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RESEARCH ARTICLE

Women's Health Research and Novel Perspectives on Sex as an Investigative Variable

The influence of sex on fiber-specific indices of oxidative capacity in human skeletal muscle

Celine Bailleul,[1]* Nathan Hodson,[1,2]* Sidney Abou Sawan,[1] Dinesh Kumbhare,[1,3] Daniel R. Moore,[1] and Jenna B. Gillen[1]

[1]*Department of Kinesiology and Physical Education, University of Toronto, Toronto, Ontario, Canada;* [2]*Department of Sport and Exercise Science, Institute of Sport, Manchester Metropolitan University, Manchester, United Kingdom; and* [3]*Toronto Rehabilitation Institute, Toronto, Ontario, Canada*

Abstract

There are reports that females compared to males display increased skeletal muscle oxidative capacity in resting mixed-muscle fiber samples from the *vastus lateralis*, including markers of mitochondrial content and capillarization. Given that sex comparisons at the mixed-fiber level may be explained by differences in muscle fiber type between males and females, it remains unclear whether the oxidative capacity of type I and/or II fibers differs between sexes. The purpose of this study was to evaluate the influence of sex on fiber-specific indices of mitochondrial content and capillarization in healthy untrained males and females. Resting skeletal muscle samples from eumenorrheic females ($n = 14$; 23 ± 5 yr; 23.3 ± 3.2 kg/m^2) and males ($n = 13$; 23 ± 4 yr; 23.1 ± 2.4 kg/m^2) were analyzed via immunofluorescence staining. There were no sex differences in indices of capillarization (all $P > 0.05$) or mitochondrial content (all $P > 0.05$) in type I or type II muscle fibers. However, we observed lower capillary density in type II vs. type I muscle fibers in males (280 ± 66 vs. 364 ± 88 capillaries/mm^2; $P < 0.001$) but not females (335 ± 77 vs. 329 ± 48 capillaries/mm^2; $P = 0.76$), owing to greater cross-sectional area (CSA) of type II vs. type I fibers in males only (males $P = 0.03$; females $P = 0.44$). Females compared to males also displayed greater proportionate area of type I fibers (44 ± 12% vs. 31 ± 14%; $P = 0.03$) and smaller CSA of type IIx fibers (3,033 ± 902 vs. 5,573 ± 1,352 μm^2; $P = 0.002$). Our results suggest that while muscle fiber type composition and size differ between males and females, there are no sex differences in mitochondrial content and capillarization of type I or II muscle fibers in untrained adults.

NEW & NOTEWORTHY Research suggests that skeletal muscle oxidative capacity in mixed-fiber muscle homogenates is greater in females than in males. In healthy, untrained individuals, we demonstrate by fiber-specific immunofluorescence that females have a greater proportionate area of type I muscle fibers but no difference in mitochondrial content or capillarization of type I or II fibers compared to males. These findings suggest that although females display a more oxidative fiber type composition, sex does not influence muscle fiber-specific oxidative capacity.

capillarization; fiber composition; mitochondria; sex differences; skeletal muscle

INTRODUCTION

Sex differences in substrate oxidation during submaximal aerobic exercise are well documented (1–3). Females have higher rates of fat oxidation and lower rates of carbohydrate oxidation compared to males during moderate-intensity continuous exercise, as evidenced by lower respiratory exchange ratio (4–9), reduced muscle glycogen utilization (10), and increased circulating and intramyocellular lipid (IMCL) oxidation (11, 12) during exercise.

Sex differences in fuel utilization during exercise have largely been attributed to higher circulating concentrations of 17β-estradiol and progesterone among females compared to males (5, 13). In addition to this, it is also possible that the higher rate of fat oxidation among females compared to males may be explained by sex differences in skeletal muscle oxidative capacity and substrate storage. Indeed, resting skeletal muscle characteristics such as greater mitochondrial and lipolytic enzymes, capillarization, and IMCL content have been associated with increased fat oxidation during exercise among other cohorts, such as endurance-trained adults (14). In direct comparisons of the vastus lateralis muscle between sexes, there are reports that females compared to males have greater capillary density (15), β-oxidation

*C. Bailleul and N. Hodson contributed equally to this work.
Correspondence: J. B. Gillen (jenna.gillen@utoronto.ca).
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enzymes (16), mitochondrial respiration (17), mitochondrial content (8, 18), and IMCL storage (11, 19, 20). These findings, albeit not universal (16, 21, 22), suggest that female skeletal muscle may be inherently more oxidative than males, at least when measured at the mixed-fiber level within the *vastus lateralis*.

Human skeletal muscle is composed of slow-twitch type I and fast-twitch type II (type IIa + type IIx) muscle fibers, which possess a predominantly more oxidative and glycolytic phenotype, respectively. Although the influence of sex on skeletal muscle oxidative capacity has been explored in mixed-fiber samples, limited research has evaluated these outcomes in a fiber-specific manner. Within the *vastus lateralis* muscle, females are reported to exhibit a greater proportionate area of type I fibers relative to type II fibers (type IIa and type IIx) (15, 23, 24) and a greater proportionate area of type I fibers relative to males (4, 11, 12, 24, 25). Considering that type I compared to type II fibers display higher mitochondrial content (26, 27), IMCL storage (28), and capillarization (27, 29), the reported increase in whole muscle indices of oxidative capacity among female skeletal muscles may be the result of greater proportionate area of type I fibers as opposed to intrinsic sex differences within each fiber type. Comparing the oxidative potential of male and female skeletal muscle in a fiber-specific manner would enhance our basic understanding of sex differences in resting skeletal muscle metabolism and may improve understanding of the mechanisms underlying sex differences in substrate metabolism during exercise.

