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

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1Department of Biological Sciences, Youngstown State University, Youngstown, Ohio; 2Department of Biological Sciences, 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


Butcher MT, Chase PB, Hermanson JW, Clark AN, Brunet NM, Bertram JEA. 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 (Vt). 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 velocity. The DDF contained a higher percentage of MHC-2A fibers with myosin (heavy meromyosin) and Vt 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 isotonic 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.


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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 fluorescently 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_f) 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 ± 4.8 yr (range: 11–22 yr) and 499.2 ± 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 (Fental-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 in vivo length. Adjacent to three fiber fascicles were dissected from the midbelly region of the physiology and functional capacity of horse digital flexors.

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Skinned fiber preparation. Freshly dissected muscle fibers from the DDF, SDF, and SOL muscles were prepared for mechanical experimentation using published methods (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. Skiing solution contained (in mM) 25 EGTA, 50 MOPS, 6 Mg (acetate)_2, 4 acetic acid, 5 ATP, and 0.03% (wt/vol) dithiotheriureol (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 ml/mg 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 PCr (CP), 100 K+ plus Na+, 3 Mg2+, 50 MOPS, 1 DTT, and 1 mg/ml creatine kinase (CK; 250 U/ml). Four stock solutions were made with the following Ca2+ concentrations (pCa): pCa 9 (relaxing solution), pCa 7, pCa 6, and pCa 5 (maximum activation), where pCa = -log10 [Ca2+]_i. [Ca2+]_i in the stock solutions was adjusted by adding appropriate amounts of Ca(acetate)2. Intermediate [Ca2+]_i (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 one end and a servomotor (step time ≤300 μs, model 312C; 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 (L0) and fiber diameter were measured in relaxing conditions using an optical micrometer in the eyepiece of the inverted microscope. L0 was determined at ×40 magnification as fiber length between end clips. Lengths of the two fixed ends were measured (in μm) at the end of each experiment and subtracted from L0 to obtain active fiber length (L_a). Fiber diameter was determined at
×320 magnification as the average of three measurements at different locations along the fiber. Sarcomere length (Ls) 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 5), 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+]i in the following order: pCa 9, 7, 6.4, 6.2, 6, 5.9, 5.8, 5.6, 5.4, 5.2, and 5 (maximum isometric force, P = P0, 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). Measurements of P0, at pCa 5, was determined at pCa 5. Measurements of VUS 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), where CSA was calculated from mean fiber diameter assuming circular geometry. To determine VUS, fibers were brieﬂy relaxed following the series of force measurements before being transferred back to maximal activation conditions (pCa 5). Measurements of VUS were made using the slack test method (13, 17). Briefly, a series of seven length steps were applied, each shortening the fiber by 0.035 to 0.12 L0, 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 at sampling frequencies (256–3,000 Hz) appropriate for optimal resolution.

Data analysis for single, permeabilized ﬁber mechanics. Force and length data records 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 P0/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 in each experimental temperature from the Hill plot regressions. VUS was calculated using both ﬁber analysis software to determine slack time and length change from force and length records and a custom slack test analysis macro (linear least squares regression) written in MS Excel for Windows (Microsoft Systems, Redmond, WA). VUS (in FLs) was determined as the slope of the linear regression between length step size (normalized to Ls) and slack time. VUS for DDF, SDF, and SOL ﬁbers was averaged at each experimental temperature for maximally activated ﬁbers (pCa 5). Values of P/CSA, force-Ca2+ slope, pCa50, and VUS for SOL ﬁbers served as a slow muscle control in these experiments.

Quality controls for high temperature. In skinned ﬁbers, prolonged activation at higher temperatures can progressively disrupt stability of the ﬁber striation pattern and ﬁber function (4, 39). In addition to constant video monitoring, Brenner cycling was particularly important in maintaining ﬁber stability at 30°C. The similarity of force during ﬁrst and last activations indicated that ﬁber structure and function remained stable throughout the protocol. Force at the end of experiments averaged 99% of that at the beginning for DDF ﬁbers, 89% for SDF ﬁbers, and 100% for SOL ﬁbers at 10°C. Average stability of force was 98% for DDF, 95% for SDF, and again 100% for SOL ﬁbers at 30°C.

