58 are specific to the MHC-1 isoform (Schiaffino et al. 1989; Sokoloff et al. 2007) and were shown to identify MHC-1 fibers in a preliminary study of sloth forelimb muscles (Spainhower et al. 2017). Four mAbs are specific to fast MHC isoforms (Schiaffino et al. 1989): 2F7 specific to MHC-2A (Zhong et al. 2008); SC-71 specific to MHC-2A/X (Rupert et al. 2014); BF-35 (old clone) specific to all MHC except 2X (Schiaffino et al. 1989); and MY32 (Sigma-Aldrich, St. Louis, MO, USA) is specific to all fast MHC isoforms. Reaction specificities against fast MHC isoforms in sloth muscles was also confirmed in a previous study (Spainhower et al. 2018). All mAbs were diluted in PBS (pH 7.4). MY32 was diluted to 1:1000, whereas antibodies from DSHB were diluted to working Ig concentrations of 2–5 μg/mL.

IHC was conducted as previously described in detail (Butcher et al. 2010; Hazimihalis et al. 2013; Rupert et al. 2014). Briefly, serial sections were blocked (2% goat serum) at room temperature for 10 min and then incubated with mAbs in a humidified chamber overnight for 12 h at 4°C. The sections were washed in phosphate buffered saline (PBS), followed by incubation with a biotinylated rabbit (anti-mouse) 2º antibody for 1 h at room temperature (washed in PBS), application of a streptavidin HRP enzyme conjugate (10 min), and reaction with DAB chromagen (5 min), all using a Histostain Plus kit (Invitrogen: Thermo Fisher Scientific). After a final wash in ddH_2O (10 min), serial sections were stained with Erlich's hematoxylin for 1–3 min to visualize nuclei and unreacted fiber morphology, rinsed in dH_2O (3 min), dehydrated in alcohol (70, 95, 100%), and cleared with xylenes before applying glass coverslips. Experimental controls were serial sections of muscle treated with PBS in place of mAbs incubation.

MHC fiber type distributions were quantified from images of IHC-stained serial sections visualized on an Olympus CX31 microscope (Olympus Microscopes, Waltham,

MA, USA) and photographed with a SPOT Idea digital camera system (Diagnostic Instruments, Sterling Heights, MI, USA). Percentages of each MHC fiber type were calculated based on counts of all reacted fibers from 3–5 representative sections of each muscle from each individual (Hazimihalis et al. 2013; Rupert et al. 2014). Muscle fibers were classified as hybrids by IHC if they showed cross-reactivity with two mAbs specific to different MHC isoforms. Fiber type distributions were quantified as mean percentages; percent MHC fiber type was averaged for each forelimb muscle and species.

Fiber size was determined by measuring cross-sectional area (CSA: in μm^2) of each MHC fiber type from cross-sectional images imported into Image J (v.1.43; NIH, Rockville, MD, USA). Fiber CSA was assessed from a random subset of ~10% of reacted fibers for each MHC fiber type in each serial section analyzed (Rupert et al. 2014), and then averaged for each forelimb muscle and species. Additionally, the total number of fibers counted for each MHC fiber type was multiplied by the corresponding mean value for fiber CSA, and each product was then divided by the sum of products to obtain the percentage of cross-sectional area (%CSA) for each MHC fiber type identified (Anapol and Jungers 1986).

Muscle Metabolism: Enzyme Assays

Enzyme kinetics for several aerobic and anaerobic pathways were quantified in a sub-sample of six hindlimb muscles from each sloth: SRT, GLM, BF, VL, TCN, and FDP. Muscles were selected based on their purported functional role, or their relative large percentages of either slow- or fast-contracting fibers to relate enzyme activity with MHC fiber type. A series of biochemical assays were run using the established methods of Kohn et al. (2011a, b). Muscle homogenates were assayed with the metabolic enzymes: citrate synthase (CS) for oxidative activity; 3-hydroxyacetyl CoA dehydrogenase (3-HAD) for fat β-oxidation activity; phosphofructokinase-1 (PFK) for glycolytic activity; lactate dehydrogenase (LDH) for lactate production; and creatine kinase (CK) for rapid ATP regeneration. Specifically, small volumes (1–10 μL) of the homogenates were combined with 0.5–1.0 mL of enzyme reagents having the below compositions.

CS assay reagent: 100 mM tris (pH 8.2), 100 μM 5,5'-dithiobis-(2-nitrobenzoate) (DTNB), 400 μM oxaloacetate, 160 μM acetyl Co A (final volume = 500 μL); **3HAD assay reagent**: 50 mM tris (Ph 8.0), 4 mM EDTA, 60 μM NADH, 50 μM acetoacetyl Co

A (final volume = 500 μL); **PFK assay reagent**: 50 mM tris (pH 8.0), 2 mM EDTA, 5 mM magnesium chloride, 0.01% BSA, 120 μM NADH, 2 mM ATP, 0.45 U/mL aldolase, 5.8 U/mL glyceraldehyde-3-phosphate dehydrogenase, 58 U/mL triosephosphate isomerase (final volume = 500 μL); **LDH assay reagents**: 50 mM tris (pH 8.0), 4 mM EDTA, 120 μM NADH, 2 mM pyruvate (final volume = 1000 μL); and **CK assay reagents**: 100 mM imidazole (pH 6.7), 2 mM EDTA, 10 mM magnesium acetate, 4 mM glucose, 2 mM ADP, 5 mM AMP, 30 mM $NADP^+$, 20 mM n-acetylcystein, 0.5 U/mL hexokinase, 0.3 U/mL glucose-6-phosphate dehydrogenase (final volume = 500 μL) (Kohn et al. 2011a, b). All reagents were purchased from Sigma-Aldrich.

Assays were reacted for 2–5 min. Substrate turnover rates were measured using spectrophotometer (SmartSpec^{TM} Plus: Bio-Rad) at 10 or 15 sec intervals (depending on the enzyme) with absorbance wavelengths read at 340 nm or 412 nm (CS activity only) (Kohn and Myburgh 2007; Kohn et al. 2011a). Trials were repeated 3x for each assay and absorbance values were plotted as a function of time and fit with linear least-squares (LLS) regressions. The slopes (Δabsorbance/min) for trial replicates were used to calculate enzyme activities with the equation:

$$(\text{slope} \times \text{dilution factor}) / (E \times [\text{muscle}]\ \text{g/L}) \qquad \text{(Eq.1)}$$

where E = molar extinction coefficient, the dilution factor is the ratio of reagent to tissue homogenate, and [muscle] is the concentration of wet muscle mass to buffer (Kohn and Myburgh 2007; Kohn et al. 2011a). Values of enzyme activity (in μmol min^{-1} g^{-1} ww) were averaged for each forelimb muscle and species.

Statistical Analyses

All muscle data are reported as species means (± SD) unless otherwise specified; values of selected muscle measurements for each individual are reported in Tables 2 and 3. MANCOVA was performed *a priori* in SPSS (v. 20: IBM, Inc., Armonk, NY, USA) to test for differences in MHC fiber type (%) and %CSA between species. For this test, data were pooled into the following seven functional groups: hip flexors, hip extensors, knee flexors, knee extensors, tarsal flexors, tarsal extensors, and digital flexors. Means for fiber CSA were similarly pooled but were not subjected to statistical testing due to the typically large SD found for this metric. MANCOVA was also used to assess statistical

significance in MHC fiber type (%), and enzyme activity (in μmol min^{-1} g^{-1} ww) between species and among the muscle sub-sample. Body mass was used as the covariate for all MANCOVA testing to account for any signal produced by body size. Following a significant MANCOVA test, MANOVA and Tukey *post-hoc* tests were employed to determine all pair-wise, mean differences after a significant finding for the effects of functional group or muscle. Enzyme activities were additionally plotted against MHC fiber type (%) expression for correlation analysis performed in MS Excel (Microsoft Corp., Redmond, WA, USA). Pearson-Moment correlation coefficients (r) were determined for each relationship found when data for both species were combined and tested individually. Significance for all statistical tests was accepted at $P \leq 0.05$.

To compare muscle metabolism in sloths with that reported for other mammals, values of enzyme activity from published literature for mammals of varying lifestyles across the broadest range of body size possible were assembled for the enzymes 3-HAD, CS, CK, LDH, as well as the corresponding body masses of the experimental animals (Table 4). Enzyme activity data were log transformed and regressed against log body mass with LLS in MS Excel to yield the slope (i.e., scaling exponent) of the relationships (Smith, 2009). A value of one was added to all data to avoid negative transformations. The regression line from each analysis was tested for a slope significantly different from zero using a one-way ANOVA. Predicted activities were calculated using the allometric equation for each enzyme analyzed and then compared to the observed (measured) activities for each animal by plotting the results against a linear regression forced through the origin. Data points below the line of unity (slope = 1.0) indicate lower activity than predicted based on body mass. Reported enzyme activities are limited to a small and dissimilar group of mammals, especially measured values for the enzyme PFK, which was excluded due to the lack of data.

RESULTS

SDS-PAGE Identification of MHC

Electrophoretic separation of protein homogenates from each muscle sampled showed expression of only two isoforms: slow MHC-1 and fast MHC-2A for both *B. variegatus* and *C. hoffmanni* as demonstrated in Fig. 1*A*. The slow isoform further separated into MHC-1 β and a variant (X1) of MHC-1, which was confirmed by peptide sequencing. The band for slow MHC-1 β consistently migrated to a low position in the gel lane and is a lighter (223.6 kD) isoform than fast MHC-2A, which migrated the least and was the most massive (223.9 kD) of the MHC bands resolved. The identity of the isoforms also was verified by conventional band migration patterns of MHC in rat m. gastrocnemius and mammalian heart (lanes not shown).

MHC Fiber Type Distribution

Three distinct MHC isoform fiber types were identified using selected mAbs specific for MHC isoforms. Figure 1*B* shows the typical reactions for each MHC isoform fiber type in sloth hindlimb muscle tissue from both species. Slow MHC-1 fibers were strongly reactive against S58 and were identified in all samples. Fast MHC-2A fibers were identified using primarily mAbs 2F7 and SC-71. Serial sections reacted against these two mAbs were identical, and allowed for clear determination of the MHC-2A fiber type. All fibers in each serial section reacted moderately or intensely against BF-35 indicating that the MHC-2X fiber type was not expressed. The third fiber type was confirmed as MHC-1/2A hybrids by co-reactions against S58, 2F7, or SC-71 in the same fibers (Fig. 1*B*).

MHC fiber type distributions were quantified from totals of n = 12,503 fibers for *B. variegatus* and n = 14,877 fibers for *C. hoffmanni*. Percentages for each MHC fiber type from all muscles sampled for both species are presented in Table 5. Figure 1*C* shows MHC fiber type distribution across functional muscle groups for both species. Notably, a change in fiber type expression occurred in muscles distal to the knee joint. For example, MHC-1 fiber type expression in *B. variegatus* for functional groups proximal to the knee joint ranged from 95–100%, and decreased to a range of 59–85% for functional groups distal to the knee joint. A similar but less obvious gradient was observed in the hindlimb of *C. hoffmanni*. Irrespective of the slower-to-faster-contracting pattern of MHC fiber type distribution, there was an overall abundance of slow MHC-1 fibers in the muscles

sampled, especially in three-toed sloths. Numerous muscles uniformly expressed MHC-1 fibers in *B. variegatus*, including: SRT, GLM, VL (all individuals); ADDM (BV4), SM (BV4 and BV8), ST (BV8), and TCN (BV4). MHC-1 fibers averaged (pooled functional group means ± SD) 98.4 ± 2.1% in the hip flexors, 97.2 ± 3.8% in the hip extensors, 95.0 ± 5.5% in the knee flexors, and 100% in the knee extensors of *B. variegatus* (Table 6; Fig. 1*C*). Decreases in MHC-1 fiber distribution in each functional group were offset by an increase in MHC-2A isoform fibers, but not MHC-1/2A hybrid fibers. The distribution of fast MHC-2A fibers was largest in the digital flexors and averaged 35.5 ± 3.8% and 46.7 ± 3.1% in *B. variegatus* and *C. hoffmanni*, respectively (Fig. 1*C*). No clear trend in MHC-1/2A expression was observed in either species, and hybrid fiber percentage distribution was relatively low (mean range: 0.6 – 7.8%, Table 5). These patterns of fiber type (%) distribution were similar to the proximal-to-distal gradient observed in sloth forelimb muscles (Spainhower et al. in press), however unlike the forelimb digital flexors, percentages of MHC-2A fibers in the hindlimb digital flexor group never exceeded MHC-1 fiber type expression for any other functional group.

MANOVA found no difference in mean MHC fiber type (%) for hip and digital flexors between *B. variegatus* and *C. hoffmanni*, whereas slow MHC-1 fiber type expression was significantly greater ($P < 0.001$) in hip extensors, knee flexors and extensors, and ankle flexors and extensors of three-toed sloths (Fig. 2*A*). Conversely, fast MHC-2A fiber type expression was significantly greater ($P \leq 0.001$) in the same functional muscle groups of *C. hoffmanni* (Fig. 2*B*). MANOVA also indicated that the digital flexors within *B. variegatus* expressed a greater percentage of MHC-2A fibers ($P < 0.05$) than their hip flexors and extensors, knee flexors and extensors, and ankle flexors (Table 6). Fiber type expression among functional muscle groups in *C. hoffmanni* showed similar, although less marked intraspecific variation, whereby the digital flexors expressed greater MHC-2A fibers ($P \leq 0.05$) than the hip flexors and extensors as well as the knee flexors of two-toed sloths (Table 6).

MHC Fiber Type Size

Figure 3 shows fiber CSA across functional muscle groups for *B. variegatus* and *C. hoffmanni*. CSA for slow MHC-1 fibers was broad ranging (mean range: 425.2 – 7787.4 μm^2) and larger than that for fast MHC-2A fibers (mean range: 326.4 – 5211.5 μm^2) in all

functional muscle groups for both species. Overall, MHC-1/2A hybrid fibers had the smallest CSA and a narrowest range (mean range: 400.1 – 4524.3 μm^2) of fiber size. Among all of the functional muscle groups, mean CSA for all three fiber types was largest in the ankle flexors of *B. variegatus* and the digital flexors of *C. hoffmanni*. The smallest fibers were observed in the knee extensors (MHC-1), knee flexors (MHC-2A), and hip extensors (MHC-1/2A) within *B. variegatus*, and in the ankle flexors for all three fiber types within *C. hoffmanni*, (Fig. 3).

Calculated %CSA values for each MHC fiber type are presented in Table 7. Similar to the trend in fiber type distribution, %CSA for slow MHC-1 fibers was greatest in proximal limb regions and declined in the distal hindlimb. For example, Fig. 2*C* shows the %CSA of MHC-1 fibers in hip flexors averaged 100 ± 0% and 85.6 ± 5.1% in *B. variegatus* and *C. hoffmanni*, respectively, compared with 68.0 ± 10.2% and 56.2 ± 2.6% in digital flexors of each species. Conversely, values of %CSA for fast MHC-2A fibers (mean range: 0 – 40.5) generally increased proximal-to-distal along the hindlimb, as did those for %CSA of MHC-1/2A hybrids, but they always were exceeded by %CSA for slow MHC-1 fibers in each muscle sampled (Table 7; Fig. 2*D*).

MANOVA found that %CSA of MHC-1 fibers was greater ($P < 0.04$) in the hip flexors, knee flexors and extensors, and ankle flexors of *B. variegatus* than that in the same functional muscle groups of *C. hoffmanni* (Fig. 2*C*). MANOVA also indicated intraspecific differences among functional groupings where %CSA of MHC-1 fibers was greater ($P < 0.05$) in the hip flexors and extensors and knee flexors and extensors than the digital flexors in *B. variegatus*, and greater ($P = 0.03$) in the hip flexors than the digital flexors in *C. hoffmanni* (Fig. 2*C*).