The primary purpose of this study was to investigate the influence of sex on fiber-specific indices of skeletal mitochondrial content and capillarization in healthy males and females. The secondary objective was to evaluate sex differences in fiber type distribution, fiber cross-sectional area (CSA), and proportionate area (relative fiber area expressed as a percentage of the total area) of type I, IIa, and IIx fibers. To complement these measures, we compared markers of mitochondrial content, mitochondrial dynamics (fission and fusion), and β-oxidation in mixed-fiber muscle homogenates. We hypothesized that females compared to males would exhibit: *1*) higher mitochondrial content and capillarization in type I fibers, with no differences in type II fibers; *2*) greater skeletal muscle proportionate area of type I fibers, smaller CSA of type IIa and IIx fibers, and no differences in fiber type distribution; and *3*) higher protein content of biomarkers of mitochondrial content, mitochondrial dynamics, and β-oxidation in mixed-fiber muscle homogenates.

MATERIALS AND METHODS

Participants and Ethics Approval

This study was conducted during COVID-19 restrictions that precluded data collection on human participants. Thus, muscle analysis was performed on a convenience sample of 27 healthy, untrained males ($n = 13$) and eumenorrheic females ($n = 14$) matched for age and body mass index (BMI) from two previously published studies in our laboratory (30, 31). Participant characteristics are represented in Table 1. Participants were deemed healthy and untrained based on self-reported physical activity habits of three or fewer exercise sessions totaling ≤150 min of moderate-intensity physical activity per week (30) or not engaging in whole body resistance exercise or plyometrics in the past 3 mo (31). Exclusion criteria included an inability to perform physical activity as determined by the Physical Activity Readiness Questionnaire (PAR-Q), regular tobacco or drug use, and/or diagnosis with a medical condition under the care of a physician. Females were eumenorrheic and not using hormonal contraceptive medication, and muscle biopsies were obtained during the early-mid follicular phase of their menstrual cycle (days 3–10) as determined by the self-reported onset of menses. Experimental protocols were approved by the University of Toronto Health Sciences Research Ethics Board (REB#36374, REB#36371), and procedures were conducted in accordance with the Declaration of Helsinki. Participants were informed of the study's experimental procedures and potential risks before providing written consent.

Table 1. *Participant characteristics*

Variable	Females ($n = 14$)	Males ($n = 13$)
Age, yr	23 ± 5	23 ± 4
BMI, kg/m^2	23.3 ± 3.2	23.1 ± 2.4
Body mass, kg	62.5 ± 9.3*	72.4 ± 9.5
Body fat, %	25.5 ± 6.7*	19.2 ± 7.1
Fat mass, kg	16.4 ± 5.3	14.4 ± 7.1
FFM, %	78.4 ± 6.7*	82.9 ± 9.9
FFM, kg	46.8 ± 6.4*	59.7 ± 7.6
Daily step count, steps/day	9,549 ± 5,870	8,316 ± 3,321

Values are means ± SD. BMI, body mass index; FFM, fat-free mass. *$P < 0.05$, significantly different from males.

Baseline Testing and Pretrial Controls

Following study enrollment, body mass and body composition (Bodpod; Cosmed USA, Inc., Concord, CA) were measured after an 8-h overnight fast. Three days prior to muscle biopsy sampling, participants wore an accelerometer (wGT3X-BT; Actigraph, Pensacola, FL) to track their habitual step count and were asked to refrain from exercise for 24 h before the biopsy. Participants were also provided with a standardized diet to consume 24 h before tissue collection that was prepared to meet individual energy needs (Bodpod-derived resting energy expenditure multiplied by an average activity factor of 1.5) and consisted of 51 ± 6% carbohydrate, 32 ± 3% fat, and 18 ± 2% protein.

Skeletal Muscle Biopsy Collection

After ~10 min of lying supine, resting skeletal muscle biopsy samples were collected from the *vastus lateralis* of participants after an 8-h overnight fast. Sample collection was performed with a Bergstrom needle modified for suction under local anesthesia (1% lidocaine) and sterile conditions. Muscle samples were cleared of any visible blood, adipose tissue, or connective tissue. One portion of the collected muscle sample was quickly snap-frozen in liquid nitrogen, and a second portion was mounted in optimal cutting temperature compound (VWR International, Mississauga, ON, Canada) and frozen in liquid nitrogen-cooled isopentane. All samples were subsequently stored at −80°C for further analyses.

Table 2. *Primary and secondary antibody conditions for immunofluorescence assays*

		Primary Antibodies				Secondary Antibodies	
IF Assay	Target	Dilution	Source	Product ID	Incubation Time	Dilution	Alexa Fluor Dyes
Fiber-type stain	MHCI	1:50	DSHB	BA-F8	2 h at RT	1:300	Alexa Fluor 488 nm
	MHCIIa	1:50	DSHB	SC-71	2 h at RT	1:300	Alexa Fluor 594 nm
	MHCIIx	1:50	DSHB	6H1	2 h at RT	1:300	Alexa Fluor 647 nm
	Dystrophin	1:25	DSHB	MANDYS1(3B7)	2 h at RT	1:300	Alexa Fluor 488 nm
	Nuclei	1:10,000		DAPI	5 min at RT		
Mitochondrial content stain	COX IV	1:500	Cell Signaling	4844	∼8 h at 4°C	1:300	Alexa Fluor 488 nm
	MHCI	1:1	Prepared by N.H.		∼8 h at 4°C	1:300	Alexa Fluor 594 nm
	Laminin	1:300	Abcam	ab11575	∼8 h at 4°C	1:300	Alexa Fluor 647 nm
	PanMHCII	1:300	Abcam	ab91506	∼8 h at 4°C	1:300	Alexa Fluor 647 nm
Capillarization stain	MHCI	1:1	Prepared by N.H.		∼8 h at 4°C	1:300	Alexa Fluor 488 nm
	Laminin	1:50	Abcam	ab11575	∼8 h at 4°C	1:300	Alexa Fluor 647 nm
	CD31	1:80	Abcam	ab28364	∼8 h at 4°C	1:300	Alexa Fluor 594 nm

IF, immunofluorescence; RT, room temperature. See GLOSSARY for other abbreviations.