Myosin isoform identiﬁcation and ﬁber typing. Myosin isoform composition was analyzed for a separate set of DDF, SDF, and SOL ﬁbers adjacent to those used for contractility measurements. MyHC isoforms of single ﬁbers for each muscle were determined by SDS-PAGE using a combination of methods described previously (46, 48). Permeabilized ﬁber segments ~2 mm in length were denatured 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’-methylen-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 glycerine, 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. MyHC bands from single ﬁbers were evaluated by comparing band migration to both molecular weight standards in the 200-kDa region and MyHC from bulk myosin samples puriﬁed from entire muscle ﬁascicles. Brieﬂy, myosin was extracted from the remaining skinned ﬁascicles by homogenizing ~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 MyHC isoforms present in DDF, SDF, and SOL ﬁbers. Percentages of each MyHC isoform were quantiﬁed from the total number of ﬁbers sampled from each muscle and each horse. Percent distribution of MyHC 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 MyHC 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 (ATTC, 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 (humeral 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 unlabeled 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:
25 KCl, 25 imidazole, 4 MgCl₂, 1 EGTA, 1 DTT, pH 7.4) was applied for 1 min to block nonspecific protein binding, followed by an AB wash to remove unbound BSA. Sheared and unlabeled F-actin was then applied for 1 min to inhibit binding of RhPh-labeled F-actin to any remaining ATP-insensitive heads. Next, 0.5 mM ATP in AB was applied to dissociate the unlabeled F-actin from active myosin heads, followed by an AB wash. Then, fluorescently labeled F-actin (∼8 nM actin monomer) was applied and allowed to bind to HMM; after 1 min, unbound filaments were removed with an AB wash. Finally, an aliquot of motility buffer (MB) was applied to flow cells to initiate RhPh F-actin motility. Motility buffer consisted of AB plus 2 mM ATP; 16.7 mM glucose, 100 μg/ml glucose oxidase, 18 μg/ml catalase, and an extra 40 mM DTT were added to minimize photo-bleaching of fluorescently labeled F-actin and limit photooxidative damage to the proteins (22).

After addition of MB, flow cells were transferred to the stage of a Diastar upright fluorescence microscope (Leica, Deerfield, IL), and 1 min was allowed for temperature equilibration (19). Flow cell temperature was maintained at either 28 (standard temperature) or 38°C (physiological core body temperature for a horse) by circulating temperature-controlled water through a copper coil surrounding the microscope objective; temperature was calibrated within flow cells directly under the objective. Image capture of F-actin motility was accomplished with a silicon intensified target camera (model VE 1000; Dage-ATI, Michigan City, IN) at 30 frames/s, with an added timedate generator signal (model WJ-810; Panasonic, Secaucus, NJ), and stored on VHS videocassettes (VCR model AG7350; Panasonic) for analysis as described (14). A count of six to 10 trial image recordings of 30–60 s duration each were made per flow cell. Three flow cells were sampled for each type of HMM (DDF, SDF, rabbit psoas) at each temperature for a total sample size of n = 18 flow cells.

Data analysis for in vitro motility assays. Recorded images of RhPh-labeled F-actin motility were digitized, and analysis of filament sliding speed (V_f) was carried out with the aid of MetaMorph software (Universal Imaging; Molecular Devices, Downingtown, PA), as described previously (28). For each flow cell, 12 stacks of frames were created from the digitized recordings; each stack consisted of 30 (1 s of recorded data) or 90 frames (3 s of recorded data) of motility data. Individual actin filament paths were then visualized by creating a superimposed image of all frames of one stack, called a projection, and subtracting the image of the first frame from that projection. For each stack, five filaments were randomly selected, and the distance traveled (d) was estimated by manually measuring the residual contour lines of their respective pathways. Finally, V_f (in μm/s) was determined as d/[t(μm of frames = 1)], where t is the frame-to-frame duration, i.e., (frame rate)⁻¹. Individual filament measurements of V_f for DDF, SDF, or rabbit psoas HMM were averaged (n = 180 filaments/muscle) for each experimental temperature and their distribution characterized by nonparametric statistics due to the large differences among means of V_f and corresponding low-error terms. V_f of rabbit psoas HMM, with a nearly 100% composition of the MHC-2X isoform (40), served as a fast myosin (control) in these experiments.

