Contractile properties of muscle fibers from the deep and superficial digital flexors of horses

M. T. Butcher,1,2 P. B. Chase,2 J. W. Hermanson,3 A. N. Clark,2 N. M. Brunet,2,4 and J. E. A. Bertram5

1Department of Biological Sciences, Youngstown State University, Youngstown, Ohio; 2Department of Biological Science, Florida State University, Tallahassee, Florida; 3Department of Biomedical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, New York; 4Program in Molecular Biophysics, Florida State University, Tallahassee, Florida; and 5Department of Cell Biology and Anatomy, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada

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Contractile properties of muscle fibers from the deep and superficial digital flexors of horses. Am J Physiol Regul Integr Comp Physiol 299: R996–R1005, 2010. First published August 11, 2010; doi:10.1152/ajpregu.00510.2009. —Equine digital flexor muscles have independent tendons but a nearly identical mechanical relationship to the main joint they act upon. Yet these muscles have remarkable diversity in architecture, ranging from long, unipennate fibers (“short” compartment of DDF) to very short, multipennate fibers (SDF). To investigate the functional relevance of the form of the digital flexor muscles, fiber contractile properties were analyzed in the context of architecture differences and in vivo function during locomotion. Myosin heavy chain (MHC) isoform fiber type was studied, and in vitro motility assays were used to measure actin filament sliding velocity (Vₛ). Skinned fiber contractile properties [isometric tension (P₀/CSA), velocity of unloaded shortening (VUS), and force-Ca²⁺ relationships] at both 10 and 30°C were characterized. Contractile properties were correlated with MHC isoform and their respective Vₛ. The DDF contained a higher percentage of MHC-2A fibers with myosin (heavy meromyosin) and Vₛ that was twofold faster than SDF. At 30°C, P₀/CSA was higher for DDF (103.5 ± 8.75 mN/mm²) than SDF fibers (81.8 ± 7.71 mN/mm²). Similarly, VUS (pCa 5, 30°C) was faster for DDF (2.43 ± 0.53 FL/s) than SDF fibers (1.20 ± 0.22 FL/s). Active isometric tension increased with increasing Ca²⁺ concentration, with maximal Ca²⁺ activation at pCa 5 at each temperature in fibers from each muscle. In general, the collective properties of DDF and SDF were consistent with fiber MHC isoform composition, muscle architecture, and the respective functional roles of the two muscles in locomotion.

Myosin; deep digital flexor; superficial digital flexor

THE DIGITAL FLEXORS OF THE EQUINE FORELIMB provide a unique opportunity to investigate the integration of molecular composition, architectural organization, and functional utilization of locomotor muscles in a large cursorial mammal. Although their tendons have essentially equivalent relationships to the main joint they act upon, the metacarpophalangeal joint (fetlock) of the distal limb, they show remarkable diversity in their muscle architecture. The “short” (humeral) compartment of the deep digital flexor (DDF) has long, unipennate fibers, whereas the superficial digital flexor (SDF) has short, multipennate fibers (8, 20, 54). Muscle architecture is an important component of musculoskeletal structure and function. For example, a short-fibered muscle with a long, compliant tendon suggests a capacity for substantial elastic energy storage, an effective means to reduce metabolic cost in locomotion (1). Recent interest in understanding how architecture of the equine digital flexors relates to their function in the distal limb during locomotion has led to in vivo studies examining 1) isometric force production for characterization of the passive and active force properties of DDF and SDF muscles (47) and 2) DDF and SDF contractile behavior and muscle-tendon unit function during walking and running (9, 10). General findings from these studies indicate that the functional roles of these two synergistic muscles differ, with the DDF (short) having a capacity to shorten to generate modest work and power during locomotion, whereas the SDF has extremely limited shortening capability, doing mostly negative work (i.e., eccentric contractions), with a capacity for high-force production (high passive and active force) and large elastic energy storage in its tendon. Amid these recent studies designed to better understand how such a diverse complex of muscles functions in vivo, basic physiological contractile properties of equine DDF and SDF muscle fibers remain largely unknown. Mechanical experiments on mammalian muscle fibers have most often been performed on skinned (permeabilized) fibers from rabbits (11–13, 35) and even smaller mammals, such as rats and mice (2, 3), to determine fundamental measurements of physiological capacity [e.g., maximum unloaded shortening velocity (V₀) or velocity of unloaded shortening (VUS)]. A thorough investigation of DDF and SDF contractile properties using single, skinned fibers would provide a valuable contribution toward understanding each muscle’s functional capacity for contraction velocity and thus work and power performance, as suggested by their respective architectures.

The velocity at which a muscle contracts (and performs mechanical work) is related to muscle fiber composition, which in turn is determined mainly by the myosin heavy chain (MHC) isoforms expressed in the fibers (35, 40). Histochemical analyses have shown that the DDF (short compartment) contains a higher percentage of fast fibers (type II), whereas the SDF has relatively more slow fibers (type I) (20). A high percentage of faster contracting fibers is common to work and power generating muscles, whereas a high percentage of slow contracting fibers is found in postural muscles (i.e., muscles that produce force economically and maintain tension for extended periods), which are generally not considered important muscles in driving locomotion. Despite a thorough histochemical analysis of muscle fiber type, the MHC composition of DDF and SDF muscle fibers cannot be determined directly from these methods; gel electrophoresis allows identification of MHC isoforms at the single fiber level (46, 52, 53). When related to MHC composition, skinned fiber mechanics studies provide impor-
tant links between muscle molecular composition, structure, and function and serve to clarify molecular bases of functional diversity among muscle fiber types (5).