Immunofluorescence Analyses

Serial cross sections (7 μm) of embedded skeletal muscle samples were prepared at −22°C and placed onto separate glass slides, with each slide designated for one of the three immunofluorescent assays listed in Table 2. Cross sections were fixed in 4% paraformaldehyde (PFA) on ice for 10 min (except during the fiber type immunofluorescent stain) and washed twice for 5 min in 1× PBST. A 5% normal goat serum (NGS) in 1× PBS blocking solution was then applied to sections and left to incubate for 60–90 min at room temperature. After slides were washed for 5 min in 1× PBS three times, sections were incubated with a primary antibody diluted in 5% NGS and 1× PBS for the respective incubation period as shown in Table 2. After incubation with the primary antibody, slides were washed for 5 min in PBST three times. Sections were incubated with their respective Alexa Fluor-conjugated secondary antibodies prepared in a solution of 1% BSA in 1× PBS for 1 h at room temperature as shown in Table 2. Then slides were washed for 5 min in 1× PBST followed by 5 min in 1× PBS. DAKO fluorescent mounting medium was applied to sections, and glass covers were carefully placed onto slides. Slides were allowed to dry overnight at 4°C for imaging at ×20 magnification under an EVOS FL Auto Cell imaging microscope (ThermoFisher Scientific, Waltham, MA). Subsequent analysis of captured images was performed by the same blinded investigator (C.B.) using ImageJ (Fiji plug-in version 1.5; National Institutes of Health, Bethesda, MD). Representative images of immunofluorescent stains are illustrated in Fig. 1.

Fiber type distribution, proportionate area, and cross-sectional area (CSA) were quantified with cross sections stained for type I, type IIa, and type IIx skeletal muscle fibers. Fiber type distribution was determined by counting all fibers on the muscle cross section (331 ± 77 fibers/participant; range: 99–829 fibers) and calculating the proportion (%) of each fiber type. Fiber type CSA was determined for 54 ± 19 type I fibers, 53 ± 17 type IIa fibers, and 47 ± 25 type IIx fibers per participant by circling the perimeter of each fiber type and calculating the mean area for each fiber type as described by Tan et al. (32). Fiber type proportionate area was quantified by multiplying the average fiber type CSA by the fiber type number count and dividing the product by the total area of quantified fibers. The threshold for fiber circularity was set at 0.6 as previously described (31), and fibers below this threshold were removed from analysis.

Fiber type-specific mitochondrial content was assessed by staining for type I fibers (MHCI), type II fibers (panMHCII), cell borders (laminin), and mitochondria (COX IV) as outlined in Table 2. Mitochondrial content was quantified separately by measuring COX IV pixel intensity in fibers expressing either MHCI or panMHCII protein: a total of 56 ± 16 type I and 64 ± 10 type II fibers per participant, which is consistent with the number of fibers analyzed by others (32). Similarly, fiber type specific capillarization was evaluated by staining for type I fibers (MHCI), cell borders (laminin), and capillaries (CD31). Type I fibers were identified by the presence of MHCI protein, whereas type II fibers were distinguished by the absence of fluorescence (i.e., lack of MHCI expression). Fiber type capillarization was determined through analysis of capillary contacts (the number of capillaries surrounding a single fiber), capillary-to-fiber (C/F_i) ratio (the number of capillaries per muscle fiber corrected by a sharing factor of the number of fibers that share a single capillary), capillary density (the number of capillaries per fiber square millimeter), and capillary-to-fiber perimeter exchange (CFPE) index (the number of capillaries shared between fibers along the perimeter of a single fiber) for 25 ± 8 type I fibers and 28 ± 6 type II fibers per participant, as described by Hepple (33).

Western Blot Analysis

Mixed-muscle fiber lysates were homogenized on ice in RIPA buffer solution (65 mM Tris Base, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) prepared by adding one tablet of Complete Mini and EDTA-free Protease Inhibitor Cocktail Tablet and one tablet of Pierce Phosphatase Inhibitor Mini Tablet (Roche Applied Science, Mannheim, Germany) to every 10-mL stock solution of buffer solution. For every 1 mg of frozen skeletal muscle samples used, 15 μL of RIPA buffer solution was added. Once homogenization was completed, samples were centrifuged for 5 min at 700 *g* at 4°C to remove the myofibrillar pellet and cell debris. The sarcoplasmic microfraction (supernatant) was pipetted into newly labeled centrifuge tubes, and the protein concentrations of extracts were determined

with a bicinchoninic acid assay (ThermoFisher Scientific, Rockford, IL). Samples were prepared to equal concentration (2 μg/μL) by diluting the extracts with lysis buffer and adding 1× Laemmli sample buffer. Western blot samples for the protein content of β-HAD, MFN2, and DRP1 were denatured via boiling for 5 min at 95°C before storage of samples at −80°C. Western blot samples for the quantification of the protein content of OXPHOS subunits were not boiled before storage of samples at −80°C as per manufacturer's instructions for OXPHOS antibody (ab110411; Abcam).