Statistical analysis. All data are expressed as means ± SE. Statistical significance of the differences among means of P0/CSA, V_U/S, force-Ca²⁺ relationship slope, and pCa₅₀ of fibers for each muscle and experimental temperature (10 and 30°C) was assessed by two-way analysis of variance (ANOVA) followed by a Holm-Sidak multiple comparison test. Considering that V_f data were not found to be distributed normally and failed to meet the assumption of equal variances, statistical significance of the differences among means of V_f for each muscle’s HMM and experimental temperature (28 and 38°C) was assessed by an ANOVA for Ranks followed by a Dunn’s multiple comparison test. Particular emphasis of both fiber mechanics and motility V_f analyses was placed on differences between means of DDF and SDF preparations. Statistical significance for all tests was accepted at P ≤ 0.05.

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.

V_US. Figure 3 illustrates the large disparity among means of V_f from motility assays, with HMM prepared from bulk muscle samples of horse DDF or SDF myosin or rabbit psoas myosin. Means of V_f were significantly different (P < 0.0001) among the three muscles for each experimental temperature (28 or 38°C). As expected for each muscle, V_f at 38°C was faster than at 28°C. Compared with rabbit psoas myosin (fast control), means of V_f 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 V_f for DDF 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 V_US) 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 SDF fibers at either temperature, although the large difference between mean P₀/CSA for SOL fibers (46.7 ± 4.5 mN/mm⁻²) and DDF fibers (93.3 ± 7.3 mN/mm⁻²) 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 and SDF fibers were similar (P = 0.77), primarily because of the significant increase in slow SOL fiber P₀/CSA with temperature.

V_US. Figure 5B shows frequency distributions of V_US for DDF, SDF, and SOL fibers at 30°C. Means (± SE) of V_US determined at maximal activation (pCa 5) are presented in Table 2. Mean V_US of DDF fibers was not significantly different compared with mean V_US for both SDF and SOL fibers at 30°C; this may be due to the apparently bimodal distribution of V_US 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 ($P < 0.006$). 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 ($1.07 \pm 0.21 \text{ FL/s}$) and VUS for SDF fibers ($1.2 \pm 0.22 \text{ FL/s}$) at 30°C, although the distribution of VUS values was broader for SDF than for SOL fibers (Fig. 5B).