In addition to fiber mechanics experiments, functional diversity present among the MHC isoforms can also be studied directly with in vitro motility assays using purified myosin [or the soluble heavy meromyosin (HMM) fragment] and fluorescence-labeled F-actin (14, 19, 39). Motility assays on preparations of HMM from horse muscles have not previously been reported. Therefore, analysis of the MHC composition and measurements of filament sliding velocity \(V_s\) of myosin from DDF and SDF muscles will be fundamental to characterization of the physiology and functional capacity of horse digital flexors.

The objective of this study was to characterize the physiological properties of DDF and SDF muscles and relate these properties to whole muscle architecture and function in vivo. Specific goals were to compare 1) relative distribution of MHC isoforms expressed in fibers from DDF and SDF muscles to the underlying histochemical composition of the muscles, 2) function of myosin (i.e., HMM) from DDF and SDF muscles in motility assays at physiological temperature, and 3) DDF and SDF fiber contractile properties that are relevant to locomotion at a near-physiological temperature. Based on the overall structure of these muscles and the existing histochemical analyses, it was hypothesized that the long-fibered DDF (short) has contractile properties relating to a faster MHC isoform composition, whereas the short-fibered, multipennate SDF has properties more consistent with the slow (MHC-1) fiber type. This study will improve our understanding of the functional roles of the equine digital flexor muscles in locomotion and how both cellular and molecular physiology contribute to this function.

**METHODS**

**Ethical approval.** All protocols for harvesting muscle tissue from horses were in accordance with the policies and standards of the Cornell University, College of Veterinary Medicine, Equine Hospital, and Institutional Animal Care and Use Committee (IACUC)-approved guidelines and protocols. Rabbit muscle tissue for motility experiments was obtained according to protocols approved by the Florida State University IACUC.

**Animals and muscle sampling.** Five adult horses (2 male thoroughbreds, 2 female thoroughbreds, and 1 female appaloosa) were used as muscle tissue donors for this study. The mean (± SD) age and body mass for the horses were 17.6 ± 2.3 yr (range: 13–24 yr) and 429.1 ± 42.9 kg (range: 454.5–545.4 kg), respectively. Horses used in this study were euthanized for reasons unrelated to this study but did not involve musculoskeletal lameness. Euthanasia was administered via barbiturate injection (Fetal-Plus; Vortech Pharmaceuticals, Dearborn, MI) at Cornell University, College of Veterinary Medicine, Equine Hospital. Following euthanasia, the DDF (humeral head, short compartment) and SDF muscles of the forelimb and the soleus (SOL) muscle \((n = 2\) horses) of the hindlimb were freshly removed, and two to three fiber fascicles were dissected from the midbelly region of each muscle and tied to Teflon strips at an in vitro length. Adjacent fascicles were sampled from superficial-to-deep regions of the muscles for fiber mechanics and fiber typing analyses.

**Skinned fiber preparation.** Freshly dissected muscle fibers from the DDF, SDF, and SOL muscles were prepared for mechanical experiment using published protocols (11, 12). Fiber fascicles were treated with a skimming solution containing 0.5% Brij-58 detergent (Pierce Ultrapure; Pierce Biotechnology, Rockford, IL) for 1 h on ice and then glycerinated with a 50% glycerol-skimming solution and stored at −20°C. Skinning solution contained (in mM) 25 EGTA, 50 MOPS, 6 Mg (acetate)\(_2\), 4 acetic acid, 5 ATP, and 0.03% (wt/vol) diithiothreitol (DTT) and 0.005% (wt/vol) leupeptin (pH 7.1, 0–2°C). Single fibers (~2 mm segments) were isolated by microdissection in a cold bath (4°C) of 50% glycerol-relaxing solution. End compliance of the fibers was minimized by chemical fixation of the fiber ends using localized micropipetting of 5% glutaraldehyde (+ 1 mg/ml fluorescein for visualization) (11). The fixed ends of the fibers (~0.5 mm) were wrapped in aluminum foil T-clips (KEM-MIL, Hayward, CA) before being transferred to the experimental apparatus for attachment and mechanical measurements. A drop of silicone was added on each clip to stabilize placement on the hooks of the motor and force transducer.

**Experimental solutions.** Relaxing and activating solutions were prepared as described previously (11). The basic composition of the solutions was (in mM) 5 MgATP, 1 P\(_i\), 10 EGTA, 15 P\(_C\) (CP), 100 K\(^+\) plus Na\(^+\), 3 Mg\(^2+\), 50 MOPS, 1 DTT, and 1 mg/ml creatine kinase (CK; 280 U/ml). Four stock solutions were made with the following [Ca\(^2+\)] concentrations ([Ca\(^2+\)]; pCa 9 (relaxing solution), pCa 7, pCa 6, and pCa 5 (maximum activation), where pCa = −log\(_{10}\) [Ca\(^2+\)]. Intermediate [Ca\(^2+\)] (pCa 6.4, pCa 6.2, pCa 5.9, pCa 5.8, pCa 5.6, pCa 5.4, and pCa 5.2) solutions were made from combinations of the stock solutions. The pH was adjusted to 7.0 for all stock solutions at 12°C. Ionic strength was 0.18 M for all stock solutions and was adjusted with Tris and acetate. Experimental solutions were used at 10 and 30°C with no further adjustments to pH and ionic strength for minor changes with temperature, as has been described (4, 46); solutions at pH 7.0 and physiological ionic strength of 0.18 M set at cooler temperatures have been shown to be adequate at higher temperatures (51). DTT and CK were added to relaxing and activating solutions on the day of each experiment.