Equal volumes of Western blot samples were loaded into lanes of 4–20% polyacrylamide Criterion TGX Precast Gel Cassettes (26 wells) (Bio-Rad Laboratories, Richmond, VA). A volume of 3 μL of a prestained protein ladder (PageRuler Plus; ThermoFisher Scientific No. 26617) was loaded into the first and last well of each gel. Gels were electrophoresed at 200 V for 45 min. Once completed, proteins were transferred onto nitrocellulose membranes at 100 V for 1 h. Ponceau S was applied to the membranes and rocked for 2 min at room temperature. Ponceau-stained membranes were imaged to verify that proteins were successfully transferred onto the nitrocellulose membranes and control for the variability of sample loading across lanes of a gel. Membranes were washed in Tris-buffered saline supplemented with 0.1% Tween 20 (TBST) for 2 × 2 min to remove Ponceau S staining before incubation on a rocker for 1 h at room temperature in a 5% BSA blocking solution made in TBST. Membranes were washed for 2 × 5 min with TBST before incubation overnight on a rocker at 4°C with primary antibodies (DRP1 no. 8570, Cell Signaling, Danvers, MA; OXPHOS ab110411, β-HAD ab154088, MFN2 ab50843; Abcam, United Kingdom) diluted 1:1,000, with the exception of COX IV (COX IV no. 4844; Abcam, United Kingdom), which was diluted at 1:10,000. After primary antibody incubation, membranes were washed for 3 × 5 min in TBST and appropriate species-specific horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit IgG no. 7074; Cell Signaling, Danvers, MA) were added at a dilution of 1:10,000 and incubated for 1 h at room temperature. After secondary antibody incubation, membranes were washed for 3 × 5 min in TBST. Finally, proteins were detected via chemiluminescence and imaged with the Fluorochem E Imaging system (Protein Simple; Alpha Innotech, Santa Clara, CA). The biomarkers of mitochondrial content and dynamics selected in this study were based on their functional roles in mitochondrial function (34). COX IV and total OXPHOS were used as indicators of mitochondrial content, given their essential roles in ATP production through the electron transport chain. DRP1 and MFN2 were chosen to assess mitochondrial dynamics for their roles as critical regulatory proteins involved in mitochondrial fission and fusion, respectively, which are crucial for maintaining mitochondrial function and health in muscle fibers.

Protein content was quantified with ImageJ (Fiji plug-in version 1.5; National Institutes of Health, Bethesda, MD) by