MHC Fiber Type Enzymatic Activity

Enzyme activities for selected hindlimb muscles are presented in Table 8. For the six muscles assayed, CK activity was the greatest (mean range: 28.0 – 192.8 μmol min^{-1} g^{-1} ww), followed by LDH activity (mean range: 46.2 – 115.2 μmol min^{-1} g^{-1} ww), and then CS activity (mean range: 0.9 – 17.0 μmol min^{-1} g^{-1} ww) in both species. Pooled means for 3-HAD, CS, PFK, and LDH activities of *B. variegatus* were consistently less than those of *C. hoffmanni*, whereas pooled mean activity for CK was greatest in three-toed sloths (Table 8). MANCOVA (factor: species) found that 3-HAD and CS activities were

significantly greater ($P < 0.001$) in *C. hoffmanni* (Fig. 4*A*) and CK activity was significantly greater ($P < 0.001$) in *B. variegatus* (Fig. 4*B*). Overall, 3-HAD and PFK showed the lowest activities, with values for each assay and individual being the lowest in *B. variegatus* (Tables 2, 3). Despite variation observed between species, MANOVA found no intraspecific differences in enzyme activity among selected muscles.

Correlation coefficients for relationships between enzyme activity and MHC fiber type (%) for both species (combined data) are presented in Table 9. CS demonstrated a narrowly non-significant negative correlation ($r_{36} = -0.313$, $P = 0.061$) with slow MHC-1 expression and a positive correlation ($r_{36} = 0.351$, $P = 0.034$) with fast MHC-2A expression. Similarly, both 3-HAD ($r_{36} = 0.378$, $P = 0.02$) and PFK ($r_{36} = 0.508$, $P = 0.001$) showed positive correlations with MHC-2A expression. CK activity showed a narrowly non-significant positive correlation ($r_{36} = 0.313$, $P = 0.061$) with MHC-1 expression and a negative correlation ($r_{36} = -0.337$, $P = 0.043$) with MHC-2A expression. MHC-1/2A fiber correlations were opposite those for pure fibers where only LDH activity correlated negatively ($r_{36} = -0.351$, $P = 0.034$) with hybrid fiber expression.

Last, muscle enzyme activities scaled according to their allometric slopes presented in Table 10. Activities across nearly a 20,000-fold change in body size for the aerobic enzymes 3-HAD and CS, and the anaerobic enzymes LDH and CK were variable as shown in Figure 5. A significant, although weak, positive relationship with body size ($P = 0.04$, $R^2 = 0.34$) was found for only 3-HAD activity (Fig. 5*A*). The slope of the relationship between CS activity (Fig. 5*B*) and body size was nearly zero, and the relationships for CK (Fig. 5*C*) and LDH (Fig. 5*D*) activity were similarly weak, but differed directionally. Figure 6 shows observed versus predicted enzyme activities for sloths compared with those in other placental mammals. In general, enzyme activities in sloths were always lower than predicted for an animal of their body mass, and tended to group randomly with enzyme activities for mammals of various body sizes. Specifically, muscles in the hindlimb of *C. hoffmanni* demonstrated faster 3-HAD (Fig. 6*A*) and CS (Fig 6*B*) activities compared muscles in their forelimb, but slower CK activity (Fig. 6*C*) compared with its forelimb and muscles from both limbs of *B. variegatus*. LDH activity (Fig. 6*D*) in each set of muscles from both species clustered closely and was similar to LDH activity observed in humans.

DISCUSSION

Suspensory locomotion is peculiar, and the contractile and metabolic characteristics of sloth hindlimb muscles are just as defining as morphological modifications that make obligatory below branch walking possible. The major findings presented in this study indicate that postural suspension is facilitated by the primary expression of slow-contracting MHC-1 fibers with a larger CSA for maximizing force output while minimizing contractile energy costs. CK showed the greatest activity of all assayed enzymes despite the primary expression of slower-contracting MHC fiber types. This combination of MHC expression and energy metabolism has not been reported in domesticated mammals and suggests that they depend on muscle function in sloths. These characteristics are similar to those reported in sloth forelimb muscles and partially support the hypothesis, but metabolic properties in hindlimb muscles are species-dependent, and the higher oxidative activity in two-toed sloths is predicted to be consequence of ecological and biomechanical differences. The intrinsic muscle properties of sloths suggest that energy metabolism is primarily influenced by functional output first and MHC expression secondarily.

The present findings are consistent with the MHC fiber type properties determined for sloth forelimb muscles (Spainhower et al. in press); however, the cellular energy systems of the hindlimb musculature deviate from those previously reported. The hindlimbs of both *B. variegatus* and *C. hoffmanni* are characterized by heterogeneous expression of slow MHC-1 and fast MHC-2A fiber types, and no expression of the fast MHC-2X or MHC-2B isoforms. The abundance of large, slow-contracting muscle fibers supports the hypothesis that sloth limb muscles have evolved energetically favorable contractile properties for sustained torque application and/or fatigue resistance. Muscle fibers expressing the MHC-1 isoform hydrolyze ATP at slower rates relative to fast MHC isoforms, which lowers the energy cost of force production in slow-contracting muscles (Bottinelli 2001, Bottinelli et al. 1994). Moreover, muscle force is proportional to CSA. The combination of slower ATP utilization and large fiber CSA has the potential to maximize contractile force while concurrently minimizing energy expenditure during suspensory locomotion by the primary recruitment of MHC-1 fibers (i.e., slow motor units). In addition, activation of a lower total volume of muscle has the advantage of

minimizing the incremental cost of contraction (Roberts et al. 1997) to provide the needed level of force to support the body weight of sloths in suspensory postures. Novel evidence on EMG muscle activation from the forelimbs of *B. variegatus* during postural support and below branch walking helps to validate this interpretation of locomotor performance in sloths (Gorvet et al. unpublished data).

The predominant expression of slow MHC-1 fibers in all functional muscle groups, coupled with atypical homogenous expression of slow-contracting fibers in several hip and knee flexor/extensors in *B. variegatus*, emphasizes how sloth hindlimb muscles are slower-contracting than those in their forelimbs. This fiber phenotype is especially notable in distal limb musculature where the average proportion of fast MHC-2A fibers is exceeded by expression of the slow MHC-1 isoform in all functional groups, despite a subtle slower-to-faster-contracting change in fiber type distribution that is retained along the length of sloth limbs. Similar observations were made in the hindlimbs of the slow loris (*Nycticebus*: Sickles and Pinkstaff 1981a, 1981b); a slow-moving primate that employs facultative suspensory behaviors. This specific pattern of fiber type distribution suggests the importance of stabilization of the hip and knee joints for below-branch arboreal maneuvering or sustained clinging in a vertical posture. The hindlimbs remain in contact with the support (in weight-bearing) while one of the forelimbs is used for reaching to acquire new grip on a branch during inverted locomotion (Mendel 1981a, 1985; personal observations). The limb cycle is continued by ipsilateral placement of a hindlimb forming a lateral couplet in a lateral sequence walk. Both *B. variegatus* and *C. hoffmanni* express similar proportions of slow MHC-1 fibers in the hip flexors, in particular, suggesting that the role of this muscle group is common to both species, and may reflect functional requirements for suspensory behaviors. Namely, the extremely slow-contracting proximal muscles may act to stabilize the hip joint via lengthening contractions so that hip extension is highly controlled during translational movement.

Sustained activation of multiple slow-contracting flexor/extensor muscles acting at the hip and knee joints in sloth hindlimbs is likely critical in minimizing oscillations that would destabilize the body (Nyakatura and Andrada 2013) and lead to a potentially fatal fall. The ability to prevent a fall could also be related to an increased (and similar) expression of fast MHC-2A fibers in the digital flexors of both species, as this feature of

the hindlimb suggests that faster-contracting digital flexors may be required for quick grasping onto the substrate and/or enhanced grip strength. Alternatively, expression of the fast MHC-2A isoform in the digital flexors, and in selected extensors muscles of the hindlimb, might also have a role in propulsion during vertical climbing. Sloths descend trees approximately once every 8 days to defecate (Montgomery and Sunquist 1975, Meritt 1985) and ascend to the rainforest canopy immediately following elimination. Vertical climbing demands strong purchase of the substrate by the claws with the hindfeet rotated in a position to maximize contact with the support. Propulsion is generated by sequential retraction of diagonal fore- and hindlimb pairs (Mendel 1985; Gorvet et al. unpublished data), where the hip, knee, and ankle joints undergo extension to push the body upward, then the contralateral forelimb pulls up against gravity.

Three-toed sloths have slightly less total muscle mass than two-toed sloths at 23.6% and 26.2% of body mass (Grand 1978), respectively, and may as well have less hindlimb muscle mass and potentially lower force production capability. Evaluation of muscle architecture (i.e., fiber pennation) and the force and velocity properties of sloth hindlimb muscles as reported for the forelimb musculature (Olson et al. 2017) are necessary to verify these possibilities. Nevertheless, *B. variegatus* has shorter hindlimbs compared with *C. hoffmanni*, and in particular, its ankle joint has a long extensor moment arm for mechanical advantage (Marshall et al. unpublished data). When combined with their observed fiber type properties, these features should facilitate large torque application at the ankle joint coupled with enhanced fatigue resistance to sustain extensor torque during slow climbing behaviors. On the other hand, two-toed sloths have less ankle joint leverage, but might have larger extensor muscles with greater physiological cross-sectional area (PCSA), and these architectural properties would offset having reduced mechanical advantage. The largest fibers were observed in the digital flexors of two-toed sloths, and this may be a compensatory modification for force in their distal hindlimbs. It is also possible the ankle extensors and digital flexors of *C. hoffmanni* supply higher power by being faster-contracting (~45% MHC-2A fibers) muscles and relatively faster shortening velocity could be another means to compensate for reduced hindlimb leverage. MHC-1 and MHC-2A isoform expression generally did not correlate with enzyme activity, suggesting that the broader distribution fast MHC-2A fibers in two-toed sloths is

mechanically-linked and represents a functional consequence. Thus, notably faster-contracting mm. gastrocnemius, soleus, and flexor digitorum profundus, a short calcaneus, and long metatarsals result in faster joint rotational velocity, and these features could facilitate faster climbing performance for predator avoidance. Two-toed sloths face predation pressure by number of predators (Goffart 1971; Moreno et al. 2006) beyond that of three-toed sloths and can benefit from faster avoidance behaviors, including climbing, whereas three-toed sloths may be limited to stealth as their main form of defense to avoid being detected by predators (Carillo et al. 2009).

However, vertical climbing is an energetically costly mode of locomotion (Cartmill 1985), and some trade-off between grip strength and power, grasp fidelity, and energy conservation could as well act as a selective pressure for retention (and limitation) of the fast MHC-2A fiber phenotype required for grasping/clinging, especially in digital flexors. Previously a distribution of MHC fiber types was observed that was in accordance with the proposed functional roles of the forelimb digital flexors (Spainhower et al. in press). Similarly, in the present study a relatively broad distribution of small fast MHC-2A fibers with a moderate-to-large CSA in the hindlimb digital flexors further reflects functional compartmentalization of these muscles. Their quick but forceful grasping is also aided by large MHC-1 fibers to ensure secure contact with the support and is possibly due to the rapid shortening of some muscle bellies of the digital flexor complex. Following contact, fast-contracting fibers and their motor units may be minimally activated while large CSA and slow-contracting MHC-1 fibers maintain digital flexion as a means of conserving energy to support the body weight. Specifically, muscle fibers from slow and fast motor units would initially shorten and then sustain grip force via isometric strong contractions with enough force to counterbalance tensile loading of the robust digital flexor tendons of tree sloths. The latter could be a mechanism critical to economical, sustained suspension in tree sloths, and the hindlimbs may play a greater role in support than the forelimbs. Future evaluation of the material properties of digital flexor tendons between limb pairs will be an initial test of the hypothesis.

Whereas requirements for locomotor stability and quick grasping may lead to similar patterns of fiber type distribution in the hip flexors and digital flexors, respectively, the differences observed between species for MHC expression in the other functional groups

are further suggestive of nuances in their lifestyles. For example, the predominant expression slow MHC-1 fibers in the hip extensors, knee flexors/extensors, and ankle flexors of *B. variegatus* consistently place a greater emphasis on slow, deliberate movements and functional limb stability. A large distribution of slow-contracting fibers in knee and ankle flexors likely provide fatigue resistance during postural hanging, behaving as anti-gravity muscle groups, while these slow fibers in the hip and knee extensors counterbalance joint flexor moments and joint stability. The predominant expression of slow MHC-1 fibers could also indicate that three-toed sloths are capable of withstanding longer bouts of static hanging or clinging than two-toed sloths, again related to its predator avoidance behaviors. Further, the functional properties of three-toed and two-toed sloths are also reflected in the specific metabolic characteristics in sloth hindlimb muscles.

Muscle Metabolic Characteristics

Sloth hindlimb muscles have lower contractile energy costs because they only express MHC-1 and MHC-2A fiber types, and not fast MHC-2X or MHC-2B isoforms. Slower isoforms utilize less energy per contraction (Schiaffino and Reggiani 2011), and by combining this MHC fiber phenotype with short, intermittent activity bouts often lasting less than a half hour (Sunquist and Montgomery 1973), sloths can take advantage of rapid CK activity for inexpensive ATP re-synthesis. This combination of fiber type properties are traits previously interpreted to have been independently acquired in three-toed and two-toed sloths (Spainhower et al. in press), providing another example of convergent evolution between sloth taxa. As opposed to typical terrestrial quadrupedal mammals, sloth movement is irregular, and they often stop for several minutes to observe the environment (Britton 1941; personal observations). CK supplies muscles with ATP during the initial seconds of movement, and because sloths typically move in very short bouts, they may rarely utilize glycolytic or oxidative cellular metabolism. This energy system appears to be more prevalent in *B. variegatus* because they have comparatively lower oxidative enzymatic activity related to spending less time in walking in suspension than two-toed sloths (Urbani and Bosque 2006), whereas significantly elevated 3-HAD and CS activities in *C. hoffmanni* may explain their proclivity to engage in more frequent activity with some bouts lasting longer than one hour (Sunquist and Montgomery 1973).

Moreover, ATP utilitization is slower in MHC-1 and -2A than in MHC-2X and -2B fibers and could be matched with lower levels of neuromuscular recruitment for sustained force production while suspended for long durations similar to upright ungulates (e.g., horses) standing for long periods of time while grazing (Hermanson and Cobb 1992). Thus, the contribution of the limb system to economize the cost of force production is two-fold: 1. cheap and rapid ATP replacement and 2. reduced muscle activation providing enough active force to balance passive suspensory support of the body weight.