**Experimental apparatus for single, permeabilized fiber mechanics.** The experimental apparatus for single, permeabilized fiber mechanics has been described in detail (13, 25). Relaxing and activating solutions were held in anodized aluminum wells (200 μl) with bottoms of glass coverslips (no. 1 thickness). The fiber could be immersed in any of 12 wells that were held in a rotating well. The temperature of the wells was measured with a k-type thermocouple and was maintained at either 10 or 30°C depending on the experiment. Temperature for individual experiments was controlled within 1°C (model ATR-4 Adaptable Thermoregulator; Quest Scientific, North Vancouver, BC, Canada). Single fibers were attached via small hooks to a force transducer (2.2 kHz resonant frequency, model 400A; Aurora Scientific) at the other end. The force transducer and motor were mounted on the modified stage of an inverted microscope. Force and length signals were digitized using custom data acquisition software (NEWDAC; see Ref. 13) on a PC-based system with a DT2831-G data acquisition board (Data Translation, Marlboro, MA). Stability of fiber structure and mechanical properties during activations were maintained as described by Brenner (6). Briefly, fibers were shortened transiently every 5 s at a rate that was at least the maximum shortening velocity, which reduced force to zero. The periodic unloading of the fiber was followed by a quick restretch to isometric length (11). Fiber striation pattern was monitored continuously by a charge-coupled device camera during experiments (model XR-77; Sony Electronics, Tokyo, Japan).

**Experimental protocol for single, permeabilized fiber mechanics.** All mechanical measurements were made at both 10 and 30°C. Isometric fiber length \(L_0\) and fiber diameter were measured in relaxing conditions using an optical micrometer in the eyepiece of the inverted microscope. \(L_0\) was determined at \(×400\) magnification as fiber length between end clips. Lengths of the two fixed ends were measured (in pCa 5) at the end of each experiment and subtracted from \(L_0\) to obtain active fiber length \(L_a\). Fiber diameter was determined at

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×320 magnification as the average of three measurements at different locations along the fiber. Sarcomere length (La) was determined by HeNe laser diffraction (12). Initial Ls was set to 2.6 μm in relaxing conditions. Following a brief initial activation (10 s, pCa 9), fibers were returned to pCa 9, Ls was adjusted to minimize passive tension, and Ls was remeasured. Mean (± SE) Ls was 2.4 ± 0.03 μm (n = 32) for DDF fibers, 2.4 ± 0.03 μm (n = 32) for SDF fibers, and 2.4 ± 0.07 μm (n = 16) for SOL fibers.

To determine isometric force (P), fibers were activated for ~30–60 s in sequentially higher [Ca²⁺] in the following order: pCa 9, 7, 6.4, 6.2, 6.5, 5.8, 5.6, 5.4, 5.2, and 5 (maximum isometric force, P = Pm, was determined at pCa 5). Measurements of P were made during the steady-state period before a Brenner cycle (25). P was normalized to fiber cross-sectional area (CSA) to give P/CSA in (mN/mm²). VUS was calculated using both fiber analysis software to determine slack time and length change from force and length records. Slack test data were digitized at sampling frequencies (256–3,000 Hz) appropriate for optimal resolution.

Data analysis for single, permeabilized fiber mechanics. Force and length data were analyzed using custom software (fiber analysis; see Ref. 13). At each pCa, passive tension measured in relaxing conditions was subtracted from calculated P/CSA, and values were averaged at each experimental temperature. Additionally, P/CSA at each pCa was also normalized to Pm/CSA at pCa 5 (for each fiber) for force-Ca²⁺ relationship analysis. Normalized P/CSA values were plotted as a function of pCa for each fiber experiment and fit with a two-parameter sigmoid Hill equation using nonlinear least squares regression (SigmaPlot 6.0; SPSS, Chicago, IL). Slope (n) and pCa50 of the force-Ca²⁺ relationships were determined for DDF, SDF, and SOL fibers for each experimental temperature from the Hill plot beginning with the greatest length change. The resulting force changes were recorded for each length step. After completion of a length step, the fiber was held at the slack length until force began to redevelop. The time between completion of the length step and the point of force redevelopment is defined as slack time. Slack test data were digitized and analyzed using a combination of methods described previously (46, 48).