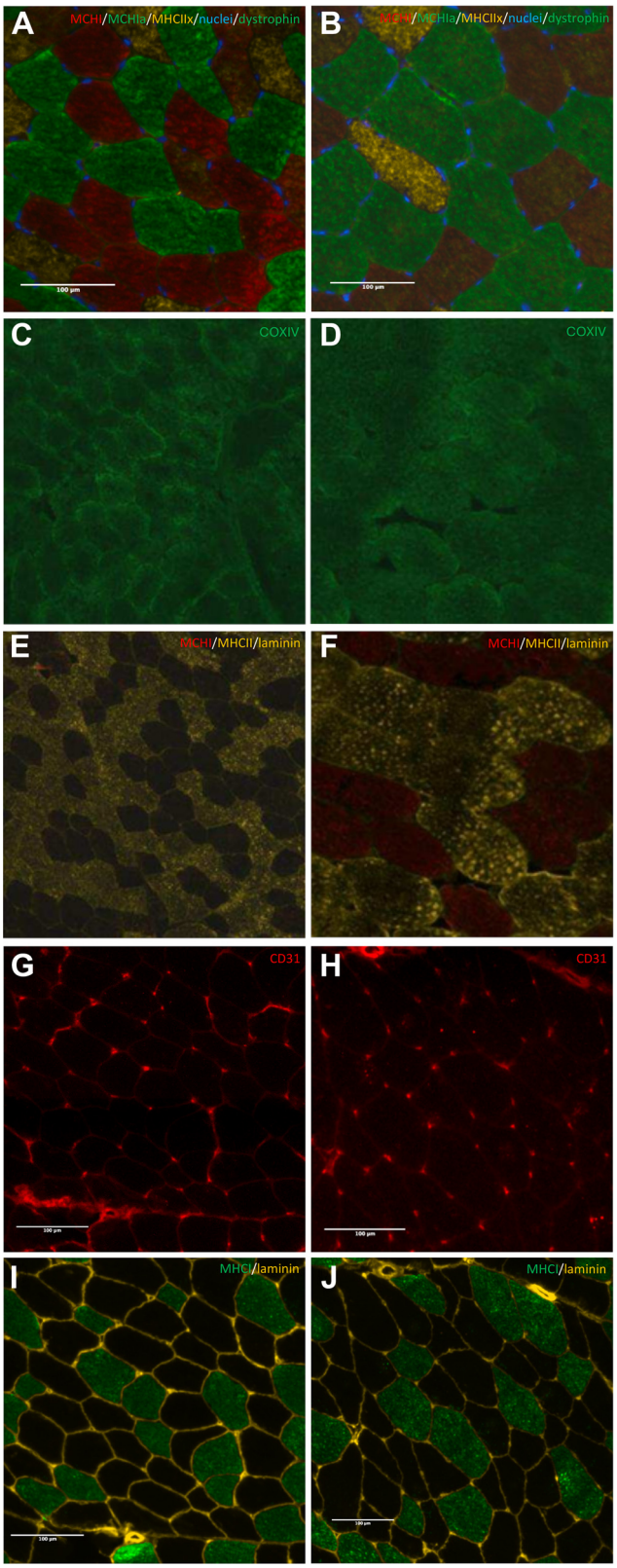

Figure 1. Fiber-type immunofluorescent stains. *A* and *B*: representative images of female (*A*) and male (*B*) myosin heavy chain (MHC) isoform immunofluorescence of MHCI (green), MHCIIa (red), MHCIIx (yellow), nuclei (blue), and dystrophin (green). *C–F*: representative images of cytochrome *c* oxidase (COX) IV (green) and MHC isoform immunofluorescence of MHCI (red), panMHCII (yellow), and laminin (yellow), from a female (*C* and *E*) and a male (*D* and *F*) participant, respectively. *G–J*: representative images of female (G and *I*) and male (*H* and *J*) MHC isoform immunofluorescence of MHCI (green), laminin (yellow), and CD31 (red). Images captured at ×20 magnification.

calculating pixel intensity. To control for potential variability in protein content between gels, the pixel intensity of bands was normalized to pixel intensity of a control sample (mixed homogenate from all samples) that was loaded on every gel. Values were then normalized to the pixel intensity of the Ponceau stain for each respective lane to account for variability in protein loading and transfer efficiency between lanes.

Statistical Analyses

All data are expressed as means ± standard deviation (SD). Baseline characteristics between sexes were compared by independent Student *t* tests. Dependent variables were inspected for normality with the Shapiro–Wilk test. A two-way mixed-model analysis of variance (ANOVA) was performed to determine the effect of fiber type (type I, type IIa, and type IIx; within factor) and sex (female and male; between factor) on fiber type distribution, CSA, and proportionate area. To evaluate differences in fiber-specific COX IV protein intensity and capillarization, a two-way mixed-model ANOVA was performed with fiber type (type I and type II) as the within factor and sex (female and male) as the between factor. In the event an ANOVA yielded a significant interaction, pairwise comparisons within each sex and between fiber types were determined post hoc with Bonferroni–Holm-corrected *t* tests as described by Holm (35). Sex differences in the protein content of mixed-fiber muscle outcomes analyzed via Western blotting were assessed by independent Student *t* tests. Statistical significance for all analyses was accepted at $P < 0.05$. Effect sizes for ANOVA tests were calculated as partial eta squared (η_p^2), where <0.05 represents a small effect, 0.06–0.13 represents a moderate effect, and >0.14 represents a large effect. All statistical analyses were performed with IBM SPSS version 25.0 (IBM Co., Armonk, NY), and figures were produced with Prism (GraphPad Software, San Diego, CA). All analyses are presented as $n = 14$ females and $n = 13$ males unless otherwise reported. Outliers were identified as falling outside ±2 standard deviations (SDs) from the mean and ±1.5 times the interquartile range above the upper quartile/below the lower quartile, as described in previous literature (36, 37). For muscle fiber type distribution, outliers and participants with missing values for a single fiber type were identified and winsorized by replacement with the subsequent minimum or maximum sex- and fiber type-specific value. Similarly, data of participants with outliers or missing values for a single fiber type for CSA were replaced via previous or next value data imputation considering the trend of fiber type CSA in the whole dataset (36, 38). Owing to weak staining that resulted in $n = 2$ being unquantifiable after multiple staining attempts, indices of fiber type capillarization were analyzed on $n = 13$ females and $n = 12$ males. Protein content analysis of mixed-fiber muscle homogenates was conducted in $n = 14$ females. For males, $n = 12$ samples were available for DRP1, MFN2, and β-HAD, whereas $n = 11$ samples were available for COX IV and total OXPHOS because of limited sample availability.

RESULTS

Participant Characteristics

Participant characteristics are displayed in Table 1. There was no difference in age ($P = 0.85$), BMI ($P = 0.83$), and daily step count ($P = 0.51$) between sexes. Compared to males, females had lower body mass ($P = 0.01$) and fat-free mass ($P = 0.01$) and greater percentage of body fat ($P = 0.03$).

Fiber Type Distribution, Cross-Sectional Area, and Proportionate Area

The hierarchy of fiber type distribution in males was type IIa > type I > type IIx, whereas in females it was type I > type IIa > type IIx. There was a main effect of fiber type ($P < 0.001$; $\eta_p^2 = 0.28$) such that the distributions of type I (36 ± 13%; $P = 0.001$) and type IIa (40 ± 10%; $P < 0.001$) fibers were greater than that of type IIx fibers (24 ± 13%; $P < 0.001$; Table 3). However, there was no main effect of sex ($P = 0.20$; $\eta_p^2 = 0.07$) or interaction between sex and fiber type on fiber type distribution ($P = 0.20$; $\eta_p^2 = 0.06$).

The hierarchy of fiber type CSA for males was type IIa > type I > type IIx, whereas in females it was type I > type IIa > type IIx. There was no main effect of sex ($P = 0.123$; $\eta_p^2 = 0.09$) but a main effect of fiber type ($P < 0.001$; $\eta_p^2 = 0.56$) and an interaction between sex and fiber type ($P = 0.005$; $\eta_p^2 = 0.19$) on CSA (Table 3). Post hoc testing revealed that type IIx fibers were 35% larger in males compared to females ($P = 0.03$), but there was no difference between sexes in the CSA of type I ($P = 0.99$) or type IIa ($P = 0.19$) fibers. Type IIa fibers were 15% larger than type I fibers ($P = 0.03$) in males, but no difference was observed between these fiber types in females ($P = 0.44$). Additionally, type IIx fibers were smaller than both type I (46%; $P < 0.001$) and type IIa (42%; $P < 0.001$) fibers in females and smaller than type IIa fibers in males (26%; $P < 0.01$).