Elevated CK and LDH activity coupled with low levels of activity for the enzymes CS, 3-HAD, and PFK are unconventional for mammals that primarily express slow MHC-1 fibers and spend the majority of their awake time being mostly inactive (Sunquist and Montgomery 1973) except for feeding and grooming. This only can be reconciled if sloths are in anaerobic metabolism at rest, and recently it was reported that sloths have a respiratory quotient (RQ) > 1 during resting metabolism (Cliffe et al. in press). Low activity for the oxidative enzymes 3-HAD and CS, as well as low body fat storage further suggests that aerobic energy pathways do not sustain ATP production in sloths. The reliance on non-oxidative pathways may be a consequence of nutrient-poor forage where lactate can serve as an energy reservoir. Sloths consume very little food (Cliffe et al. 2015) and must extract as much energy as possible from their diet consisting primarily of leaves from species of cecropia trees (Meritt Jr. 1985; Urbani and Bosque 2006). It is proposed that sloths could be converting lactate into supplemental glucose by it being shuttled to the liver and used as a substrate for gluconeogenesis. Newly synthesized glucose can be transported back to muscle tissue and stored as glycogen, thereby augmenting contractile fuel economy. This possibility will be confirmed by future evaluation of lactate transport proteins, activities of gluconeogenesis pathway enzymes, and glycogen stores in fresh liver and muscle tissue. *In vivo* creatine phosphate stores also need to be evaluated to verify its availability for the CK pathway to be the main phosphagen energy system utilized by sloth limb muscles. Additionally, this interpretation may be particularly important for *B. variegatus* since it selectively consumes only leaves (Montgomery 1983, Chiarello 2008), showed significantly greater CK activity, and is nearly half the adult body mass of *C. hoffmanni* (Britton 1941), which has a more diverse diet (Miller 1935, Meritt Jr. 1985) and showed greater CS activity.

These physiological differences may also account for slight behavioral differences between three-toed and two-toed sloths (Sunquist and Montgomery 1973); higher quality forage and significantly elevated aerobic activity in two-toed sloths coincides with an overall faster fiber phenotype.

Muscle fiber size influences the rates of oxygen diffusion in that fiber size is inversely related to oxidative activity (Schiaffino and Reggianni 2011), and is possibly the reason for decreased oxidative activity in the slow isoform fiber type. MHC-1 fibers have the greatest %CSA in sloth limb muscles and this finding is supported by several studies (e.g., Armstrong and Phelps 1984, Jouffroy et al. 2003, Hesse et al. 2010) using histochemical methods of fiber typing that report that the predominant MHC fiber type expressed tends to be the largest in size. A larger fiber is capable of producing greater force, but at the cost of decreased gas exchange between the muscle fiber and the surrounding capillary beds. Elevated CK activity in *B. variegatus* also could be a consequence of the predominant expression of large MHC-1 fibers, which may be less oxidative than smaller, fast MHC-2A fibers as suggested by the positive correlation between CS activity and MHC-2A expression. This is further supported by the positive relationship between MHC-2A expression and CS activity. The generally smaller, fast MHC-2A fibers diffuse oxygen more rapidly, and these fibers can be more oxidative than slow MHC-1 fibers (Hepple et al. 2000). Slow-contracting fibers can utilize anaerobic metabolism as shown in the present study just as fast-contracting fibers can rely on oxidative metabolism as demonstrated by the expression of highly oxidative fast MHC-2X fibers in the black wildebeest (Kohn et al. 2011b). In accordance with these findings as well as those for several other African antelopes (Kohn 2014), the data herein convincingly suggest that functional requirements influence MHC fiber type properties, rather than dogma established from studies of domesticated animals implying that energy metabolism depends on MHC fiber type expression. This supposition may be particularly relevant for suspensory habits and the consequent functional demands placed on the limb muscles in slow moving, grasping/clinging mammals.

Lastly, one of the more interesting findings of the present study and a previous study (Spainhower et al. in press) is that muscle-specific enzyme activity does not depend on body size in mammals. The scaling relationships demonstrated in Figures 5 and 6, again

support the conclusion that muscle metabolism in sloths is suppressed for mammals of their range of body mass. Notably, tree sloths, in particular *B. variegatus*, demonstrate the lowest field metabolic rate of any non-hibernating mammal (Pauli et al. 2016). Only 3-HAD in *C. hoffmanni* hindlimbs exceeded the predicted levels of enzyme activity, and it was the only enzyme to show any dependence on body size. This finding is less surprising since larger animals can store more fat and utilize it as an energy source, but the lack of relationship for CS, CK, and LDH reinforces that metabolic properties of muscle fibers are likely more dependent on lifestyle (and/or locomotor habits) than body size. For instance, elevated CK activity is observed in both sloths and ambush predator felids, yet fast MHC-2X is the predominant fiber type of a lion m. vastus lateralis (Kohn et al. 2011a) versus 100% MHC-1 in that of *B. variegatus*. Energy metabolism may be similar in sloths and felids because CK activity meets the requirements of intermittent contractile performance, whereas MHC fiber type differs because sloth extensor muscles mainly stabilize the limb flexors for suspensory habits, but felid muscles must generate power for rapid acceleration and quickly subduing prey. Functional similarities may therefore be the main determinant of their muscle metabolism and not 'fiber type,' as established by dogma in domesticated mammals. Moreover, sloths do not share low, variable body temperature and low basal metabolism with most placental mammals, and these characteristics are critical to the suggestion that metabolic energy conservation is fundamental to sloth survival at the extremes of mammalian function and existence. Slow and intermittent movement, enhanced muscle force production at low shortening velocities, and suppressed muscle metabolism all appear to be systemic physiological modifications that convergently evolved in order to enhance energy conservation in tree sloths, potentially beyond those observed in any other placental mammals.

CONCLUSIONS

The hindlimb musculature of sloths is part of an integrated system that is modified for large metabolic energy savings by reliance on low rates of energy expenditure provided by slow movement, low body temperature/basal metabolism, and the primary expression of large, slower-contracting MHC-1 fibers for sustained force production. However, fiber type expression and metabolic properties differ by species, which was not previously observed in the forelimb. In contrast to nearly identical intrinsic forelimb muscle

characteristics, a slower fiber phenotype in *B. variegatus* hindlimb muscles indicates a greater emphasis on stability, whereas relatively greater expression of fast MHC-2A fibers in the hindlimbs of *C. hoffmanni* potentially permits greater contractile velocity for power output for climbing and arboreal maneuvering. Despite these differences, similar independently acquired metabolic characteristics may synergistically lower muscle metabolism to a level where rapid anaerobic pathways within slow and fast MHC fiber types provide adequate ATP production to keep pace with the energy demands of the suspensory habits in both species. Moreover, muscle metabolism is not a physiological parameter determined by body size in mammals, and therefore the fiber type properties observed in the tree sloth lineage appear to have a functional basis more strongly influenced by a slow lifestyle to make suspensory habits possible.

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APPENDIX

INTRODUCTION

This study investigates the fiber type distribution, myosin heavy chain (MHC) isoform composition, and metabolic characteristics of sloth skeletal muscle, and how these properties relate to suspensory behaviors in tree sloths. Muscle tissue specimens will be collected from Hoffmann's two-toed (*Choloepus hoffmanni*) and brown-throated three-toed (*Bradypus variegatus*) sloths. These species were chosen because they are sympatric in Costa Rica, not endangered, and exhibit obligatory suspensory locomotion and/or inverted (below branch) posture (Mendel, 1981, 1985). Suspensory abilities require great strength and endurance, yet muscle mass in tree sloths is low, accounting for 23.6% of total body mass (Grand, 1978), compared with reported average muscle masses of 33% for other arboreal mammals (Muchlinski et al., 2012). However, their overall limb morphology is extremely modified for suspensory behaviors (Miller, 1935), and while descriptions of limb morphology are available, the intrinsic properties of sloth muscles remain poorly understood. Metabolic properties and myosin fiber type distribution in the limb musculature of *C. hoffmanni* and *B. variegatus* will be determined to validate our understanding of how mammalian muscles become adapted for strong, sustained contractions required for prolonged grip force during suspensory behaviors. In the following sections, the use of tree sloths as an animal model for improving our understanding of the range of contractile performance in mammalian skeletal muscle, and how suspensory behaviors relate to metabolic energy savings, will be established by discussing their origin and evolution, ecology, behavior, physiology, limb myology, physiological (metabolic) properties of muscle fiber types, and preliminary data on sloth muscle characteristics.

ORIGIN AND EVOLUTION

Sloths belong to the order Pilosa and superorder Xenarthra. Xenarthrans (anteaters, armadillos, and sloths) represent a basal clade of placental mammals namely sharing xenarthrous (extra) intervertebral, or zygapophyseal, joints among the lumbar vertebrae. Also, the transverse processes of the anterior caudal vertebrae are fused to the pelvis, and this rigid structure provides additional stability in the trunk (Engelmann, 1985). Members of this clade exhibit fossorial, terrestrial, and arboreal habits, and among them, the sloths

are most distinctive for their suspensory habits and adaptations for these behaviors. *Choloepus* (Family: Megalonychidae) represents the only extant taxon descending from the Megalonychids (and possibly the Mylodonts), which alongside the Megatheriids, make up the extinct giant ground sloths. Despite their structural and functional similarities, recent evidence suggests that *Choloepus* and *Bradypus* (Family: Bradypodidae) are only distantly related, and that *Bradypus* split from the giant ground sloths nearly 40 million years ago (Gaudin, 2004), thus representing an astonishing example of convergent evolution. Morphological similarities between genera are likely the result of canalized traits in the last common ancestor that exhibited a fossorial to semi-arboreal lifestyle (Nyakatura, 2012).

ECOLOGY AND BEHAVIOR

C. hoffmanni occurs in two disjoined populations: the northern population ranges from Nicaragua to as far south as western Venezuela, whereas the southern population occupies north-central Peru and ranges through the regions of western Brazil and central Bolivia (Plese and Chiarello 2014). The range of *Bradypus* is broad, extending north into Honduras and south into northern Argentina (Wetzel and Avila-Pires, 1980; McCarthy et al., 1999). Populations of *C. hoffmanni* are sympatric (overlapping) with those of *B. variegatus* primarily in their specified northern regions. Generally, sloths are arboreal folivores that spend the majority of their lives in the canopies of neotropical rainforests of the Americas. The average home range is 1.97 ha for *C. hoffmanni* and 1.60 ha for *B. variegatus*. Specifically, sloths predominantly inhabit the crowns of multiple tree species and change trees by traversing interconnected crowns and vines, but they will descend to the forest floor to defecate once every 7–10 days (Montgomery and Sunquist, 1975). Both species show a preference for trees with large crowns (greater than 200 m^2) that have high exposure to light (Montgomery and Sunquist, 1978). However, *C. hoffmanni* prefers moist over dry habitats, whereas *B. variegatus* shows a preference for dry sites (Handley, 1976).

Unless they are searching for a mate, sloths rarely leave their home range. They are not social animals and instead are known for solitary habits (Hayssen, 2010). Notably, population density estimates based on fecal census taking showed that the number of *C. hoffmanni* per unit area is 1.1 per hectare (Montgomery and Sunquist, 1975). *B.*

variegatus lives at higher population densities than *C. hoffmanni*, which is reported to be 8.5 individuals per hectare (Montgomery and Sunquist, 1975). Sympatric species of three-toed sloths, of which there are currently four recognized species of *Bradypus*, tolerate one another presumably because they each prefer feeding on the foliage of different tree species (see below); foraging habits that are continued through the genealogy of an individual (Montgomery and Sunquist, 1978).

Canopy dwelling species often have the advantage of having relatively few natural predators. Descriptions of harpy eagle and jaguar depredation are available for *B. variegatus* and *C. hoffmanni* (Galetti and de Carvalho, 2000; Garla et al., 2001; Touchton et al., 2002; Carillo et al., 2009). Accounts of ocelot, margay, and anaconda attack also exist for *C. hoffmanni* (Goffart, 1971; Moreno et al., 2006). Recently it has been suggested that the selective pressure of depredation partially explains the slow and deliberate movements of sloths (Nyakatura and Andrada, 2013). Moving cautiously may be a strategy used to avoid being seen by predators. This type of cryptic locomotion was first described in slow lorises (Demes et al. 1990), and the strategy seems appropriate for deterring attacks by birds-of-prey and may also provide some insurance against attack by a carnivorous felid while on the ground, a circumstance where sloths are particularly vulnerable to attack.

Nocturnal behavior is also an advantageous strategy for avoiding predators. *C. hoffmanni* is strictly nocturnal; activity usually begins one hour after sunset and ceases by sunrise with no reports of crepuscular (i.e., twilight activity) habits (Sunquist and Montgomery, 1973). In contrast, *B. variegatus* is diurnal being active both day and night, with the height of its activity occurring from noon to 4PM (Sunquist and Montgomery, 1973; Urbani and Bosque, 2007). Whereas activity patterns in *Choloepus* appear to be controlled by light-dark cycles, activity patterns in *Bradypus* are less clear, but may be related to digestion (i.e., they are active when hungry and inactive when satiated) (Sunquist and Montgomery, 1973). In general, sloths have extraordinarily low metabolic energy requirements, and activity patterns reflect this trend in energy savings. Sunquist and Montgomery (1973) reported that an average activity level of 10.1 hours for *Bradypus* is more than that for *Choloepus*, which is active for 7.6 hours on a daily basis. Much of their active time is spent grooming and removing debris by using their claws to

comb their fur (Merrit, 1985). Additionally, bouts of activity rarely last more than 6 hours total. Whereas nearly half of the individual activity bouts are less than half an hour for *Bradypus*, bouts were more evenly distributed from 0–1/2 hour (25.4%) to 2–6 hours (23.1%) for *Choloepus* (Sunquist and Montgomery, 1973). Despite these differences, sloths generally tend to act "slothfully," and this behavior is further emphasized by their energetically restrictive diet of primarily leaves.

Sloths are primarily high canopy folivores. Reports show that foliage amounts to 99.4% whereas fruit makes up 0.6% of their diet (Urbani and Bosque, 2006). However, the diet of *C. hoffmanni* is more varied and additionally consists of buds, flowers, twig tips, and young stems (Meritt, 1985). In captivity, *C. hoffmanni* is also tolerant of small amounts of meat protein in their diet. *B. variegatus* on the other hand, almost exclusively feeds on leaves. Interestingly, the digestive tract of sloths is long and ruminant-like in its modifications, consisting of a six to eight chambered 'stomach.' Food passage rates in sloths are the slowest recorded for any mammal, ranging from 2.5 to 50 days from ingestion to voiding waste (Montgomery and Sunquist, 1978). Because of this extremely slow digestion rate, and despite the fact that food intake for *Bradypus* is reported to be a mere 17 g dry weight kg^{-1} day^{-1}, their stomachs are constantly full of food material (Cliffe et al., 2015). Nearly half of sloth fecal matter is composed of undigested foliage, and because of this it is a significant long-term stable nutrient source for trees (Montgomery and Sunquist, 1975), making sloths important contributors to nutrient cycling, an ecological component that plays an essential role in maintaining a healthy, productive rainforest ecosystem.

Sloths forage by traversing the canopy in search of trees with abundant leaf content. They move about while hanging suspended from branches and vines. Of the various forms of arboreal locomotion, suspensory behaviors are peculiar, and only a few species of mammals (e.g., some primates and sloths) are known for maneuvering about trees in a suspended posture. For example, the slow loris (*Nycticebus*) exhibits facultative suspensory locomotion, but this behavior is only supplemental to above branch or pronograde locomotion (Ishida et al., 1990). Sloths exhibit obligatory suspensory locomotion that is below branch (anti-pronograde) and have undergone extreme morphological modification such that they are highly adapted to this arboreal lifestyle.

For example, long, re-curved claws wrap above and around the support in a hook-like fashion (Britton, 1941). Interlocking (by keel-and-groove articulations) interphalangeal joints and metacarpo/metatarsophalangeal joints permit flexion/extension only, and preclude lateral deviation and independent positioning of digits, while highly mobile carpal (wrist) and tarsal (ankle) joints accommodate lateral and medial movements of the feet (Mendel, 1981b). Together these characteristics represent arboreal adaptations and will be described in more detail below (see section: Morphology and Physiology).