Myosin isoform identification and fiber typing. MHC isoform composition was analyzed for a separate set of single fibers for each muscle by SDS-PAGE using a combination of methods described previously (46, 48). Permeabilized fiber segments ~2 mm in length were denaturated for 2 h at room temperature in 20 μl of Laemmli solution (23) with the following composition: 62.5 mM Tris, 2.3% (wt/vol) SDS, 10% glycerol, and 5% (vol/vol) β-mercaptoethanol. Denatured fiber samples were stored overnight at −20°C. Typically, 5–10 μl of the denatured fiber sample was loaded onto the gels (mini slab gels). The acrylamide: N,N'-methylene-bisacrylamide ratio of the gels was 37.5:1, with total polyacrylamide percentage equaling 4 and 6% in the stacking and separating gels, respectively. Gel apparatus chambers (Mini-Gel System; Bio-Rad Laboratories, Hercules, CA) were filled with electrode buffer containing 25 mM Tris, 192 mM glycine, and 0.1% SDS (pH 8.3) (46). Electrophoresis was run for 22–24 h at 100 V at 4°C (48). Gels were silver stained (Sigma ProteoSilver Stain Kit; Sigma Chemical, St. Louis, MO) for identification of protein bands.

MHC bands from single fibers were evaluated by comparing band migration to both molecular weight standards in the 200-kDa region and MHC from bulk myosin samples purified from entire muscle fascicles. Briefly, myosin was extracted from the remaining skinned fascicles by homogenization ~30 mg of muscle on ice in 1 ml of a high-salt buffer containing (in mM) 250 sucrose, 300 KCl, 5 EDTA, and 10 Tris. Precipitated myosin was then centrifuged for 10 min, and the pellet was collected, solubilized in Laemmli solution for 1 h at room temperature, and stored overnight at −20°C. Typically, 3–5 μl of purified myosin samples were loaded in the end lanes of gels and used as references for identifying MHC isoforms present in DDF, SDF, and SOL fibers. Percentages of each MHC isoform were quantified from the total number of fibers sampled from each muscle and each horse. Percent distribution of MHC isoforms for the overall number of fibers sampled from each muscle (i.e., population) was also quantified.

Fiber type staining (histo/immunohistochemistry) was performed using established methods (20). Briefly, histochemical staining followed protocols for myosin ATPase originally derived from Brooke and Kaiser (7) and Padykula and Herman (29). Slow and fast properties of fibers were estimated by myosin ATPase staining of continuous serial tissue sections. For immunohistochemistry, serial sections were first reacted with one of several primary MHC isoform-specific antibodies for 16–18 h at 4°C, followed by reaction against secondary antibody (anti-mouse) and 3,3'-diaminobenzidine-tetrahydrochloride staining with a Zymed Histostain Plus kit (Zymed Laboratories, San Francisco, CA). Primary antibodies were SS5 (provided by Dr. Frank Stockdale, Stanford Medical School), which reacts with vertebrate slow myosin (MHC-1), and MY32 (Sigma Chemical), which reacts against vertebrate fast myosins. Additionally, the SC71 antibody (ATTCC, Raleigh, NC), which has yielded positive reaction against the fast MHC-2A isoform, was used.

Myosin preparation and in vitro motility assay. Myosin from DDF (hemural head, short compartment) and SDF muscles from a thoroughbred horse and myosin from the psoas muscle and F-actin from the back and leg muscles of New Zealand White rabbits were prepared according to established methods (24, 30). Chymotryptic digestion of myosin from each muscle was used to obtain the soluble HMM fragment, and samples were stored at 4°C. In addition, F-actin was fluorescently labeled with rhodamine-phalloidin (RhPh) (19, 22). On the day of the experiments, ATP-insensitive myosin heads were removed from HMM by ultracentrifugation in the presence of unleveled F-actin and MgATP (22), followed by adjustment of the HMM concentration (range: 0.20–0.35 mg/ml) for each muscle’s HMM sample. Motility experiments took place within 1–2 days after HMM purification using an assay protocol derived with slight modification from that described previously (14).

Motility assays were carried out in flow cells constructed of a glass coverslip coated with a thin layer of 0.1% nitrocellulose in amyl acetate mounted on an untreated microscope slide using spacers cut from no. 1.5 glass coverslips and silicone high-vacuum grease applied by syringe. Motility solutions were applied at room temperature in a standardized order (14). Briefly, an HMM sample was adsorbed (2 applications 1 min apart) onto the coated inner flow cell surface. Next, 0.5 mg/ml bovine serum albumin (BSA) in actin buffer (AB; in mM:...
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RESULTS

MHC isoform composition. Myosin ATPase and antibody staining on large cross-sections of DDF (see Fig. 1) and SDF muscle fibers showed that distributions of slow (MHC-1) and fast (MHC-2A) fibers from the population sampled (n = 1,000 fibers/muscle) in this analysis were 29 and 71%, respectively, for DDF and 60 and 40%, respectively, for SDF. Electrophoretic (SDS-PAGE) results of single fibers showed good consistency with fiber type staining analyses. For each muscle sampled, protein bands from only two MHC isoforms, MHC-1 and MHC-2A, were clearly identified (see Fig. 2). Slow MHC-1 migrated faster than MHC-2A and generally migrated to the same position for DDF, SDF, and SOL muscle samples from horse. SDS-PAGE identification of MHC isoforms in single, permeabilized fibers from DDF, SDF, and SOL is illustrated in Fig. 2, and summary statistics from this separate analysis of individual fibers are presented in Table 1. The typically slow SOL muscle contained 100% MHC-1 fibers. Distributions for MHC-1 and MHC-2A isoforms from the population of fibers sampled were 12.7 and 83.5%, respectively, for DDF (n = 79 fibers) and 44.3 and 48.1% for SOL (n = 79 fibers). A small percentage of hybrid MHC-1–2A fibers were also identified in DDF (3.8%) and SDF (7.6%).