Force-$\text{Ca}^{2+}$ relationship parameters. Figure 6 shows averaged force-$\text{Ca}^{2+}$ 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-$\text{Ca}^{2+}$ relationships were significantly different between SOL and DDF fibers ($1.63 \pm 0.12$ vs. $3.00 \pm 0.22$, $P = 0.002$) and SOL and SDF fibers ($1.63 \pm 0.12$ vs. $2.54 \pm 0.31$, $P = 0.045$) 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-$\text{Ca}^{2+}$ relationships for individual fibers from each muscle provided measurements of the pCa$_{50}$ for each relationship. Like force-$\text{Ca}^{2+}$ relationship slope, mean pCa$_{50}$ for SOL fibers was significantly different from DDF ($6.18 \pm 0.03$ vs. $6.01 \pm 0.03$, $P = 0.01$) and SDF fibers ($6.18 \pm 0.03$ vs. $6.05 \pm 0.05$, $P = 0.04$) at 10°C. Significant differences between mean pCa$_{50}$ of SOL fibers and DDF ($6.23 \pm 0.05$ vs. $6.08 \pm 0.06$, $P = 0.03$) or SDF ($6.23 \pm 0.05$ vs. $6.00 \pm 0.05$, $P = 0.001$) 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) 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 may be a feature fundamental to Equus given the broad ranges of age, breed, and training level 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>DDF MHC-1, %</th>
<th>DDF MHC-2A, %</th>
<th>DDF MHC-1-2A, %</th>
<th>n</th>
<th>SDF MHC-1, %</th>
<th>SDF MHC-2A, %</th>
<th>SDF MHC-1-2A, %</th>
<th>n</th>
<th>SOL MHC-1, %</th>
<th>SOL MHC-2A, %</th>
<th>SOL MHC-1-2A, %</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>87.5</td>
<td>12.5</td>
<td>8</td>
<td></td>
<td>50.0</td>
<td>50</td>
<td></td>
<td></td>
<td>100</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>37.5</td>
<td>50.0</td>
<td>12.5</td>
<td>16</td>
<td>46.7</td>
<td>46.7</td>
<td>6.7</td>
<td>15</td>
<td>100</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>100</td>
<td>100</td>
<td>8</td>
<td>8</td>
<td>85.7</td>
<td>14.3</td>
<td></td>
<td>7</td>
<td>23</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>17.4</td>
<td>82.6</td>
<td>23</td>
<td>23</td>
<td>50.0</td>
<td>46.2</td>
<td>3.8</td>
<td>26</td>
<td>13</td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>100</td>
<td>100</td>
<td>24</td>
<td>24</td>
<td>21.7</td>
<td>65.2</td>
<td>13.0</td>
<td>23</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>%Population</td>
<td>12.7</td>
<td>83.5</td>
<td>3.8</td>
<td>44.3</td>
<td>48.1</td>
<td>7.6</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MHC, myosin heavy chain; DDF, deep digital flexor; SDF, superficial digital flexor; SOL, soleus. MHC-1-2A is a hybrid-slow, fast-fiber type.
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), \( V_f \) at body temperature of the horse (38°C) provides realistic estimates of physiological myosin function (i.e., contractile velocity) in the digital flexor muscles. \( V_f \) for DDF was significantly faster (2×) than \( V_f \) 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 \( V_f \) 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 \( V_f \) and thus potential for faster contractile properties at the fiber level, consistent with our main hypothesis.

DDF and SDF fibers showed similar Ca\(^{2+}\) 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 Ca\(^{2+}\) generally correlate with MHC isoform, whereas thin filament proteins troponin and tropomyosin are also important for properties at subsaturating Ca\(^{2+}\) 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

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**Fig. 4.** Example chart record of single skinned DDF fiber force at 30°C, illustrating the time course of the overall experimental protocol. Fibers were initially activated in high Ca\(^{2+}\) solution (pCa 5; left). Following activation, passive tension (pCa 9, relaxing) was minimized, and isometric sarcomere length (L_s) and thus final fiber length (L_0) were set. Isometric force (P) was measured over a range of increasing [Ca\(^{2+}\)] (decreasing pCas), ending in the highest (P_0 at pCa 5, maximal activation). Measurements of P were taken after force reached steady state at each Ca\(^{2+}\). Changes in gain.

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**Fig. 5.** Frequency distributions for isometric tension (P_0/CSA; A) and V_{US} (B) of SOL (light gray), SDF (black), and DDF (dark gray) fibers at pCa 5 and 30°C. Histogram A does not indicate a bimodal distribution of P_0/CSA among DDF, SDF, and slow (MHC-1) SOL fibers. Note in histogram B the apparent bimodal distribution of V_{US} values from individual DDF fibers.
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 Vₚₛ 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 Vₚₛ 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 Vₚₛ were not statistically significant between the DDF and SDF, Vₚₛ 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 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, 49, 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 Vₚₛ of 0.33 FL/s (15°C) for MHC-2A fibers from the same study (38) is identical to the mean Vₚₛ 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 Vₚₛ 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 Vₚₛ, consistent observations for a known fiber type between cows and horses likely reflect the strong dependence of Vₚₛ 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

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**Table 2. Means ± SE of P₀/CSA and Vₚₛ 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>Vₚₛ, FL/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDF (6,459 ± 512)</td>
<td></td>
<td></td>
</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; Vₚₛ, 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 isoforin 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 multipennate 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 muscle 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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**DISCLOSURES**

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

**REFERENCES**


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