Sloths move by placing their feet in contralateral (opposite side), diagonal couplets and also utilize a lateral sequence 'walking' gait when progressing at slower speeds and a diagonal sequence gait when progressing at moderate speeds (Mendel, 1981a, 1985). While their feet are poorly equipped for upright, terrestrial support and locomotion, sloths are observed moving on the ground. Mendel (1981a) noted that sloths do not drag their ventrum on the ground and their step lengths are short, averaging 21 cm. Three limbs are usually in contact with the ground, though at the end of the swing phase of a forelimb, the contralateral hind limb will enter its swing phase. While climbing vertically, contralateral fore- and hindlimbs are nearly in simultaneous swing phase, and thus the remaining two limbs that are in contact with the substrate (i.e., stance phase) support the body weight (Mendel 1981a, 1985). Arboreal locomotion in both *C. hoffmanni* and *B. variegatus* is slow and deliberate, with step length averaging 30 cm, which is slightly greater than step length for their terrestrial walking (Mendel, 1981a). Foot placement is delicate, presumably to avoid generating a substrate reaction force large enough to break the arboreal support that would result in a deadly fall (Nyakatura, 2013). Stance phase usually lasts twice as long as the swing phase, and because sloths cannot introduce an aerial phase to their stride (i.e., sloths cannot run), they are limited to decreasing stride duration and increasing stride length when switching from moderate to higher walking speeds (Nyakatura et al., 2010). Furthermore, whereas *C. hoffmanni* will descend a tree headfirst, *B. variegatus* descends tail-first (Mendel 1985). Descending trees headfirst is rare among arboreal mammals.

When not foraging or in search of a potential mate, sloths spend much of their time resting. Observations by Urbani and Bosque (2007) indicate that *B. variegatus* spent nearly 73% of its time resting, and over 60% of its stationary time is spent in a huddled

posture. If they are not suspended from a support, then they may assume a posture similar to sitting cross-legged. They also will squat in the fork of a tree while grasping the trunk with its limbs. Furthermore, EEG recordings reported by de Moura Filho et al. (1983) show that *Bradypus* spent only about 10% of a 24-hour period in deep sleep, and that the rest of the time it is either in light sleep (~56%), or awake (~34%). Thus behavioral sleep, where the sloth is resting with its eyes closed and head resting on the chest, predominates daily activities. When not sleeping, a sloth will spend much of its active time grooming. It will remove food from its head/face and scratch its fur for up to six-minute bouts (Meritt, 1985).

MORPHOLOGY AND PHYSIOLOGY

Sloths are medium-sized arboreal mammals. On average, adult male three-toed sloths are slightly larger (4.1 kg) than adult females (3.9 kg) (Pedrosa et al., 2002; Muhlbauer et al., 2006). Two-toed sloths are larger than three-toed sloths, reaching body masses of 6.5–7.5 kg (Britton, 1941). Notably, the faces of *Bradypus* and *Choloepus* are unmistakable. Whereas *Bradypus* has a blunt face, *Choloepus* has an elongated face and a fleshy, wet nose. Furthermore, *Choloepus* possesses an anterior caniniform tooth, which is absent in *Bradypus* (Webb, 1985).

Beyond simple interspecies differences (and/or sexual dimorphism) in body mass and facial features, *Bradypus* and *Choloepus* also differ in a number of morphological features, most notably by the number of digits remaining on their forefeet as their common names suggest. *Bradypus* (three-toed sloths) has three partially fused digits on their forefeet (digits II–IV) whereas *Choloepus* (two-toed sloths) has two unfused digits (digits II and III); however, both genera possess three digits (digits II–IV) on their hind feet. The bony digital elements of the hind feet are partially fused to fully fused, and the feet as a collective unit function like hooks for suspensorial posture and locomotion (Mendel, 1981b, 1985). Additionally, the volar surfaces of *Bradypus* and *Choloepus* differ in that the volar pads of *Bradypus* are hairy whereas *Choloepus* possesses hairless, glabrous volar pads. Despite these differences, both genera utilize these structures as friction pads that aid in gripping arboreal supports. Though they share functional similarity, deviations in digit retention and volar surface morphology are suggestive of independently evolved suspensory function (Mendel, 1985).

Both genera of sloths are also rare examples of taxa that deviate from the nearly exclusive mammal rule of having seven cervical vertebrae regardless of neck length, a trait shared only with manatees and dugongs (the Sirenia). The neck of *Bradypus* has 8 to 9 cervical vertebrae and is capable of rotating the head nearly 180º to face forwards during below branch walking (Miller, 1935; Mendel, 1985). In contrast, the neck of *Choloepus* has 5 to 8 cervical vertebrae (number varies per individual and species) with a limited range of motion in rotation (Miller, 1935). As a consequence, *Choloepus* hyperextends its neck/head and must look upside down while traveling below orthogonal branches. Another interesting morphological difference is that fore- and hindlimb length is nearly equal in *Choloepus*, and it is often found hanging from all four limbs with its trunk near horizontal to the arboreal substrate (Hayssen, 2011). *Bradypus* on the other hand, has longer forelimbs than hindlimbs, and it regularly assumes a seated posture in a corner between trunk and branch, or it will suspend from branches at angles less than ninety degrees to the tree trunk (Hayssen, 2010). Either suspensory behavior is facilitated by a shift in elbow extensor/flexor muscle mass ratio, which is four times smaller than that of upright quadrupeds, thus demonstrating a morphological adaptation for resisting the inverted gravitational vector resulting from below branch behaviors (Fujiwara, 2011). The favoring of flexor over extensor mass may have further implications for myosin heavy chain (MHC) isoform expression and fiber type distribution (see section: Properties of Muscle Fiber Types, *Contractile physiology*).

A suite of physiological adaptations complements the morphological modifications described, and these are suggested to be adaptations for energy-metabolic savings during suspensory behaviors. It has already been established that sloth metabolic rates are much lower than that of other placental mammals. For example, *B. variegatus* has the lowest recorded field metabolic rate of 162 kJ/day*kg$^{0.734}$ among non-hibernating mammals (Pauli et al., 2016). A low metabolic rate accompanies lower body temperature. Daily body temperature rhythms for sloths are consistently lower than averages for placental mammals and are reported to be 34.5°C for *Choloepus* and 32.7°C for *Bradypus* (Pauli et al., 2016). In addition to these critical physiological parameters, sloths possess *retia mirabilia* in each limb. *Retia* are complex vascular bundles that serve as countercurrent heat exchangers that cool the distal limbs and likely reduce metabolic requirements of

tissue for oxygen. Specifically, *retia mirabilia* may function to reduce metabolic requirements of limb muscles when blood supply is limited due to prolonged muscle contraction for grasping (e.g., slow lorises), hanging stabilization (e.g., sloths), and postural maintenance (O'Dea, 1990). The convergent traits of *rete miribile* and low metabolic rate between slow lorises and sloths are both interesting and informative regarding structure-function in slow climbing, slow moving mammals.

MYOLOGY

The effect of gravity has played a crucial role in the evolution of tetrapod locomotion. Because the gravity force vector always points towards the Earth, thus pulling objects downward and giving them weight, the antigravity function of muscles depends on the orientation of the body and is facilitated by either flexors or extensors (Jouffroy et al., 1990). Whereas in pronograde arboreal locomotion the gravity vector is a compressive force that favors the recruitment of extensor muscles, the effect of gravity becomes a *tensile* force in suspensory locomotion, thus favoring the recruitment of flexor muscles in an antigravity role (Jouffroy and Stern, 1990). A tensile limb system is unusual among mammals, and it indicates a deviation from typical (upright) functional locomotor requirements. Aside from sloths, one other notable example of a tensile limb system is the forelimb of gibbons (*Hylobates*), although these mammals employ brachiation whereby they either swing (consecutive) or ricochet between handhold contacts with tree branches. Certain species of neotropical primates such as woolly and spider monkeys (Family: Atelidae) also will engage in forelimb suspensory behaviors while maneuvering in trees. These modes of arboreal locomotion are more specialized, and while limited data are available for skeletal features of tensile limb systems, the influence of suspension on the intrinsic properties of limb muscles has not been investigated. Moreover, few descriptions of myology exist for sloths and most of these are historical references (e.g., Macalister, 1869; Humphry, 1870; Windle and Parsons, 1899) that are incomplete and use outdated anatomical nomenclature. The focus herein will be on the hindlimb as studies of sloth forelimb myology, muscle architecture, and MHC fiber type distributions are in progress (Olson et al., in review; Spainhower et al., unpublished data).

The following anatomical descriptions are for the hindlimb musculature in *Bradypus* and are based primarily on observations made by Humphry (1870) and Macalister (1869)

with updated nomenclature provided by Butcher et al. (in preparation). All muscles of the hindlimb are organized into functional groupings. Moreover, the muscles identified below are selected to be representative of each functional group based on ease of observation and available muscle mass, and do not necessarily reflect the complete anatomy of the sloth hindlimb.

Hip Adduction

The thigh is adducted at the hip by *mm. adductor brevis, adductor magnus, adductor longus,* and *gracilis*. As the nomenclature implies, the primary function of the adductor muscle group is adduction of the thigh at the hip joint and secondarily may act to extend the hip joint (e.g., *adductor magnus*). *M. gracilis* is the broadest (sheet-like) muscle of the hip adductors, and it also participates in knee flexion due to its attachments distal to the knee joint at the medial tibial condyle and crural fascia just proximal to the ankle joint. During below branch walking, the femur is held in a laterally rotated position, but it must be adducted toward the branch while the limb segment is moved primarily in a sagittal plane during vertical climbing. Emphasis would be placed on this muscle group during vertical clinging and potentially during suspensory activities as well to maintain hip joint stability.

Hip Rotation and Stabilization

The deep rotators *mm. piriformis* and *quadratus femoris* are present, as are *mm. gemelli*, the latter muscles being approximately equal in size and indistinguishable from one another. It is noted that in some individuals the presence of distinct bellies for both the *m. piriformis* and *m. quadratus femoris* muscles is not clear. The *m. quadratus femoris* is the more variable muscle (Mackintosh, 1875). The *mm. gemelli* bellies share a common insertion with the other deep rotators. As a functional group, these muscles cause thigh axial rotation, or lateral rotation of the femur, as well as acting to stabilize the hip joint.

Hip Flexion

Flexion of the thigh at the hip joint is performed by *mm. psoas major, sartorius, iliacus*, and *rectus femoris*. The primary action of *m. psoas major* (no distinction of a minor head is clearly observed) is to flex the hip. The biarticular *m. sartorius* is a long, strap-like muscle that causes flexion at the hip and knee joints and also rotates the thigh laterally

and the leg medially. This positioning of the hindlimb likely plays an important role for vertical climbing.

Hip Extension and Knee Flexion

The thigh is extended at the hip joint by action of the *mm. gluteus superficialis, gluteus medius*, and *gluteus profundus*. *M. gluteus superficialis* is a thin, triangular muscle that is superficial to the other two gluteal bellies. *Mm. gluteus medius* (most massive) and *gluteus profundus* are largely inseparable from one another and have the usual mammalian attachments. In addition to extending the thigh, the gluteal muscles also facilitate thigh abduction at the hip joint. The hamstrings, *mm. semitendinosus*, *semimembranosus*, and *biceps femoris* are all bi-articular, and act in both hip extension and knee flexion. *Mm. semitendinosus* and *semimembranosus* arise by a common tendon from the large ischial tuberosity and together descend the posteromedial thigh. *M. biceps femoris* is a large muscle consisting of two heads: the long (pelvic) head acts as a hip extensor and knee flexor whereas the femoral head acts solely as a knee flexor. Additionally, *mm. sartorious* (described above: Myology, *Hip Flexion*), *gastrocnemius*, and *flexor digitorum superficialis* (or *m. plantaris*) act to flex the leg at the knee joint.

Knee Extension

Extension of the leg at the knee joint is performed by the four muscle heads of the *m. quadriceps femoris*: *rectus femoris*, *vastus lateralis*, *vastus intermedius*, and *vastus medialis*. Each muscle head has a distinct origin on the innominate bone and/or proximal femur. The four bellies merge distally and continue as a single quadriceps tendon before inserting on the patella, which then attaches distally to the tibial tuberosity via the patellar ligament. This pulley-like arrangement provides greater muscle moment arm (i.e., mechanical advantage) during knee extension than if the quadriceps tendon were to insert directly onto the tibia after crossing the femoral condyles closer to the joint center of rotation.

Ankle Flexion

M. tibialis cranialis acts to flex the ankle joint and invert the foot. This well-developed muscle is comprised of one tibial and two fibular heads. The three heads merge and continue as a tendon that inserts on the remnant of metatarsal I, in addition to having

minor attachments to the medial tarsal bones. *Mm. fibularis longus* and *fibularis quartus* flex the ankle joint and evert the foot, whereas *mm. fibularis brevis* and *extensor digitorum longus* both facilitate ankle flexion only.

Ankle Extension

Extension of the foot at the ankle joint is performed by *mm. gastrocnemius* and *soleus*. This action is also facilitated by the digital flexors (described below: Myology, *Digital flexion*). *M. gastrocnemius* is the most superficial muscle of the posterior leg compartment and consists of two distinct heads with fleshy, proximal origins on the distal femur. The medial head is larger than the lateral head and together form a V-shaped muscle-tendon unit by merging distally and inserting via a single tendon on the calcaneus. Deep to *m. gastrocnemius* lies *m. soleus*, which also inserts on the calcaneus via the common calcaneal tendon. *M. tibialis caudalis* is distinct from the other ankle extensors by inserting on the remnant of metatarsal I via a long, gracile tendon. It has a reduced belly and is commonly fused with bellies of the *mm. flexores digitorum profundi* complex. This muscle also supinates (or inverts) the foot.

Digital flexion

M. flexor digitorum superficialis and *mm. flexores digitorum profundi* (*m. flexor digitorum lateralis* and *m. flexor digitorum medialis*) comprise the well-developed digital flexor group. Smooth, coordinated movement of the digits is an essential component of suspensory function, and therefore the digital flexors are of high importance. *M. flexor digitorum superficialis* originates from the femoral epicondyles and continues as a modest tendon that merges near its origin with the common tendon of *mm. flexores digitorum profundi*. This robust common tendon passes deep to *m. soleus*, and then splits into three tendons that pass to the digital volar surfaces via specializations of the palmar ligaments (volar plates) that form grooves (i.e., tendon tunnels) for the thick flexor tendons to insert onto the distal phalanges.

Digital Extension

Extension of digits II-IV (in *Bradypus*) is caused mainly by the action of the diminutive *m. extensor digitorum brevis*. It passes distally along the cranial leg compartment and continues as a short tendon that inserts onto the proximal phalanges of digits II-IV.

PROPERTIES OF MUSCLE FIBER TYPES

Contractile physiology and function

Four adult myosin heavy chain (MHC) isoforms have been identified in mammalian limb muscles using protein gel electrophoresis (SDS-PAGE), and they are in order from slowest-to fastest contracting: MHC-1, MHC-2A, MHC-2X, and MHC-2B (Bottinelli, 2001; Toniolo et al., 2004). Each isoform corresponds to a generalized 'fiber type' of similar nomenclature (e.g., Type I and Type 2A fibers). MHC isoform expression influences intrinsic fiber contractile properties, namely ATP turnover rate (myosin ATPase activity), shortening velocity (V_0, in fiber lengths/second), and power (W, in watts) (Bottinelli, 2001). For example, myosin ATPase activity is faster for isoforms MHC-2A, X, and B than the MHC-1 isoform. Iorga et al. (2007) showed that in rabbit muscle the ATP binding rate is 2–5 times slower and ATP hydrolysis rate is 5–6 times slower in MHC-1 fibers compared with MHC-2X fibers. Moreover, myosin ATPase rate directly influences rates of actomyosin cross-bridge cycling, and thus magnitudes of V_0 and W (Barany, 1967; Schiaffino and Reggiani, 2011). Slow-contracting MHC isoforms hydrolyze ATP at a slower rate and are better suited for prolonged and/or repetitive contractions. In contrast, fast MHC isoforms and their corresponding fiber types 2A, 2X, and 2B, are capable of higher force and power output, but because their myosins hydrolyze ATP more quickly, these fiber types are fatigable.