Vf. Figure 3 illustrates the large disparity among means of Vf from motility assays, with HMM prepared from bulk muscle samples of horse DDF or SDF myosin or rabbit psoas myosin. Means of Vf were significantly different (P < 0.001) among the three muscles for each experimental temperature (28 or 38°C). As expected for each muscle, Vf at 38°C was faster than at 28°C. Compared with rabbit psoas myosin (fast control), means of Vf for DDF and SDF were 3.7–8.3 times lower at 28°C and 2.3–5.4 times lower at 38°C. Mean Vf for SDF myosin was consistently 2.3 times lower than for DDF myosin regardless of experimental temperature.

Fiber contractile properties: maximum isometric tension (P0/CSA). Figure 4 illustrates the force response of a single fiber to sequentially increasing [Ca2+]over the time course of an entire experimental protocol at 30°C; parameters for maximum Ca2+ activation (P0/CSA and VUS) were obtained during the latter part of the protocol (pCa 5). Frequency distributions of P0/CSA for DDF, SDF, and SOL fibers at 30°C are shown in Fig. 5A. Means (± SE) of P0/CSA at both 10 and 30°C are presented in Table 2. Mean P0/CSA of DDF fibers was just significantly different (P = 0.03–0.05) compared with SOL or fast fibers at either temperature, although the large difference between mean P0/CSA for SOL fibers (46.7 ± 4.5 mN/mm2) and DDF fibers (93.3 ± 7.3 mN/mm2) at 10°C was highly significant (P < 0.0001). Means of P0/CSA were also found to be just significantly different between SOL and SDF fibers (P = 0.04) at 10°C, although the values at 30°C for SOL fibers were lower (P = 0.77), primarily because of the significant increase in slow SOL fiber P0/CSA with temperature.

VUS. Figure 5B shows frequency distributions of VUS for DDF, SDF, and SOL fibers at 30°C. Means (± SE) of VUS determined at maximal activation (pCa 5) are presented in Table 2. Mean VUS of DDF fibers was not significantly different compared with mean VUS for both SOL and SDF fibers at 30°C; this may be due to the apparently bimodal distribution of VUS values from DDF (Fig. 5B), as could be
expected from the mixture of fiber types found by histochemical and SDS-PAGE analyses (Figs. 1 and 2 and Table 1). However, differences in mean VUS at 10°C were statistically significant between SOL and DDF fibers (P < 0.005). As expected, mean VUS increased significantly with temperature for DDF, SDF, and SOL fibers. This increase, in large part, accounted for the similarity (P < 0.89) between VUS for SOL fibers (1.07 ± 0.21 FL/s) and VUS for SDF fibers (1.2 ± 0.22 FL/s) at 30°C, although the distribution of VUS values was broader for SDF than for SOL fibers (Fig. 5B).

Force-Ca^{2+} relationship parameters. Figure 6 shows averaged force-Ca^{2+} relationships for DDF, SDF, and SOL fibers at both experimental temperatures. The slopes of these relationships for DDF and SDF fibers appear similar at 10 and 30°C, but both appear different from SOL. Statistical comparisons of the curve parameters determined for individual fiber experiments revealed that the mean slopes (n) of the force-Ca^{2+} relationships were significantly different between SOL and DDF fibers (1.63 ± 0.12 vs. 3.00 ± 0.22, P = 0.002) and SOL and SDF fibers (1.63 ± 0.12 vs. 2.54 ± 0.31, P = 0.045) at 10°C, whereas neither DDF nor SDF slopes differed from that of SOL fibers at 30°C. The slopes of the DDF and SDF fibers were also not significantly different at either temperature. Mean slopes for each muscle did not vary significantly with temperature (e.g., a slope of 2.5 for SDF fibers was determined for both 10 and 30°C).

Hill regression analysis used to determine force-Ca^{2+} relationships for individual fibers from each muscle provided measurements of the pCa_{50} for each relationship. Like force-Ca^{2+} relationship slope, mean pCa_{50} for SOL fibers was significantly different from DDF (6.18 ± 0.03 vs. 6.01 ± 0.03, P = 0.01) and SDF fibers (6.18 ± 0.03 vs. 6.05 ± 0.05, P = 0.04) at 10°C. Significant differences between mean pCa_{50} of SOL fibers and DDF (6.23 ± 0.05 vs. 6.08 ± 0.06, P = 0.03) or SDF (6.23 ± 0.05 vs. 6.00 ± 0.05, P = 0.001) fibers were also found at 30°C, whereas mean pCa_{50} was not statistically significant between DDF and SDF fibers for either temperature (Fig. 6). Within each muscle, mean values of pCa_{50} again did not vary significantly with temperature, ranging from pCa 6.0 to pCa 6.2 for all three muscles.

DISCUSSION

The main objective of this study was to characterize and compare cellular and molecular contractile properties between equine DDF and SDF muscles and relate these properties to their muscle architecture and function in vivo. These muscles have similar apparent action around the metacarpophalangeal joint but substantially different architecture. Motility and fiber mechanical studies were conducted at two experimental temperatures so that comparisons could be made with past studies (typically at subphysiological temperatures) and to establish functional parameters at temperatures that more closely approximate physiological function.