The hierarchy of fiber type proportionate area for males was type IIa > type I > type IIx, whereas in females it was type I > type IIa > type IIx. There was a main effect of fiber type ($P < 0.001$; $\eta_p^2 = 0.46$) and an interaction between sex and fiber type ($P = 0.016$; $\eta_p^2 = 0.15$) on fiber type proportionate area (Fig. 2). Post hoc testing revealed that females had a greater proportionate area of type I fibers compared to males (44 ± 12% vs. 31 ± 14%; $P = 0.03$). Within the male participants, the proportionate area of type IIa fibers was greater than that of type IIx (47 ± 14% vs. 22 ± 15%; $P = 0.02$) and type I (31 ± 14%; $P = 0.07$) fibers. Within female participants, the proportionate areas of both type I (44 ± 12%; $P < 0.001$) and type IIa (40 ± 7%; $P < 0.001$) fibers were greater than that of type IIx (16 ± 9%) fibers, but there was no difference between type I and type IIa fibers ($P = 0.25$).

Fiber-Specific Mitochondrial Content

There was no main effect of fiber type ($P = 0.5$; $\eta_p^2 = 0.02$) or sex ($P = 0.48$; $\eta_p^2 = 0.02$), or interaction between sex and fiber type ($P = 0.42$; $\eta_p^2 = 0.03$), on fiber-specific COX IV intensity (Table 3).

Fiber Type Capillarization

Analyses of capillary contacts, C/F_i ratio, and CFPE index revealed no main effects of fiber type (capillary contacts: $P = 0.65$, $\eta_p^2 = 0.01$; C/F_i ratio: $P = 0.41$, $\eta_p^2 = 0.03$; CFPE index: $P = 0.28$, $\eta_p^2 = 0.05$) or sex (capillary contacts: $P = 0.67$, $\eta_p^2 = 0.14$; C/F_i ratio: $P = 0.06$, $\eta_p^2 = 0.146$; CFPE index: $P = 0.49$,

Table 3. *Muscle fiber-type characteristics in females and males*

Variable	Female			Male		
	Type I	Type IIa	Type IIx	Type I	Type IIa	Type IIx
Fiber type distribution, %	40 ± 11δ	38 ± 5δ	22 ± 12	32 ± 13δ	43 ± 14δ	25 ± 15
Cross-sectional area, μm^2	4,822 ± 1,390#	4,651 ± 1,397#	3,033 ± 902*	4,814 ± 1,440	5,573 ± 1,352†#	4,298 ± 1,412
Proportionate area, %	44 ± 12#*	40 ± 7#	16 ± 9	31 ± 14	47 ± 14#	22 ± 15
COX IV protein intensity, a.u.	12.71 ± 2.81	12.72 ± 2.47	13.50 ± 2.43	13.32 ± 2.29		
Capillary contacts	3.73 ± 0.86	3.53 ± 0.88	4.26 ± 1.26	4.32 ± 0.68		
C/F$_i$ ratio	1.39 ± 0.35	1.31 ± 0.35	1.64 ± 0.51	1.62 ± 0.29		
Capillary density, capillaries/mm^2	329 ± 48	335 ± 77	364 ± 88^	280 ± 66		
CFPE index	5.3 ± 0.63	5.13 ± 0.98	5.6 ± 1.40	5.32 ± 0.88		

Data are expressed as means ± SD. Fiber type distribution (%) and cross-sectional area (μm^2) and proportionate area (%) of type I, type IIa, and type IIx fibers measured in biopsy samples of females ($n = 14$) and males ($n = 13$). Mitochondrial content as determined by COX IV protein intensity (a.u.) measured in biopsy samples of females ($n = 14$) and males ($n = 13$) in type I and type II fibers. Fiber-type specific capillary contacts, capillary-to-fiber (C/F$_i$) ratio, capillary density, and capillary-to-fiber perimeter exchange (CFPE) index measured in biopsy samples of females ($n = 13$) and males ($n = 12$) in type I and type II fibers. Significantly different: *$P < 0.05$, vs. male in respective fiber type; #$P < 0.05$, vs. type IIx fibers in respective sex; †$P < 0.05$, vs. type I in respective sex, ^$P < 0.05$, vs. type II fibers in respective sex; δ$P < 0.05$, vs. type IIx fibers irrespective of sex.

$\eta_p^2 = 0.02$), or interaction between sex and fiber type (capillary contacts: $P = 0.44$, $\eta_p^2 = 0.03$; C/F$_i$ ratio: $P = 0.66$, $\eta_p^2 = 0.01$; CFPE index: $P = 0.78$, $\eta_p^2 = 0.003$), as illustrated in Table 3. Measurement of capillary density revealed that there was no main effect of sex ($P = 0.71$; $\eta_p^2 = 0.006$) but a main effect of fiber type ($P = 0.005$; $\eta_p^2 = 0.3$) and an interaction between sex and fiber type ($P = 0.001$; $\eta_p^2 = 0.37$), as illustrated in Table 3. Post hoc testing revealed that capillary density was greater in type I versus type II fibers in males (364 ± 88 vs. 280 ± 66 capillaries/mm^2; $P < 0.001$) but not females (329 ± 48 vs. 335 ± 77 capillaries/mm^2; $P = 0.76$).

Western Blotting

There were no differences between males and females for protein content of β-HAD ($P = 0.37$), MFN2 ($P = 0.90$), or DRP1 ($P = 0.65$) (Fig. 3). Similarly, there were no differences between sexes in the protein content of complex II subunit 30 kDa ($P = 0.30$), complex III subunit Core 2 ($P = 0.53$), complex IV subunit II ($P = 0.93$), and ATP synthase α-subunit ($P = 0.71$) (Fig. 3).