A myosin molecule consists of two heavy chain myosin molecules that wrap together to form a rod with two club-like heads at one end. Myosin therefore can express homogeneous or heterogeneous MHC isoforms. Whereas fibers expressing a single MHC isoform are classified as 'pure' MHC fiber types, fibers expressing more than one MHC isoform are classified as 'hybrid' MHC fibers. Contractile properties of hybrid MHC fibers are intermediate to those of pure myosin fibers expressing the same isoforms (Rupert et al., 2014). Functional transitions between isoforms that result in hybrid fibers demonstrate the fluidity of fiber type distributions and show that muscles possess a large capacity for plasticity in response to use or disuse. Fibers change in response to exercise (e.g., strength training) with increases in cross-sectional area (CSA) that lead to muscle hypertrophy and greater force output, in addition to MHC isoform transitions. In general, myosin fiber type transitions proceed in a stepwise direction with MHC expression

shifting to the isoform that is immediately slower or faster contracting. For example, a faster-to-slower contracting transition with repeated use of a muscle would be the following along with selective hypertrophy in fiber CSA: MHC-2B>MHC-2X>MHC-2A>MHC-1 (Schiaffino and Reggiani, 2011). Most often MHC-2A fibers undergo hypertrophy and may be considered the most functional fiber type. Conversely, immobilized muscles become progressively faster in the direction MHC-1>MHC-2A>MHC-2X>MHC-2B and fiber CSA decreases representing muscle atrophy (Schiaffino and Reggiani, 2011).

The energy metabolism of muscle fibers is also conventionally thought to be related to their MHC isoform content. For example, fast MHC fiber types can contract more rapidly and are subject to fatigue, thus they are believed to rely more on glycolytic metabolic pathways (Schiaffino and Regianni, 2011). Cheetahs best demonstrate this convention by being capable of sprinting at 100 km h^{-1}, but they can sustain this effort for only <300 m before exhaustion. These carnivorous felids have a primary expression of fast MHC-2X fibers in their hindlimb muscles, indicative of their role in propulsion for high-speed (pursuit) prey hunting (Goto et al., 2013). A greater expression of MHC-2X fibers additionally is a strong predictor for maximum running speed (Kohn et al., 2011a). However, whereas muscle fiber type distribution can largely be constrained by phylogeny (i.e., ancestral retentions), the metabolic properties of muscle fibers can be modulated and 'tuned' for specific behaviors, as discussed below (Properties of Muscle Fiber Types: *Metabolic Properties of Muscle Fibers*) (Kohn et al., 2011a).

In contrast to limb muscles that have a large distribution fast MHC fiber types, muscles predominated by slower-contracting MHC fibers are optimal for postural maintenance and sustained movement because of their oxidative metabolism and fatigue resistant properties. Slow lorises best demonstrate this convention, and the available data for *Nycticebus coucang* serve a proxy for the muscle physiology expected in sloths. The hindlimb muscles in *Nycticebus* contain no fast MHC-2X or 2B fibers, and instead have broad distributions of slow, oxidative MHC-1 fibers and fast, oxidative MHC-2A fibers (reported as fast oxidative-glycolytic fibers: FOG) (Sickles and Pinkstaff, 1981). More than half of their hindlimb muscles express >50% slow MHC-1 fibers with higher percentages observed in proximal and deep muscles than in distal muscles, while their

distribution of fast MHC-2A fibers shows the opposite trend (Sickles and Pinkstaff, 1981). These findings match well with the slow and deliberate vertical climbing behavior utilized by *Nycticebus*, which would best be achieved by large percentages of slow MHC-1 fibers for sustained grasping/clinging to trees.

Fiber type heterogeneity also is an essential functional feature of the mammalian musculoskeletal system. This allows muscles to act in a variety of roles, and because limb muscles typically accommodate multiple tasks (e.g., braking, propulsion, and posture), they rarely express a single myosin fiber type. Again using data for the slow loris, it is observed that the hip and thigh muscles are predominated by MHC-1 fibers, whereas muscles of the leg have nearly equal percentages of MHC-1 and MHC-2A fibers (Sickles and Pinkstaff, 1981). The difference in fiber type distributions between proximal and distal limb muscles (a slower-to-faster gradient) likely reflects functional differences. That is, hip and thigh muscles can act as stabilizers for postural support and may not be involved in either potentially rapid movements of the digits or higher force contractions of muscles that flex the digits for grasping. However, MHC fiber expression of distal limb muscles that have a strict postural or proprioceptive role, as hypothesized for the *m. soleus* in horses (Meyers and Hermanson, 2006; Butcher et al., 2010), is an exception to this principle.

Muscle function is a major determinant of intrinsic properties, but size also matters in physiology, and body size also influences MHC fiber type expression and muscle contractile velocities. The general principle is that as body size increases, muscles become slower-contracting, and this has important consequences for animal thermoregulation. Therefore, percentage distributions of slow MHC-1 fibers increase while those of fast MHC fiber types decrease, especially for fast MHC-2B, which is generally not expressed as a functional protein in placental mammals beyond several kilograms in body mass (Pellegrino et al., 2003). Exceptions to the latter observation are expression of MHC-2B fibers in marsupial muscles (Hazimihalis et al., 2013), domesticated pig limb muscles (Toniolo et al., 2004), and the extraocular muscles of mammals (Schiaffino and Reggiani, 2011). Notably, V_0 of all four MHC isoform fibers scales inversely with increasing body size (Pellegrino et al., 2003). This relationship is more extreme in slow MHC-1 fibers than that for either fast MHC-2A, 2X, or 2B fibers

(Toniolo et al., 2007). This indicates that slow MHC-1 fibers in small mammals have a much faster intrinsic shortening velocity than the same isoform (orthologous) fibers in large mammals. This observation is often interpreted as small mammals requiring greater intrinsic power to cycle their short limbs at higher frequencies. Despite having fewer MHC-1 fibers with relatively high $V_{0,}$ these fibers along with a large distribution of fast MHC fiber types, all have high mitochondrial density and do well to resist fatigue (Leiber, 2002). Thus, the metabolic properties of muscle fibers can be specialized for functional demand (Kohn et al., 2011a).

Metabolic Properties of Muscle Fibers

Muscles rely on oxidative and glycolytic metabolic pathways for ATP generation and re-synthesis. Conventional physiology informs us that slow MHC-1 and fast MHC-2A fibers are relatively small in their cross-sections but contain large numbers of mitochondria, and thus use oxidative metabolic pathways for sustained and/or repetitive muscle contractions. On the other hand, fast MHC-2X and 2B fibers typically have a larger CSA, contain fewer mitochondria, and require rapid ATP replenishment to match high rates of energy expenditure for power output. Moreover, muscles get their reddish color from myoglobin content, which can be a preliminary indicator of muscle function (e.g., muscles having a postural vs. dynamic role). Because MHC-1 fibers are rich in myoglobin and 2A isoform fibers also have an appreciable myoglobin content, muscles containing a predominance of MHC these fiber types are typically deep red in color. Conversely, pink (or white) muscles typically contain broad distributions of MHC-2X and 2B fibers and get their color due to lower myoglobin content and glycogen storage.

Although fiber type distribution may largely be constrained by phylogeny, mammalian skeletal muscle is still adaptable. Energy metabolism can be modulated so that muscle tissue simultaneously exhibits oxidative and fast-twitch properties to match functional demands for force/power output while resisting fatigue (Kohn et al., 2011a; Kohn et al., 2011c; Rupert et al., 2015). For example, Kohn et al. (2011b) found a large percentage of MHC-2X fibers that had both large glycolytic and oxidative capacities in *mm. vastus lateralis* and *longissimus lumborum* in several African ungulates. These authors also reported that the same muscles in the lion (*Panthera*) expressed relatively more MHC-2X fibers with similar glycolytic capacities, but lower oxidative enzymatic activities than

those in springboks and wildebeests (Kohn et al., 2011a). The collective findings from these studies indicate that MHC content is not strictly correlated with muscle metabolism, and fiber type properties transition (or shift) as a result of functional use. Therefore, it is possible for mammals to have MHC fiber types with fast contractile properties and high fatigue resistance. The prey-predator interaction between cursorial ungulates and felids demonstrates this form of selection on muscle physiology as it relates to running performance and fitness. That is, ungulates require the capacity for both high speed and high endurance to outlast pursuit predators, while the cats require greater capacity for high velocity/power to ambush and chase down prey.

To better understand the metabolic capacities of muscle tissue, enzymatic activity is investigated by measuring substrate turnover. Specifically, enzyme kinetics for metabolic markers of phosphofructokinase (PFK), lactase dehydrogenase (LDH), and creatine kinase (CK) activities indicate anaerobic metabolism (i.e., glycolysis), whereas citrate synthase (CS) and 3-hydroxyacetyl coA dehydrogenase (3HAD) activities indicate aerobic metabolism (i.e., Krebs cycle) (Kohn et al., 2011a). This quantitative method extends beyond traditionally qualitative assessments via histochemical staining for NADH (e.g., NAD, SDH, and α-GPD) for several reasons. For instance, conventional staining techniques are statistically limited because they are based on mitochondrial density and NADH concentration within a fiber, parameters that are informative but not necessarily definitive. In contrast, because quantitative measurements are amenable to statistical analysis, enzymatic activity can be included in more sophisticated study designs that address the relationship between myosin fiber type and aerobic/anaerobic capacities.

Lastly, direct measurements of metabolic enzyme activity are critical to our understanding of how muscles can produce substantial force and simultaneously maintain force production for extended period of time without fatigue. For example, the predominant expression of fast, and large MHC-2X fibers in a muscle would suggest that it is capable of producing high force. If the same muscle also showed high enzymatic activity for oxidative metabolism, then it would be capable of high force production and the ability of sustaining that level of force during prolonged and/or repeated contractions. Finding additional examples of animals and muscle groups beyond those discussed above

(see Kohn et al., 2011a,b,c) in which MHC fiber type expression does not predict oxidative/glycolytic muscle metabolism, would not only continue to improve knowledge of the range of contractile performance of mammalian muscle, but may also shift the current paradigm of muscle 'fiber type' properties.

PRELIMINARY DATA

Prior to the upcoming hindlimb study, our lab has collected data on MHC fiber type distribution and muscle fiber size in sloth forelimb musculature. Previous analyses were limited due to the techniques available at that time and characterized sloth limb muscles as the "red" variety (Goffart, 1971). This observation was made at a time when the role of myosin isoforms with respect to contractile properties was not well understood, and thus previously published information has become outdated. However, in a qualitative manner, data from Goffart (1971) suggests that sloth limb muscles are moderately-to-highly oxidative in their energy metabolism, recalling that darker muscles are indicative of higher levels of myoglobin, and thus greater oxygen storage in the tissue. Our novel data support and update the previous findings by showing the expression of only slow MHC-1 and fast MHC-2A in sloth forelimb muscles (Spainhower et al., 2017).

Shoulder, brachial, and antebrachial muscles in both *B. variegatus* and *C. hoffmanni* are comprised predominately of MHC-1 fibers, followed by an appreciable percentage of MHC-2A fibers, and a small percentage of MHC-1/2A hybrid fibers. Furthermore, it is observed the percentage decrease of slow MHC-1 fibers in the shoulder/brachial muscles is proportional to increase of fast MHC-2A fibers in the distal carpal/digital flexors. Notably, and unconventionally, MHC-1 fibers were found to be larger in CSA than MHC-2A fibers (Spainhower et al., 2017). Because fiber force is proportional to CSA, large MHC-1 fibers likely produce moderate force and resist fatigue via oxidative metabolic pathways and slower ATP turnover rates. Additionally, a substantial amount of extracellular matrix is observed, and we speculate that the connective tissue consists primarily of type I collagen and is part of an extensive intramuscular tendon that augments levels of muscle *passive tension* during suspensory behaviors. It is reasonable to form the supposition that sloth hindlimb muscles will share similar morphological and physiological properties as those found in forelimb muscles.

Objectives and Hypotheses

The objective of this study is to evaluate MHC isoform expression and metabolic enzyme activity in sloth hindlimb muscles. The collected data will be used determine the fiber type properties that help make suspensory behaviors possible. Because sloths are able to hang suspended for extended periods of time without fatigue, it is hypothesized that sloth limb muscles have evolved modifications to muscle contractile properties and energy systems that provide fatigue resistance for maintaining moderate-to-high levels of force production. This hypothesis will be tested by determinations of MHC content, and fiber type distribution, CSA, and metabolic profiles. Specifically, the following outcomes are predicted: (i) primary expression of slow MHC-1 and a large distribution of MHC-1 fibers, (ii) expression of the fast MHC-2A isoform only and not MHC-2X or B, (iii) a proximodistal, slower-to-faster isoform gradient along the length of the limbs, and (iv) high enzyme activity for oxidative markers and low activity for glycolytic markers for each MHC fiber type identified. The latter prediction would indicate predominant use of oxidative metabolic pathways in sloth skeletal muscle tissue for sustaining force during prolonged muscle contraction. Furthermore, because sloths experience tensile loading on their limbs, the inverted nature of suspensory behaviors should favor greater modification of the hindlimb flexor musculature over that of the hindlimb extensors. Fiber typing analyses therefore may be expected to reveal greater numbers of slow MHC-1 fibers in flexors versus extensors. This study will also investigate this possibility by comparing fiber type distribution between sloth hindlimb flexor and extensor muscle groups.