MHC isoform composition. Large mammals typically express three conventional MHC isoforms (MHC-1, MHC-2A, MHC-2X).
MHC-2X) in their skeletal muscles (15). For example, the equine gluteus medius, a powerful hindlimb muscle for locomotor propulsion, has been shown to contain a relatively high percentage of fast MHC-2X fibers in addition to MHC-2A and MHC-1 fibers (36, 42). Other, more specialized muscles of the distal hindlimb or forelimb, however, do not show concurrent expression of all three isoform fiber types [soleus (21, 27) and interosseus (43)] and may be expected to have a primary composition of MHC-1 and MHC-2A fibers, as evidenced in the forelimb digital flexor muscles of horses in this study.

Fiber typing results for DDF and SDF by electrophoresis (Table 1 and Fig. 2) and histo/immunohistochemical techniques (Fig. 1) compare well with available fiber type data for these two muscles. Inferring MHC isoform, the DDF (short) was reported to contain 44% MHC-1 and 51% MHC-2A fibers, whereas the SDF contained 59% MHC-1 and 39% MHC-2A fibers (20). The same study also indicated that each muscle contained a very small percentage of MHC-2X fibers (reported as type IIB, consistent with classification at that time). A recent immunohistochemical study of fiber types in thoroughbred horses specifies a composition of 76% MHC-2A fibers in the DDF (head of this complex muscle not noted), 57% MHC-2A fibers in the SDF, and 0% MHC-2X fibers in both muscles (21). Despite slight differences in the calculated percentages of each fiber type among the previous and present studies, all confirm a primary composition of MHC-1 and MHC-2A isoforms in the DDF and SDF muscles. The very small percentage of fibers previously inferred to be MHC-2X in DDF short compartment (20) and the lack of MHC-2X fibers identified in this study and in a recently published report (21) suggest that the MHC-2X isoform may not significantly contribute to contractile function of the forelimb DDF and SDF muscles during locomotion. Furthermore, age, breed, and training effects may be factors that influence fiber type distributions observed in horses (21, 37). Although assessment of these factors is beyond the scope of this study, we speculate that the consistent composition of MHC-1 and MHC-2A fibers in the specialized digital flexor muscles of horses used in studies (20, 21) for which percentage fiber type has been determined.

Table 1. %Distributions of MHC fiber type from SDS-PAGE analysis on single skinned DDF, SDF, and SOL fibers from the 5 donor horses

<table>
<thead>
<tr>
<th>Horse</th>
<th>MHC-1, %</th>
<th>MHC-2A, %</th>
<th>MHC-1-2A, %</th>
<th>n</th>
<th>MHC-1, %</th>
<th>MHC-2A, %</th>
<th>MHC-1-2A, %</th>
<th>n</th>
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<th>MHC-2A, %</th>
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MHC, myosin heavy chain; DDF, deep digital flexor; SDF, superficial digital flexor; SOL, soleus. MHC-1-2A is a hybrid-slow, fast-fiber type.

Fig. 2. Composite of MHC isoform gels (SDS-PAGE) of single skinned DDF, superficial digital flexor (SDF), and soleus (SOL) muscle fibers. Samples from individual gels are separated by vertical heavy gray lines. On the left are 2 sample gel lanes run with bulk bundle myosin preparations (illustrated are SOL and DDF) that were used as MHC isoform references (see METHODS); the 1st lane shows the slow MHC-1 isoform from a SOL myosin sample, whereas the 2nd lane clearly shows both the MHC-1 isoform and a fast MHC-2A isoform from a DDF myosin sample. The latter is representative of bulk myosin from either DDF or SDF bundles, although the proportions of the 2 MHC bands vary. All other lanes show MHC bands present in example single fibers from DDF (2 fibers), SDF (2 fibers), or SOL (2 fibers). MHC-1 and MHC-2A were the only 2 isoforms identified by SDS-PAGE in the population of DDF and SDF fibers sampled from 5 horses. Some hybrid fibers of the MHC-1–2A isoforms (coexpression) were found in the sample and are illustrated by a single SDF fiber (SDF D). Only MHC-1 was found in SOL fibers. Representative examples of 2 SOL fibers from different horse donors (SOL D and SOL E) are shown on the far right.

Fig. 3. Filament sliding velocity (Vf; means ± SE) measured from motility assays for horse DDF, SDF, or rabbit psoas muscle heavy meromyosin (HMM). *Vf for DDF and SDF HMM was significantly lower than Vf of rabbit psoas measured at the same temperature (28 or 38°C). ‡Vf for SDF HMM was significantly lower than Vf of DDF measured at the same temperature (28 and 38°C). Number of individual actin filaments analyzed was n = 180 for each temperature and each muscle.
Myosin functional capacity and fiber contractile properties. MHC isoform composition in muscle fibers is the primary determinant of skeletal muscle contractile properties and performance (5, 40). At the molecular level (purified HMM), Vf at body temperature of the horse (38°C) provides realistic estimates of physiological myosin function (i.e., contractile velocity) in the digital flexor muscles. Vf for DDF was significantly faster than Vf for SDF at 38°C (Fig. 3). These motility data indicate a good correlation between MHC isoform composition of a muscle and functional properties of its constituent myosin molecules. The large mean difference likely reflects functional heterogeneity in MHC isoforms between the DDF and SDF muscles. MHC-2A isoforms hydrolyze ATP at faster rates than MHC-1 isoforms, and thus actin filament interactions (i.e., cross-bridge cycling) occur at faster rates. Slower detaching myosin heads of MHC-1 isoforms in a heterogeneous HMM sample create internal drag forces and slow Vf by counteracting the faster actomyosin cycling rates of MHC-2A isoforms (18). Therefore, the DDF with a comparatively higher percentage of the MHC-2A isoform has a higher mean Vf and thus potential for faster contractile properties at the fiber level, consistent with our main hypothesis.