DISCUSSION

The primary objective of this study was to investigate the influence of sex on fiber-specific indices of skeletal muscle mitochondrial content and capillarization in healthy males and eumenorrheic females. To complement these measurements, we also quantified sex differences in fiber type distribution, CSA, and proportionate area as well as mixed-muscle fiber indices of oxidative capacity. Contrary to our hypothesis, we observed no sex differences in indices of mitochondrial content or capillarization in type I or type II fibers. Similarly, there were no sex differences in indices of mitochondrial content, mitochondrial dynamics, or β-oxidation in mixed-fiber muscle homogenates. However, we observed lower capillary density of type II fibers versus type I fibers in males but not females, owing to the greater CSA of type IIa versus type I fibers among males only. In addition, females displayed greater proportionate area of type I fibers compared to males, a result that was attributed to sex differences in fiber type CSA and distribution. Collectively, these results suggest that while there are sex differences in skeletal muscle fiber type size and proportionate area, there are no differences in mitochondrial content and capillarization of type I or II fibers among healthy untrained males and females.

Previous research has investigated sex differences in mixed-muscle fiber mitochondrial content of the vastus lateralis, with some reports demonstrating greater COX IV protein content (18) and mitochondrial volume density (8) in females compared to males. However, to the best of our knowledge, no study has investigated whether these sex differences in mitochondrial content are found within type I and/or type II muscle fibers. Using immunofluorescent staining of COX IV, we observed no difference in mitochondrial content of type I or type II fibers among healthy untrained males and females. We also failed to demonstrate sex differences in indices of mitochondrial content at the mixed-fiber level, as reflected by similar content of proteins in the electron transport chain, including COX IV. Others have also reported no sex difference in the protein content of COX IV and other electron transport chain proteins in resting mixed-fiber muscle homogenates from males and females (16, 22). The discrepancy between our findings and those of Scalzo et al. (18) and Montero et al. (8), who reported increased mitochondrial content among females compared to males, may be due to methodological differences between studies such as a lower fitness of participants in the present investigation. Moreover, Montero et al. (8) observed sex differences in skeletal muscle mitochondrial volume density (MitoVD) via transmission electron microscopy, which may be a more sensitive technique for quantifying potentially small differences in mitochondrial content between sexes. Nonetheless, the present findings suggest that there is no difference in mitochondrial content of type I or II fibers, or mixed-fiber muscle homogenates, between untrained males and females by immunofluorescent staining of COX IV. Future investigations should investigate fiber-specific mitochondrial content among trained males and females who, based on previous research (8), may differ in mitochondrial content at the mixed-fiber level.

While sex differences in fiber-specific mitochondrial content were not observed within the present study, we did observe that females displayed a greater proportionate area