Table 1. *Morphometric data of individuals used for muscle sampling*

Animal	Sex	Age	Limb (L/R)	Body Mass (kg)
†Bv1	M	Sub-adult	R	3.4
‡Bv2	M	Adult	R	4.0
Bv4	M	Adult	R	4.5
Bv7	F	Sub-adult	R	3.2
Bv8	M	Sub-adult	R	3.6
Ch4	M	Sub-adult	R	3.8
Ch5	unknown	Adult	R	+4.9
Ch6	unknown	Adult	R	+4.9

†Used for muscle sample supplementation of m. sartorius in Bv7

‡Used for muscle sample supplementation of mm.sartorious, semitendinosus, and flexor digitorum profundus in Bv4, and m. tibialis cranialis in Bv8

+Average body mass for Ch1, Ch2, Ch3, and Ch4 (Spainhower et al. in press) supplemented missing data

Table 2. *Raw data (muscle sub-sample) for each individual of brown-throated three-toed sloth (Bradypus variegatus)*

Muscle/ Individual	MHC fiber type (%)			Enzyme activity (µmol min^{-1} g^{-1} ww)				
	MHC-1	MHC-2A	MHC-1/2A	3-HAD	CS	PFK	LDH	CK
SRT								
BV1	100	0	0	1.3	3.0	1.1	101.6	178.1
BV2	100	0	0	2.1	11.0	1.4	51.9	47.4
BV8	100	0	0	2.8	4.1	0.9	69.2	232.5
GLM								
BV4	100	0	0	0.1	4.6	3.2	110.8	157.9
BV7	100	0	0	0.6	1.4	0.5	30.6	83.6
BV8	100	0	0	2.4	2.2	1.4	108.9	161.7
BF								
BV4	96.7	0	3.3	1.4	1.0	1.2	61.1	76.0
BV7	87.1	11.7	1.2	1.2	1.4	0.4	29.2	145.8
BV8	84.6	14.8	0.5	2.6	0.7	0.7	69.0	33.5
VL								
BV4	100	0	0	1.7	2.5	1.7	101.1	192.4
BV7	100	0	0	4.4	1.3	0.6	21.2	83.6
BV8	100	0	0	5.7	10.0	0.5	162.3	274.4
TCN								
BV2	65.1	34.1	0.8	0.7	1.5	0.7	42.0	54.5
BV4	100	0	0	1.5	2.2	1.8	65.0	192.4
BV7	79.6	18.5	1.9	1.1	0.52	0.8	43.2	140.3
FDP								
BV2	56.9	35.6	7.5	1.23	0.74	4.19	91.59	239.23
BV7	63.3	33.4	3.3	0.62	1.8	0.47	32.26	126.96
BV8	65.1	31.1	3.8	0.64	0.044	1.31	14.71	212.13

SRT, m. sartorius; GLM, m. gluteus medialis; BF, m. biceps femoris; VL, m. vastus lateralis; TCN, m. tibialis cranialis; FDP, m. flexor digitorum profundus

Table 3. *Raw data (muscle sub-sample) for each individual of Hoffman's two-toed sloth (Choleopus hoffmanni)*

Muscle/ Individual	MHC fiber type (%)			Enzyme activity (µmol min^{-1} g^{-1} ww)				
	MHC-1	MHC-2A	MHC-1/2A	3-HAD	CS	PFK	LDH	CK
SRT								
CH4	81.0	17.1	1.9	2.83	5.78	1.58	23.95	45.28
CH5	88.5	11.5	0	5.7	11.27	2.38	46.14	17.92
CH6	92.6	7.4	0	4.41	11.01	1.86	133.87	20.85
GLM								
CH4	72.4	23.7	3.8	2.05	14.56	1.02	68.76	60.86
CH5	70.5	29.5	0	6.47	23.29	1.71	127.45	29.12
CH6	63.4	33.8	2.8	4.37	13.21	1.36	149.3	45.64
BF								
CH4	73.4	23.4	3.2	3.74	12.07	1.3	34.51	33.55
CH5	70.2	28.1	1.7	4.8	12.89	1.51	63.5	22.87
CH6	61.6	37.9	0.6	4.95	12.88	1.66	106.81	67.24
VL								
CH4	62.7	34.6	2.7	4.38	21.6	2.29	40.19	33.87
CH5	58.7	41.3	0	5.67	17.45	1.43	71.28	26.99
CH6	70.9	25.7	3.4	4.09	8.9	1.72	89.6	30.92
TCN								
CH4	47.3	50.9	1.8	3.57	7.83	2.52	28.19	118.79
CH5	57.5	42.5	0	3.93	13.48	2.53	69.61	43.89
CH6	64.7	34.0	1.3	4.06	10.58	1.46	77.81	18.7
FDP								
CH4	49.7	48.9	1.4	3.34	8.12	3.38	49.89	90.25
CH5	54.8	44.4	0.8	5.06	16.68	3.64	68.76	46.64
CH6	55.1	44.9	0	3.93	15.22	4.31	106.81	27.3

SRT, m. sartorius; GLM, m. gluteus medialis; BF, m. biceps femoris; VL, m. vastus lateralis; TCN, m. tibialis cranialis; FDP, m. flexor digitorum profundus

Table 4. *Mammalian taxa and reported enzyme activities used in scaling analysis*

Taxa	Body Mass (kg)	3-HAD	CS	LDH	CK	Source
Rat (unspecified)	0.02	--	17	448	3403	Stewart et al. 2005
Northern short-tailed shrew	0.02	--	97	138	1865	Stewart et al. 2005
Sprague-dawley rat	0.20	--	23	--	--	Kohn and Myburgh 2007
3-toed sloth						
-forelimb	4.00	1.8	10.3	99.0	202.7	Spainhower et al. in press
-hindlimb	3.74	1.3	2.8	75.4	151.4	Present study
2-toed sloth						
-forelimb	5.23	2.2	5.8	42.0	169.0	Spainhower et al. in press
-hindlimb	4.53	4.3	13.2	75.4	43.4	Present study
Caracal	12.5	1.7	6.2	346.0	274.0	Kohn et al. 2011a
Mountain reedbuck	28.0	6.0	27.1	192.1	329.2	Kohn 2014
Cheetah	36.7	4.3	6.3	1632.3	N/A	Williams et al. 1997
Blesbok	58.5	3.6	28.3	184.5	328.3	Kohn 2014
Human	70.0	5.2	23	55	146	Kohn et al. 2011a
Lion	120	2.1	6.7	227	281	Kohn et al. 2011a
Black wildebeest	145	5.6	26.4	287.1	374.4	Kohn et al. 2011b
Reindeer	200	49.9	73.2	621.2	N/A	Essen-Gustavsson and Rehbinder 1985
Greater kudu	300	5.1	14.4	261.5	465.8	Kohn 2014

Table 5. *Mean distributions of MHC fiber type (%) in sloth hindlimb muscles*

Muscle	Functional group	*B. variegatus* (*N* = 5)			*C. hoffmanni* (*N* = 3)		
		MHC-1	MHC-2A	MHC-1/2A	MHC-1	MHC-2A	MHC-1/2A
SRT[a,b]	HF	100	0	0	85.5 ± 6.1	13.7 ± 5.1	0.8 ± 1.1
GLM[a,b]	HE	100	0	0	68.5 ± 4.8	29.3 ± 4.8	2.2 ± 1.7
ADDM	HE	94.2 ± 4.8	5.3 ± 4.3	0.5 ± 0.8	69.1 ± 5.7	29.1 ± 7.3	1.8 ± 1.8
SM	HE, KF	97.7 ± 3.8	2.1 ± 3.4	0.2 ± 0.4	68.2 ± 10.2	29.9 ± 11.1	2.0 ± 2.7
ST	HE, KF	97.2 ± 1.9	0	2.8 ± 1.9	71.7 ± 12.0	27.8 ± 11.5	0.6 ± 0.6
BF[a,b]	KF†	89.9 ± 6.2	8.3 ± 7.7	1.8 ± 1.5	66.9 ± 5.6	31.6 ± 6.7	1.5 ± 1.1
VL[a,b]	KE	100	0	0	62.3 ± 4.9	36.0 ± 6.3	1.7 ± 1.6
TCN[a,b]	AF	80.1 ± 16.4	19.0 ± 15.9	0.9 ± 0.9	57.2 ± 6.9	41.8 ± 6.8	1.0 ± 0.8
EDLO	AF	90.9 ± 6.6	7.5 ± 6.2	1.5 ± 0.5	40.9 ± 10.1	51.3 ± 10.1	7.8 ± 9.1
GAS[a]	AE	83.6 ± 9.8	15.4 ± 10.5	1.0 ± 0.8	59.9 ± 7.0	38.6 ± 6.5	1.6 ± 1.4
SOL[a]	AE	77.3 ± 17.5	21.4 ± 17.9	1.3 ± 0.5	51.1 ± 14.3	48.9 ± 14.3	0
FDP[a,b]	DF	59.4 ± 5.4	35.5 ± 3.8	5.1 ± 2.2	52.7 ± 3.1	46.7 ± 3.1	0.6 ± 0.6

Values are means ± SD; *N* = number of individuals used for MHC fiber type (%) quantifications
Muscle abbreviations: SRT, m. sartorius; GLM, m. gluteus medialis; ADDM, m. adductor magnus; SM, m. semimembranosis; ST, m. semitendinousis; BF, m. biceps femoris; VL, m. vastus lateralis; TCN, m. tibialis cranialis; EDLO, m. extensor digitorum longus; GAS, m. gastrocnemius; SOL, m. soleus; FDP, m. flexor digitorum profundus
Functional groups: HF, hip flexors; HE, hip extensors; KF, knee flexors; KE, knee extensors; AF, ankle flexors; AE, ankle extensors; DF, digital flexors
[a]Muscle subset used for SDS-PAGE quantification of MHC isoform content
[b]Muscle sub-sample used for enzyme analysis and statistical analyses
†combined mass of vertebral and femoral heads primarily flexes the knee joint

Table 6. *Mean distributions of MHC fiber type (%) among muscle functional groups*

Functional group	*B. variegatus* (*N* = 5)			*C. hoffmanni* (*N* = 3)		
	MHC-1	MHC-2A	MHC-1/2A	MHC-1	MHC-2A	MHC-1/2A
HF	100*	0*	0	85.5 ± 6.1*	13.7 ± 5.2*	0.8 ± 1.1
HE	97.2 ± 3.8*	2.1 ± 3.4*	0.7 ± 1.3	69.2 ± 8.0*	29.1 ± 8.4*	1.9 ± 2.0
KF†	95.0 ± 5.5*	3.6 ± 5.8*	1.4 ± 1.6	72.8 ± 11.2*	25.9 ± 11.1*	1.5 ± 1.9
KE	100*	0*	0	63.7 ± 5.6	34.3 ± 7.1	2.0 ± 1.6
AF	85.5 ± 13.2*	13.3 ± 13.0*	1.2 ± 0.8	49.7 ± 11.7	46.2 ± 9.4	4.1 ± 6.8
AE	80.4 ± 13.9	18.4 ± 14.3	1.1 ± 0.7	56.1 ± 9.1	42.7 ± 9.3	1.1 ± 1.4
DF	59.4 ± 5.4	35.5 ± 3.8	5.1 ± 2.2	52.7 ± 3.1	46.7 ± 3.1	0.6 ± 0.6

Values are means ± SD; *N* = number of individuals used for MHC fiber type (%) quantifications
Functional groups: HF, hip flexors; HE, hip extensors; KF, knee flexors; KE, knee extensors; AF, ankle flexors; AE, ankle extensors; DF, digital flexors
*significantly different ($P \leq 0.05$) from values for DF within species
†includes m. biceps femoris: combined mass of vertebral and femoral heads primarily flexes the knee joint

Table 7. *Means of %CSA for MHC fiber types in sloth hindlimb muscles*

Muscle	Functional group	*B. variegatus* (*N* = 5)			*C. hoffmanni* (*N* = 3)		
		MHC-1	MHC-2A	MHC-1/2A	MHC-1	MHC-2A	MHC-1/2A
SRT[a,b]	HF	97.0 ± 5.2	0	3.0 ± 5.2	85.6 ± 5.1	12.2 ± 1.5	2.2 ± 3.9
GLM[a,b]	HE	100	0	0	66.6 ± 8.6	21.5 ± 6.8	11.9 ± 10.9
ADDM	HE	92.1 ± 7.0	7.3 ± 6.8	0.6 ± 1.1	67.8 ± 10.3	21.7 ± 9.5	10.4 ± 9.2
SM	HE, KF	94.3 ± 9.8	5.0 ± 8.7	0.7 ± 1.2	77.7 ± 8.2	21.0 ± 9.3	1.2 ± 2.2
ST	HE, KF	98.1 ± 1.9	0	1.9 ± 1.9	75.2 ± 11.9	21.2 ± 6.7	3.7 ± 5.2
BF[a,b]	KF†	87.3 ± 7.7	7.5 ± 9.0	5.2 ± 4.0	76.4 ± 0.7	17.8 ± 3.9	5.8 ± 3.7
VL[a,b]	KE	100	0	0	58.5 ± 4.5	31.5 ± 5.0	10.0 ± 9.0
TCN[a,b]	AF	81.2 ± 19.3	15.6 ± 16.9	3.2 ± 2.8	63.1 ± 9.3	30.0 ± 6.7	6.9 ± 6.2
EDLO	AF	86.4 ± 7.1	7.7 ± 7.2	5.9 ± 3.3	46.9 ± 12.3	43.1 ± 14.2	10.0 ± 7.7
GAS[a]	AE	81.2 ± 5.1	16.2 ± 4.9	2.6 ± 2.3	66.1 ± 3.8	24.0 ± 4.2	9.9 ± 1.8
SOL[a]	AE	78.8 ± 10.6	12.2 ± 12.6	9.0 ± 4.8	61.3 ± 17.4	38.3 ± 17.4	0
FDP[a,b]	DF	68.0 ± 10.2	28.0 ± 8.1	2.4 ± 4.1	56.2 ± 2.6	40.5 ± 2.1	2.5 ± 4.3

Values are means ± SD; *N* = number of individuals used for %CSA quantifications
Muscle abbreviations: SRT, m. sartorius; GLM, m. gluteus medialis; ADDM, m. adductor magnus; SM, m. semimembranosus; ST, m. semitendinosus; BF, m. biceps femoris; VL, m. vastus lateralis; TCN, m. tibialis cranialis; EDLO, m. extensor digitorum longus; GAS, m. gastrocnemius; SOL, m. soleus; FDP, m. flexor digitorum profundus
Functional groups: HF, hip flexors; HE, hip extensors; KF, knee flexors; KE, knee extensors; AF, ankle flexors; AE, ankle extensors; DF, digital flexors
[a]Muscle subset used for SDS-PAGE quantification of MHC isoform content
[b]Muscle sub-sample used for enzyme assays and statistical analyses
†combined mass of vertebral and femoral heads primarily flexes the knee joint

Table 8. *Means of enzyme activities (in µmol min^{-1} g^{-1} (ww)) for selected sloth hindlimb muscles*

	B. variegatus (*N* = 5)					*C. hoffmanni* (*N* = 3)				
Muscle	3-HAD	CS	PFK	CK	LDH	3-HAD	CS	PFK	CK	LDH
SRT	2.1 ± 0.8	6.0 ± 4.4	1.1 ± 0.2	152.6 ± 95.1	74.3 ± 25.2	4.3 ± 1.4	9.4 ± 3.1	1.9 ± 0.4	28.0 ± 15.0	67.9 ± 58.1
GLM	1.0 ± 1.2	2.8 ± 1.6	1.7 ± 1.4	134.4 ± 44.0	83.4 ± 45.8	4.3 ± 2.2	17.0 ± 5.5	1.4 ± 0.3	45.2 ± 15.9	115.2 ± 41.7
BF	1.7 ± 0.8	4.6 ± 4.7	0.8 ± 0.4	116.3 ± 36.1	53.1 ± 21.1	4.5 ± 0.7	16.0 ± 6.5	1.5 ± 0.2	41.2 ± 23.1	68.3 ± 36.4
VL	0.9 ± 0.8	1.0 ± 0.4	0.9 ± 0.6	183.5 ± 95.7	94.9 ± 70.7	4.7 ± 0.8	12.6 ± 0.5	1.8 ± 0.4	30.6 ± 3.5	67.0 ± 25.0
TCN	1.1 ± 0.4	1.4 ± 0.8	1.1 ± 0.4	129.1 ± 69.7	50.1 ± 14.0	3.9 ± 0.3	10.6 ± 2.8	2.2 ± 0.6	60.5 ± 52.1	58.5 ± 26.6
FDP	0.8 ± 0.3	0.9 ± 0.9	2.0 ± 2.0	192.8 ± 58.6	46.2 ± 40.3	4.1 ± 0.9	13.3 ± 4.6	3.8 ± 0.5	54.7 ± 32.2	75.2 ± 29.0
Pooled means	**1.3 ± 0.8**	**2.8 ± 3.1**	**1.3 ± 1.0**	**151.5 ± 65.9**	**67.0 ± 39.1**	**4.3 ± 1.1**	**12.1 ± 4.6**	**2.1 ± 0.9**	**43.4 ± 26.6**	**75.4 ± 37.0**

Values are means ± SD; **In bold are pooled means ± SD**; *N* = number of individuals
3-HAD, 3-hydroxyacetyl Co A dehydrogenase; CS, citrate synthase; PFK, phosphofructokinase; CK, creatine kinase; LDH, lactate dehydrogenase.