DDF and SDF fibers showed similar Ca2+ dependence of steady-state isometric force (Fig. 6), whereas DDF fibers developed higher isometric tension than SDF fibers and on average shortened twice as fast at 30°C (Table 2). Overall, these findings may be explained by the heterogeneous composition of MHC-1 and MHC-2A fibers in both muscles, with the DDF having a higher percentage of fast MHC-2A fibers. However, although our study does not allow us to rule out the possibility that observed differences in contractile properties between DDF and SDF resulted from intrinsically different properties of the same fiber type, previous studies of skinned fibers (5, 32, 49, 50) indicate that contractile properties at saturating Ca2+ generally correlate with MHC isoform, whereas thin filament proteins troponin and tropomyosin are also important for properties at subsaturating Ca2+ levels. Despite the evaluation of differences in contractile properties being limited to a set of mean measurements between two muscles instead of a specific fiber type, comparisons between the mixed fiber type DDF and SDF muscles are consistent with the main objective of this study. Furthermore, we should not expect to find differences in properties of the same fiber type...
Cows and horses likely reflect the strong dependence of VUS on remarkable. Means of P₀/CSA and VUS for DDF, SDF, and (i.e., orthologous MHC isoforms) of similar size is equally contractile properties of a known fiber type between species slow SOL fibers at 10°C in this study. The similarity of isometric tension values of 84 and 97 mN/mm² for DDF and SDF fibers at 10°C are well within the range of contractility of these parameters for the horse. Mean P₀/CSA data bolster confidence in validity and, where applicable, fiber properties reported in this study and previously published for mean fiber types (i.e., within a species, different muscles) are generally similar (32, 49, 50). Accord between DDF and SDF fibers sampled from different muscles and compared within a species.

Contractile property data available for muscle fibers of large mammals indicate that properties of homologous MHC isoform fiber types (i.e., within a species, different muscles) are generally similar (32, 34, 45, 50). Accord between DDF and SDF fiber properties reported in this study and previously published data bolster confidence in validity and, where applicable, repeatability of these parameters for the horse. Mean P₀/CSA for DDF and SDF fibers at 10°C are well within the range of isometric tension values of 84 and 97 mN/mm² (15°C) reported for horse muscle fibers (38) identified as MHC-1 and MHC-2A, respectively, by SDS-PAGE analysis of myosin light chains (44). A mean VUS of 0.33 FL/s (15°C) for MHC-1 fibers from the same study (38) is identical to the mean VUS of slow SOL fibers at 10°C in this study. The similarity of contractile properties of a known fiber type between species (i.e., orthologous MHC isoforms) of similar size is equally remarkable. Means of P₀/CSA and VUS for DDF, SDF, and SOL fibers at 10°C (Table 2) are in good agreement with comparable data measured for MHC-1 and MHC-2A fibers from skeletal muscles in 400- to 500-kg cows (51). In the case of VUS, consistent observations for a known fiber type between cows and horses likely reflect the strong dependence of VUS on body size (32, 38, 41, 49, 50, 53). Although the consistency among fiber contractile property data from similarly sized mammals (and measured under similar conditions) is notable, more studies are needed for confirmation of these observations.

Relatively few studies have investigated contractile properties of skinned muscle fibers at near-physiological temperatures (31, 34, 45, 46, 55). Data reported herein are the first measured contractile properties of muscle fibers from a large mammal at higher temperature. Overall P₀/CSA increased moderately with temperature for DDF and SDF (ratio of P₀/CSA at 30:10°C = 1.11 DDF and 1.12 SDF). Changes in isometric tension with temperature are consistent with those observed in other studies of skinned fibers, although means of P₀/CSA at 30°C for DDF and SDF fibers are relatively lower. Differences in temperature dependence of isometric tension for horse muscle fibers compared with rabbit (31, 34) and human (4, 46) fibers may be related to variation in temperature sensitivity depending on either the temperature range studied or species studied. The modest increase in P₀/CSA in DDF and SDF muscles may also be attributed to reduced temperature sensitivity of isometric tension in faster contracting fiber types at temperature of 30°C and above (33, 34, 45, 46, 51). This is reasonable given the marked presence of the fast MHC-2A isoform in both DDF and SDF fibers and the higher temperature sensitivity of MHC-1 fibers (ratio of P₀/CSA at 30:10°C = 1.67 SOL). Comparison of VUS between DDF and SDF fibers at 30°C is also indicative of the influence of a composition of MHC-1 and MHC-2A isoforms. The apparent bimodal distribution of VUS values for individual DDF fibers is suggestive of a higher percentage of fast MHC-2A fibers in this muscle (Fig. 5B). Although differences in mean VUS were not statistically significant between the DDF and SDF, VUS measurements have been shown to be more variable at higher experimental temperatures (4), and MHC-1 fibers demonstrate a greater temperature sensitivity of shortening velocity (33), which may