Table 9. *Pearson-moment correlation statistics for enzyme activity and MHC fiber type (%) in selected sloth hindlimb muscles*

	MHC-1		MHC-2A		MHC-1/2A	
Enzyme	*r*	*P-value*	*r*	*P-value*	*r*	*P-value*
3-HAD	-0.336	0.044*	0.378	0.022*	-0.249	0.142
CS	-0.313	0.061	0.351	0.034*	-0.232	0.172
PFK	-0.493	0.002*	0.508	0.001*	0.054	0.753
CK	0.313	0.061	-0.337	0.043*	0.108	0.527
LDH	0.267	0.114	-0.239	0.158	-0.351	0.034*

MHC expression data from muscles SRT, GLM, BF, VL, TCN, and FDP
Degrees of freedom (df = 34) for all tests; *Bradypus* and *Choloepus* data combined
*significant relationship ($P \leq 0.05$) between enzyme activity and MHC fiber type (%)

Table 10. *Regression statistics for log transformed enzyme activity and body mass*

Enzyme	*N*	Slope	R^2	*P-value*
3-HAD*	13	0.31	0.34	0.04
CS	16	0.02	0.003	0.85
CK	13	-0.17	0.08	0.34
LDH	15	0.17	0.1	0.27

N is the number of species for which data are available
P-values are from ANOVA for tests of slopes
*significantly different ($P \leq 0.05$) from slope = 0

Figure 1. MHC expression and fiber type distribution patterns for hindlimb muscle functional groups in three-toed (*B. variegatus*) and two-toed sloths (*C. hoffmanni*). *A*: Silver-stained SDS-PAGE gel identifying MHC isoforms in selected hindlimb muscles of sloths. Gel lanes are representative for all muscles sampled from each species (Lanes 1 & 2, Bv: *B. variegatus*; Lane 2, Ch: *C. hoffmanni*). The fastest migrating (lowest position in gel lanes) MHC isoform was identified as a variant of MHC-1 β by peptide sequencing. *B*: Representative IHC serial cross-sections (e.g., m. flexor digitorum profundus, FDP) reacted against mAbs BAD5 (anti MHC-1) (left) and SC71 (anti MHC-2A/X) (right). Hybrid MHC-1/2A fibers (open diamond) reacted strongly against BAD5 and moderately against SC71. *same fiber in the serial sections. Scale bar = 100 μm. *C*: Stacked columns for mean (%) distribution of MHC fiber types in forelimb muscle functional groups of *B. variegatus* (*Bv*) and *C. hoffmanni* (*Ch*). Standard deviations (SD) not shown. *Intraspecific differences between the digital flexor group and the indicated functional group within species ($P \leq 0.05$). Functional groups are: (Hip Flexors) m. sartorius; (Hip Extensors) mm. gluteus medius, adductor magnus, semimembranosus, semitendinosus; (Knee Flexors) mm. semimembranosus, semitendinosus, biceps femoris; (Knee Extensors) m. vastus lateralis; (Ankle Flexors) m. tibialis cranialis; (Ankle Extensors) mm. gastrocnemius, soleus; and (Digital Flexors) m. flexor digitorum profundus.

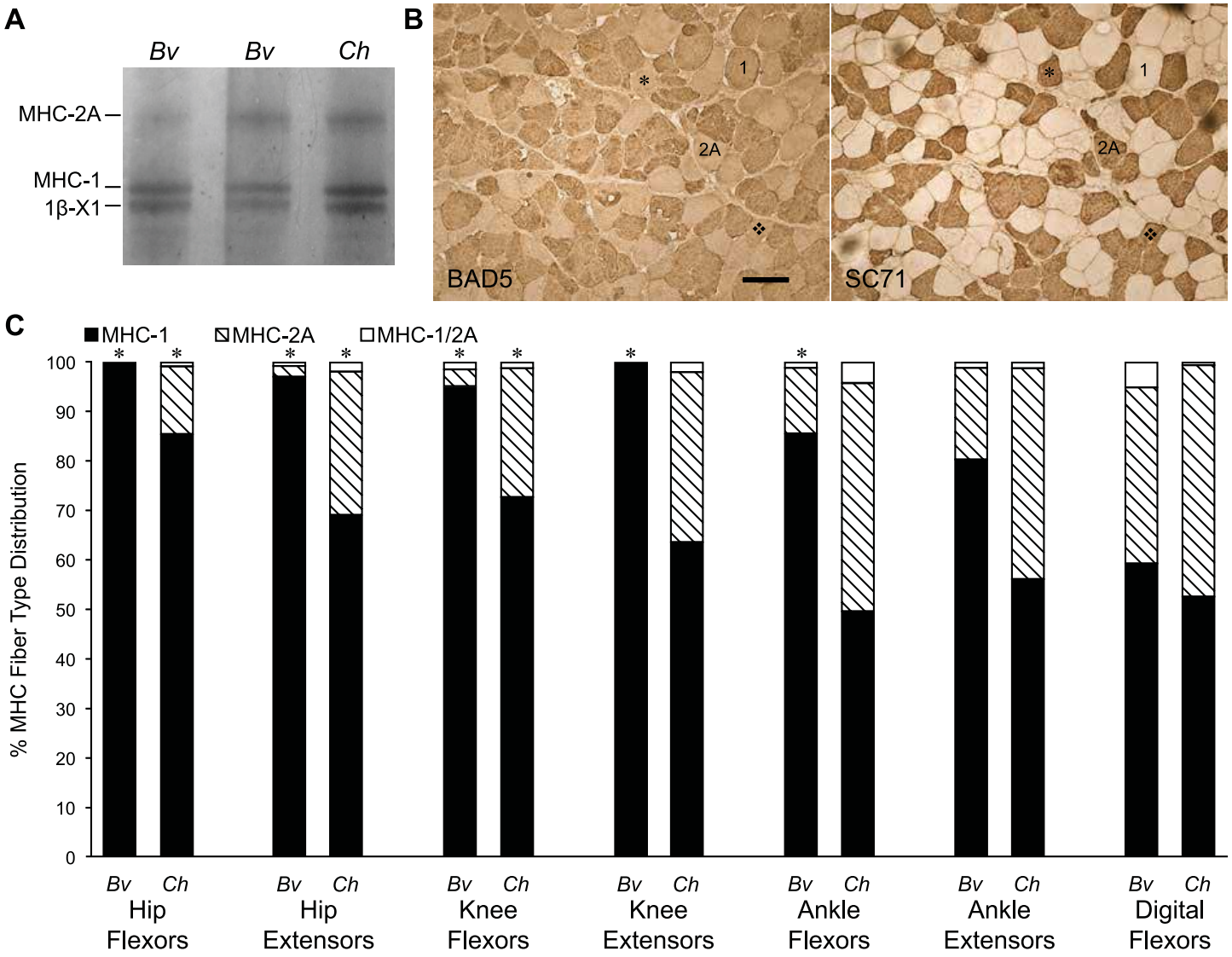

A
Bv
Bv
Ch
MHC-2A
MHC-1
1β-X1
B
1
2A
BAD5
SC71
C
MHC-1
MHC-2A
MHC-1/2A
% MHC Fiber Type Distribution
100
90
80
70
60
50
40
30
20
10
0
Bv Ch
Hip Flexors
Hip Extensors
Knee Flexors
Knee Extensors
Ankle Flexors
Ankle Extensors
Digital Flexors

Figure 2. Inter- and intraspecific variation in MHC fiber type and size for hindlimb muscle functional groups in *B. variegatus* and *C. hoffmanni*. *A*: MHC-1 (%) distribution in muscle functional groups for *B. variegatus* (open circles) and *C. hoffmanni* (hatched circles). Slow fiber expression was greater in all functional groups for three-toed sloths, and generally decreased from proximal-to-distal groups. *B*: MHC-2A (%) distribution in sloth muscle functional groups. Fast MHC-2A fiber type expression was greater in all functional groups for two-toed sloths and generally increased from proximal-to-distal groups. *C*: %CSA for MHC-1 fibers in sloth muscle functional groups. MHC-1 %CSA was greater in three-toed sloths and generally decreased from proximal-to-distal groups. *D*: %CSA for MHC-2A fibers in sloth muscle functional groups. MHC-2A %CSA was greater in two-toed sloths and generally increased from proximal-to-distal groups. *Interspecific differences within a functional group ($P \leq 0.05$). Functional groups are the same as those in Fig. 1*C*. Error bars are SD.

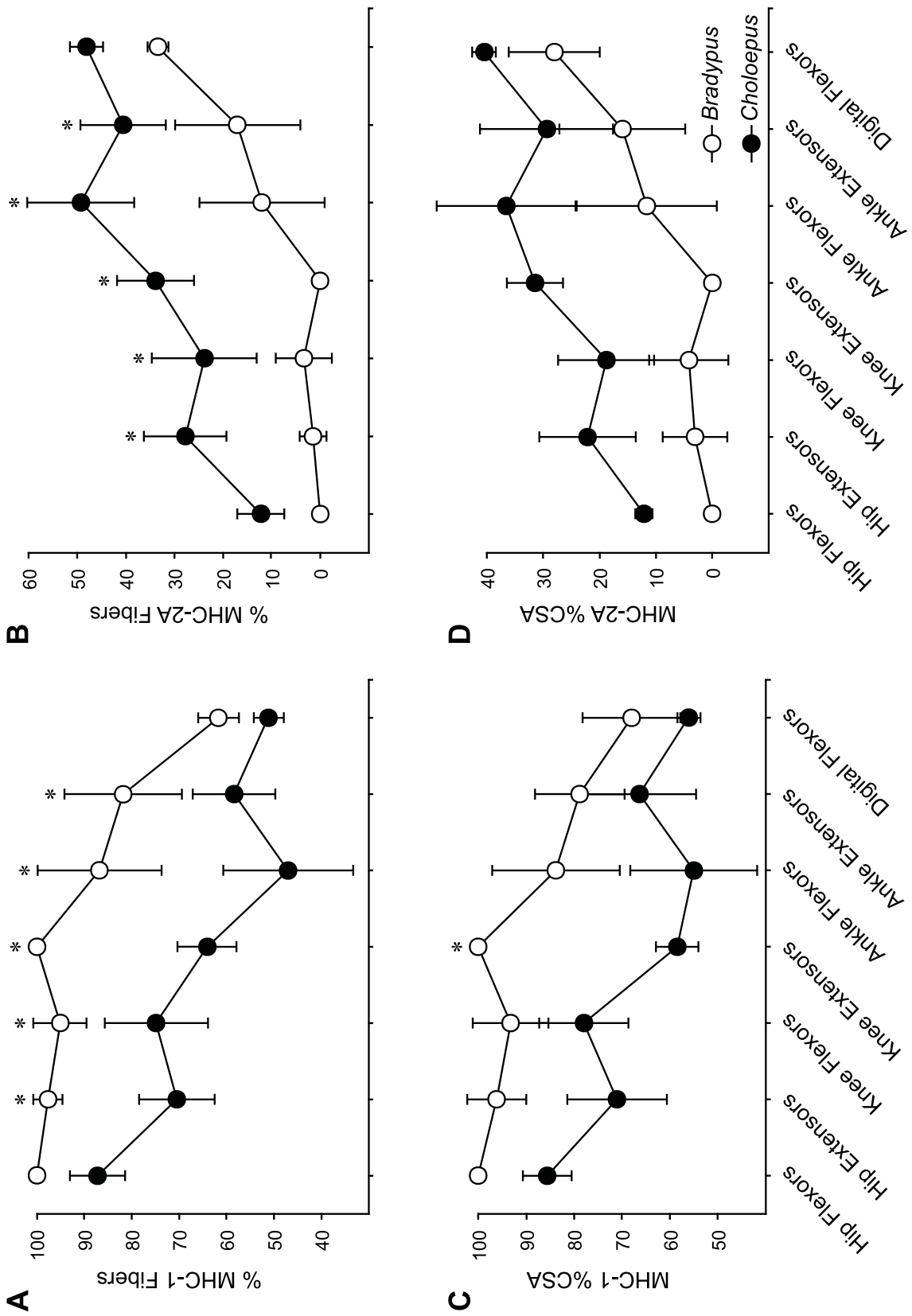

A
% MHC-1 Fibers
100
90
80
70
60
50
40
*
B
% MHC-2A Fibers
60
50
40
30
20
10
0
C
MHC-1 %CSA
100
90
80
70
60
50
D
MHC-2A %CSA
40
30
20
10
0
Bradypus
Choloepus
Hip Flexors
Hip Extensors
Knee Flexors
Knee Extensors
Ankle Flexors
Ankle Extensors
Digital Flexors

Figure 3. Mean MHC fiber type CSA for hindlimb muscle functional groups in *B. variegatus* and *C. hoffmanni*. CSA was measured from a total of n = 808 fibers for *B. variegatus* (*Bv*) and n = 1,006 fibers for *C. hoffmanni* (*Ch*). Functional groups are the same as those in Fig. 1*C*. Error bars are SD.

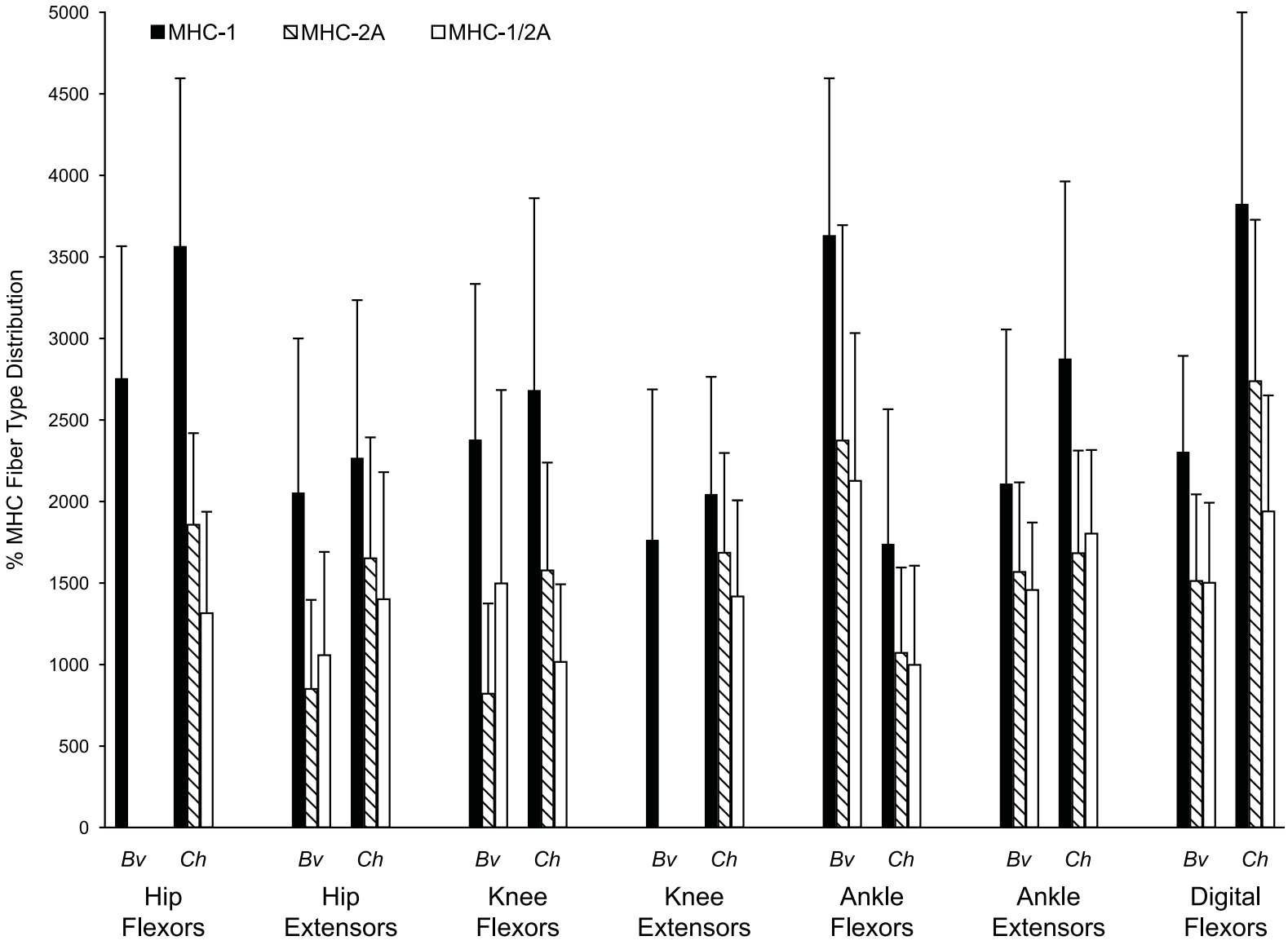

MHC-1
MHC-2A
MHC-1/2A
% MHC Fiber Type Distribution
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Bv
Ch
Hip Flexors
Hip Extensors
Knee Flexors
Knee Extensors
Ankle Flexors
Ankle Extensors
Digital Flexors

Figure 4. Interspecific variation and MANCOVA results for mean enzyme activities *between B. variegatus* and *C. hoffmanni*. Bars are means and error bars are SD. *A*: Mean activities for oxidative enzymes 3-HAD and CK were lower in *B. variegatus* (open bars) than *C. hoffmanni* (hatched bars), whereas mean PFK activity (glycolytic) was similar between species. (*B*) Mean CK activity was greater in *B. variegatus* than *C. hoffmanni* whereas LDH activity (non-oxidative) was similar between species. *significantly different ($P < 0.001$) from *B. variegatus* in each panel.

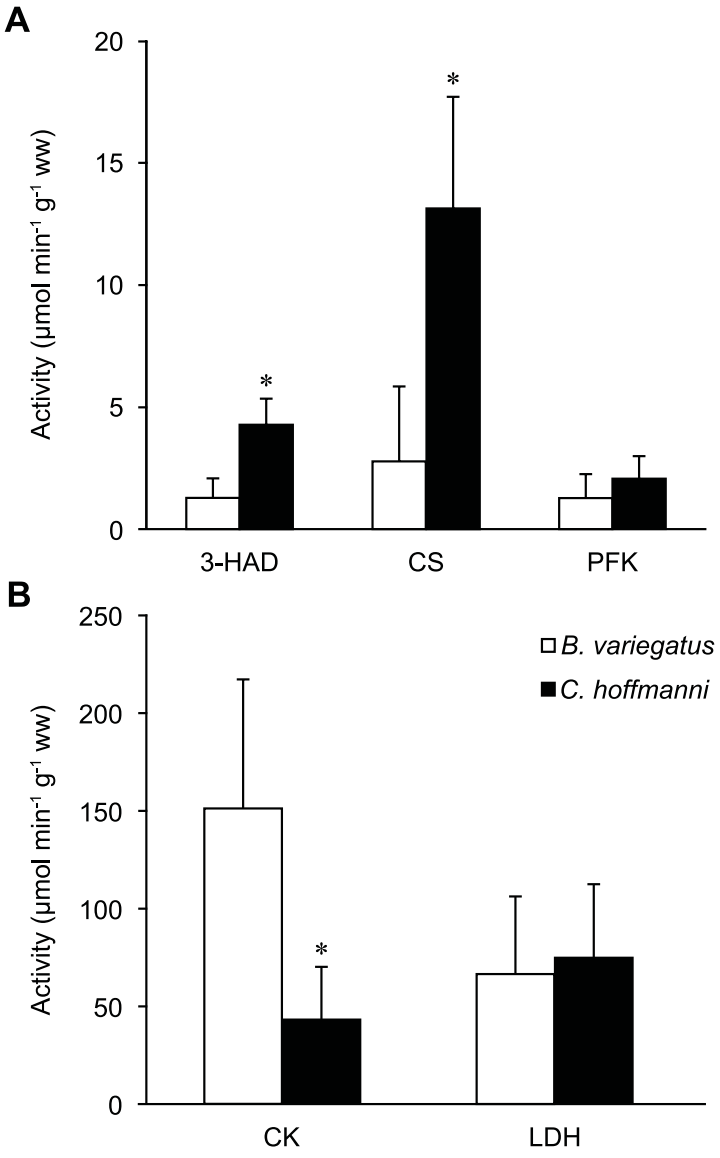

A
20
15
10
5
0
Activity (μmol min-1 g-1 ww)
*
*
3-HAD
CS
PFK
B
250
200
150
100
50
0
Activity (μmol min-1 g-1 ww)
B. variegatus
C. hoffmanni
*
CK
LDH

Figure 5. Allometric relationships for muscle enzyme activities and body mass across a range of placental mammals. *A*: 3-HAD activity as function of body mass. *B*: CS activity as function of body mass. *C*: CK activity as function of body mass. *D*: LDH activity as function of body mass. Data points for each relationship are log transformed mean enzyme activity and log transformed body mass for each species, and fitted with linear least-squares (LLS) regressions. Equations for each relationship are shown on the plots. Represented taxa are identified by their symbol the in legend and it is the same in each panel.

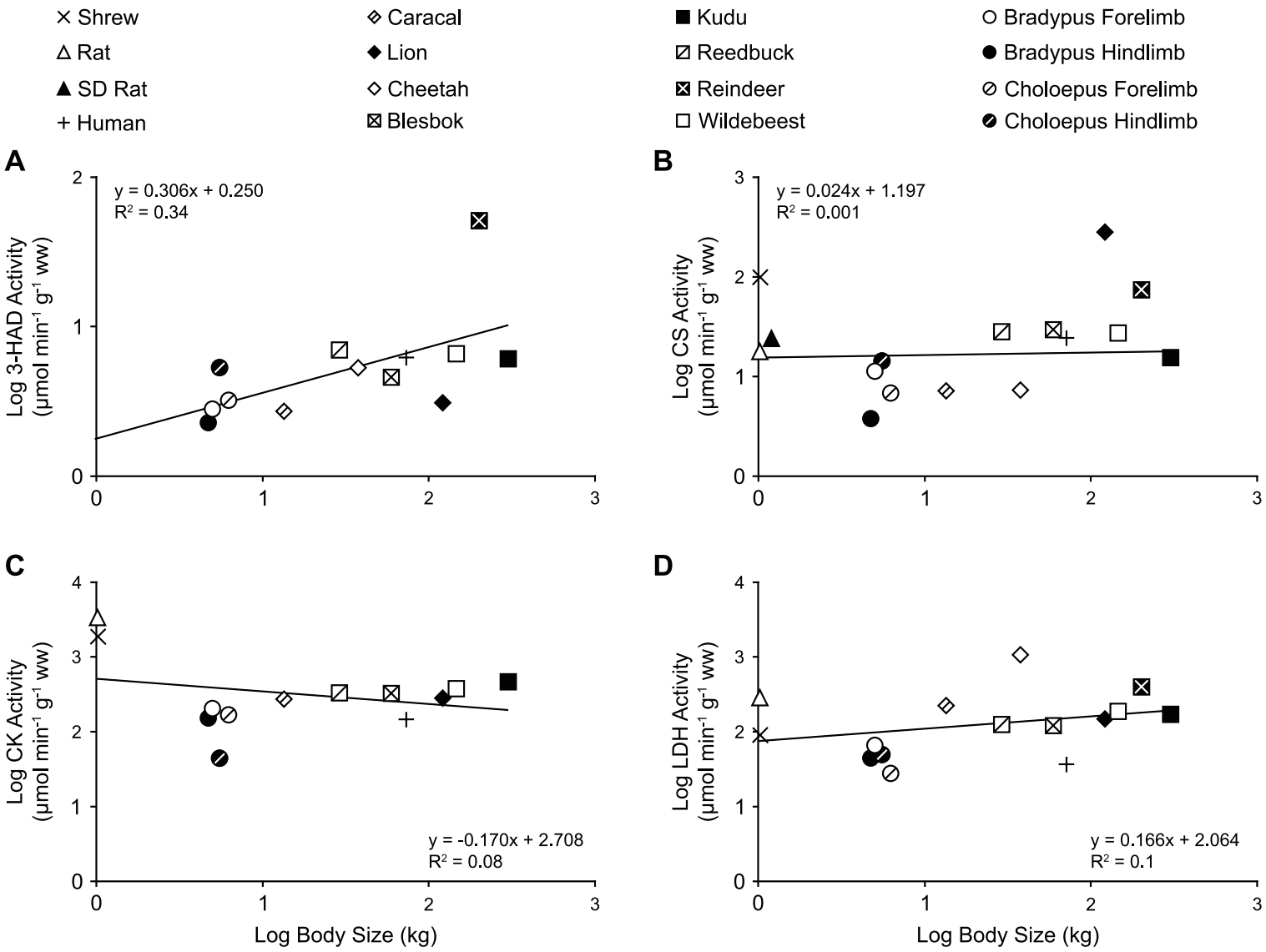
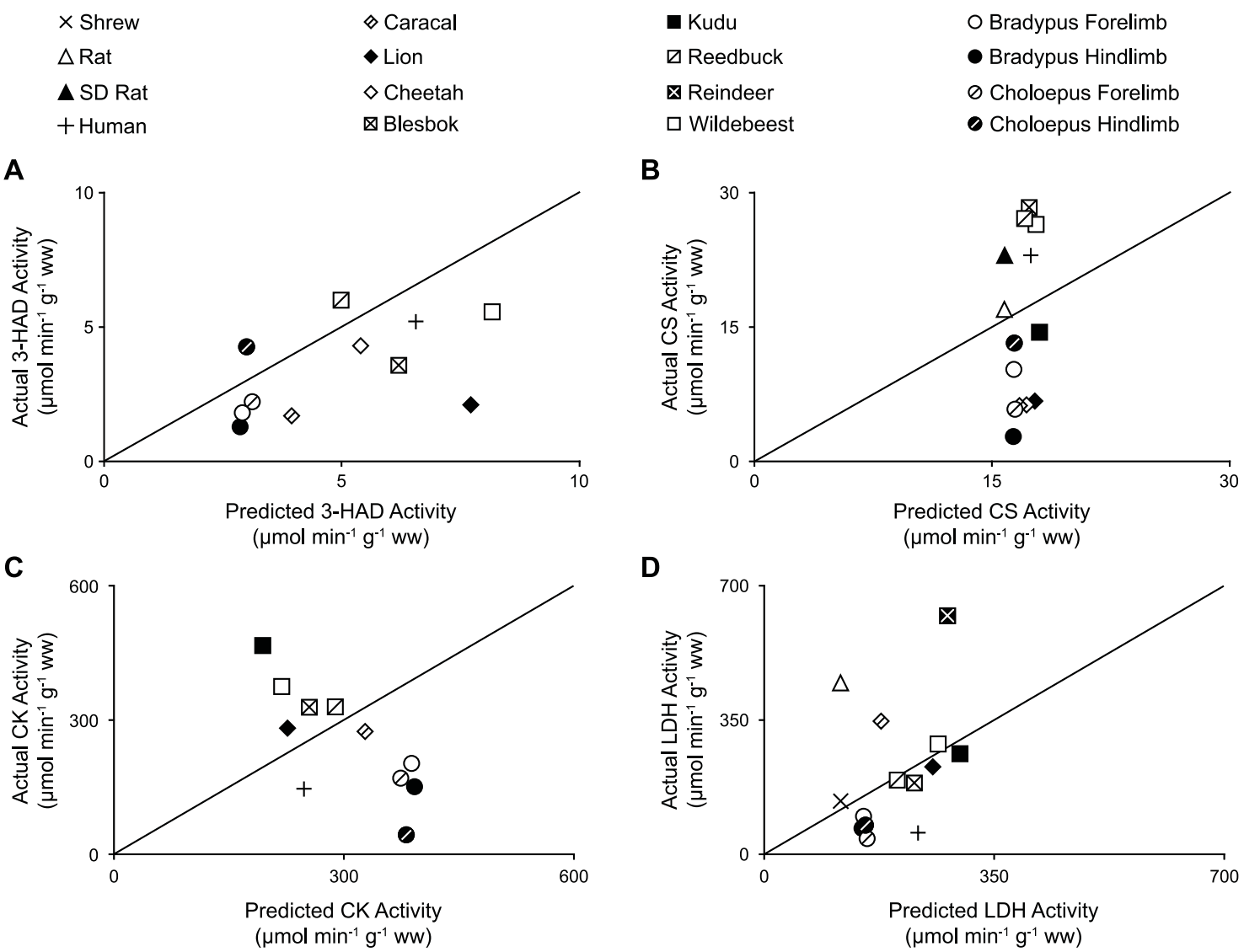

× Shrew
△ Rat
▲ SD Rat
+ Human
◈ Caracal
◆ Lion
◇ Cheetah
⊠ Blesbok
■ Kudu
▨ Reedbuck
⊠ Reindeer
□ Wildebeest
○ Bradypus Forelimb
● Bradypus Hindlimb
⊘ Choloepus Forelimb
◍ Choloepus Hindlimb
A
y = 0.306x + 0.250
R² = 0.34
Log 3-HAD Activity (µmol min⁻¹ g⁻¹ ww)
B
y = 0.024x + 1.197
R² = 0.001
Log CS Activity (µmol min⁻¹ g⁻¹ ww)
C
y = -0.170x + 2.708
R² = 0.08
Log CK Activity (µmol min⁻¹ g⁻¹ ww)
Log Body Size (kg)
D
y = 0.166x + 2.064
R² = 0.1
Log LDH Activity (µmol min⁻¹ g⁻¹ ww)
Log Body Size (kg)

Figure 6. Observed versus predicted muscle enzyme activities across a range of placental mammals. Measured *A*: 3-HAD activity; *B*: CS activity; *C*: CK activity; *D*: LDH activity. Fitted lines of unity in each plot are linear least-squares regressions forced through the origin with a slope = 1.0. Represented taxa are identified by the same symbols as those in Figure 5. Apart from 3-HAD activity in the *C. hoffmanni* hindlimb (vertically hatched circle) being greater than predicted, sloth data are below the body size prediction for each relationship shown.


× Shrew
△ Rat
▲ SD Rat
+ Human
◇ Caracal
◆ Lion
◇ Cheetah
⊠ Blesbok
■ Kudu
▨ Reedbuck
⊠ Reindeer
□ Wildebeest
○ Bradypus Forelimb
● Bradypus Hindlimb
⊘ Choloepus Forelimb
◒ Choloepus Hindlimb
A
Actual 3-HAD Activity (µmol min-1 g-1 ww)
Predicted 3-HAD Activity (µmol min-1 g-1 ww)
0
5
10
B
Actual CS Activity (µmol min-1 g-1 ww)
Predicted CS Activity (µmol min-1 g-1 ww)
0
15
30
C
Actual CK Activity (µmol min-1 g-1 ww)
Predicted CK Activity (µmol min-1 g-1 ww)
0
300
600
D
Actual LDH Activity (µmol min-1 g-1 ww)
Predicted LDH Activity (µmol min-1 g-1 ww)
0
350
700