Table 2. Means ± SE of P₀/CSA and VU5 measured at pCa 5 for DDF, SDF, and SOL (slow muscle control) fibers

<table>
<thead>
<tr>
<th>Muscle (Fiber CSA, μm²)</th>
<th>P₀/CSA, mN/mm²</th>
<th>VUS, FL/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDF (6,459 ± 512)</td>
<td></td>
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</tr>
<tr>
<td>30°C</td>
<td>104 ± 8.8 (n = 22)†</td>
<td>2.4 ± 0.5 (n = 12)</td>
</tr>
<tr>
<td>10°C</td>
<td>93.3 ± 7.3 (n = 24)†</td>
<td>0.7 ± 0.2 (n = 13)*</td>
</tr>
<tr>
<td>SDF (4,615 ± 341)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30°C</td>
<td>81.8 ± 7.7 (n = 24)</td>
<td>1.2 ± 0.2 (n = 12)</td>
</tr>
<tr>
<td>10°C</td>
<td>73.1 ± 7.5 (n = 22)*</td>
<td>0.6 ± 0.2 (n = 11)*</td>
</tr>
<tr>
<td>SOL (1,775 ± 102)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30°C</td>
<td>78.1 ± 6.0 (n = 11)</td>
<td>1.1 ± 0.2 (n = 9)</td>
</tr>
<tr>
<td>10°C</td>
<td>46.7 ± 4.5 (n = 11)</td>
<td>0.3 ± 0.1 (n = 9)</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of fibers analyzed. P₀/CSA, isometric tension; VUS, velocity of unloaded shortening. *Significantly different from SOL at P < 0.05 for corresponding temperature. †Significantly different from SDF at P < 0.05 for corresponding temperature.
have disproportionately increased \( V_{US} \) of slow contracting fibers.

**Architecture and physiology of the digital flexor muscle fibers in horse locomotion.** In the distal forelimb of the horse, the DDF and SDF muscles share a similar mechanical advantage about the metacarpophalangeal joint (fetlock); however, muscle architecture of each muscle-tendon unit differs greatly, as do their functional roles in locomotion. The comparatively fast contracting DDF muscle (short compartment, humeral head), with its long fibers in a unipennate arrangement (20, 54), shortens to generate modest amounts of mechanical work during locomotion, thus contributing to flexion of the digit late in the swing phase (9, 10). In vivo performance of the DDF is consistent with a muscle composed of long and predominantly fast MHC-2A isoform fibers. In particular, fast isoform fibers have intrinsically faster rates of cross-bridge cycling and the capacity to shorten at velocities several times faster than slow contracting fibers (38, 49, 50), where mechanical power generation is greater (5).

In contrast to the DDF, during locomotion the short multi-pennate fibers of the SDF (8, 20, 54) undergo isometric and lengthening (eccentric) contraction, allowing the muscle to produce high force economically and its tendon to store large amounts of elastic strain energy (9, 10). A muscle that has a role of high-force production by little to no change in length of its fibers is less dependent on fast MHC isoforms and fast shortening velocities of the muscle fibers for peak in vivo performance. Compared with the DDF, a higher percentage of slow MHC-1 fibers and slower contractile properties are also considered to be consistent with the functional role of the SDF muscle in locomotion. Despite the capacity to shorten faster than slow isoform fibers, mammalian fast MHC-2A fibers have higher oxidative capacity (i.e., mitochondrial content) than MHC-1 fibers (16, 26). Therefore, in the context of force-producing muscles as opposed to a power-generating muscle, both MHC-1 and MHC-2A fibers arranged in a multipennate architecture can provide high-force capacity by isometric or eccentric contractions with the benefit of consuming less metabolic energy.

**Perspectives and significance.** The diverse in vivo contractile behaviors of the DDF and SDF muscles match well with each muscle’s architecture and physiological properties, with direct consequences for the energetic cost of locomotion in horses. A primary composition of MHC-1 and MHC-2A isoform fibers alone arguably reflects an evolutionary specialization for metabolic energy savings, although it appears that fiber architecture may be more influential in determining the functional role of each muscle in locomotion. The relative distributions of slow and fast MHC isoforms and the lack of substantial differences in fiber contractile properties between DDF and SDF further suggest that architecture of specialized distal limb muscles may be an evolutionary constraint on contractile physiology and function. The distal limbs of cursorial animals display numerous morphological specializations for economical running that are perhaps best exemplified by the equine SDF (and interosseous), an oxidative muscle with extremely short fibers arranged in a multipennate architecture. Muscles like the SDF are much less suited for mechanical work and power generation but are rather highly specialized for economical, high-force production. Therefore, distinctions in physiology and function displayed in equine digital flexors may represent features of limb morphology that are fundamental to the evolution of all economical, long-distance running animals.

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Present address for N. M. Brunet: Donders Institute for Brain, Cognition, and Behaviour, Centre for Cognitive Neuroimaging, P. O. Box 9101, NL-6500 HB Nijmegen, The Netherlands.

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**DISCLOSURES**

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

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