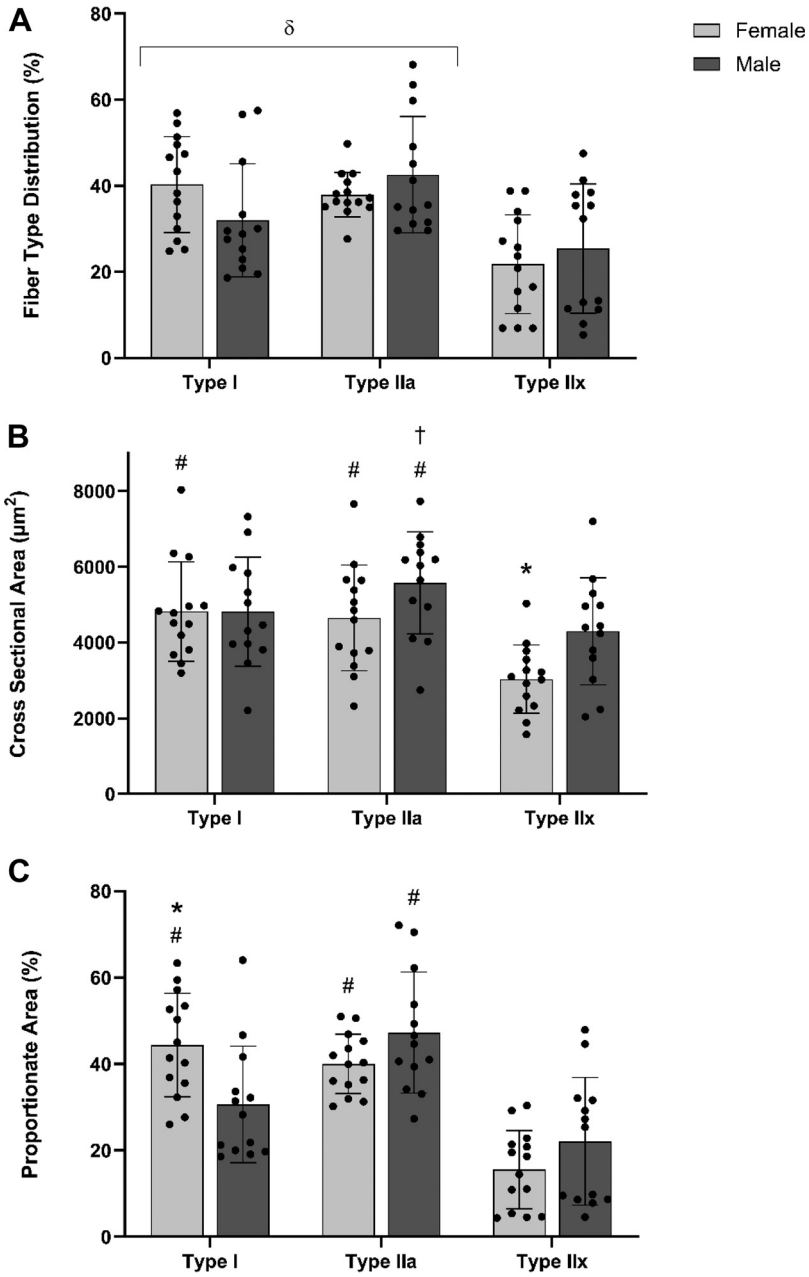

Figure 2. Fiber type composition. Fiber type distribution (%; *A*), cross-sectional area (μm^2; *B*), and proportionate area (%; *C*) of type I, type IIa, and type IIx fibers measured in biopsy samples from the vastus lateralis of females ($n = 14$) and males ($n = 13$). Significantly different ($P < 0.05$): *compared to males for respective fiber type, #compared to type IIx fibers for respective sex, †compared to type I in respective sex, and δ compared to type IIx fibers irrespective of sex, as determined by post hoc analysis following significant ($P < 0.05$) interaction of fiber type × sex of ANOVA. Data are expressed as means ± SD.

of oxidative type I fibers compared to males (44% vs. 31%). In addition, males had greater proportionate area of type II versus type I fibers (47% vs. 31%), whereas there was no significant difference between these two fiber types in females (40% type II vs. 44% type I). These findings are consistent with others who have demonstrated greater proportionate area of type I fibers among females compared to males in sedentary (25), untrained (4, 11), recreationally active (13), and trained (11, 12) adults. For example, Staron and colleagues (24) demonstrated among a large cohort of females ($n = 55$) and males ($n = 95$) that females had a greater proportionate area of type I fibers when assessed via ATPase histochemical analysis (~44% vs. 36%) and relative MHCI isoform percentage by SDS-PAGE electrophoresis (~41% vs. 34%). Additionally, Nuzzo (39) concluded through a meta-analysis of 16 studies that females compared to males had a greater proportionate area of type I fibers (~47% vs. 38%), whereas males had a greater proportionate area of type IIa fibers compared to females (~50% vs. 54%). Given the lack of sex difference in mitochondrial content at the fiber-specific level in the present study, it remains possible that an increased proportionate area of type I fibers among females contributes to previously reported sex differences in skeletal muscle oxidative capacity.

Sex differences in skeletal muscle capillarization have been previously investigated at a mixed-fiber level. For example, Roepstorff and colleagues (12) reported a 23% higher capillary density within the vastus lateralis among endurance-trained females compared to males. However, this is not a universal finding, as no difference has been

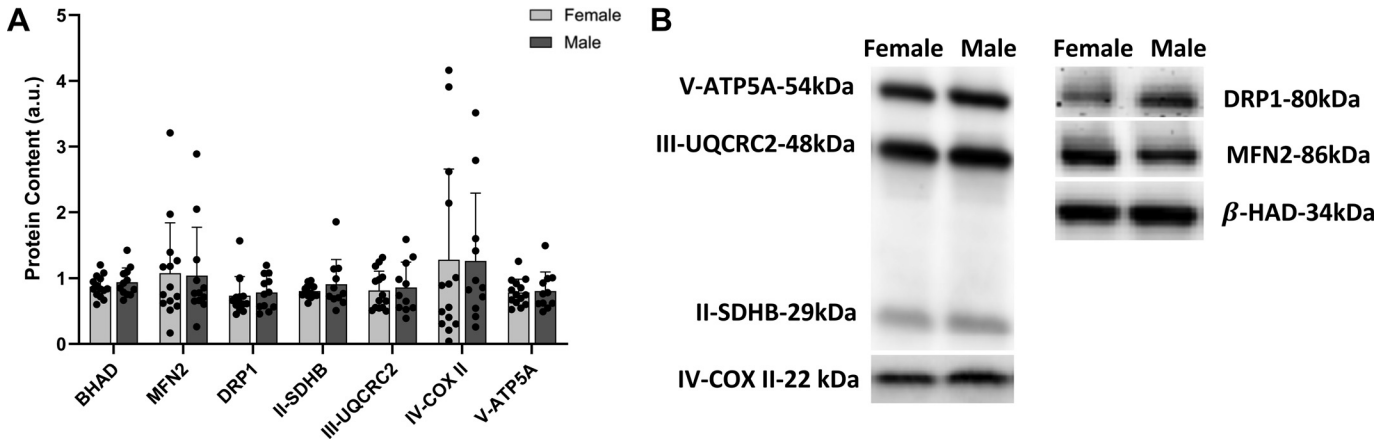

Figure 3. Whole muscle protein content of biomarkers associated with β-oxidation, mitochondrial dynamics, and mitochondrial content. *A*: measured in biopsy samples from the vastus lateralis of females ($n = 14$) and males ($n = 12$ for β-HAD, MFN2, and DRP1; $n = 11$ for COX IV and total OXPHOS) analyzed via Western blotting. *B*: Western blots from a representative male and female participant. Data are expressed as means ± SD. a.u., Arbitrary units.

observed between sexes in other cohorts of endurance-trained adults (8, 40) or sedentary adults over 65 yr (41). To the best of our knowledge, our analysis is the first to evaluate indices of skeletal muscle capillarization in a sex- and fiber-specific manner or among untrained adults. We observed no difference between males and females in capillary contacts, C/F_i ratio, and CFPE index of type I or type II fibers in the present study. However, it should be noted that there was a trend for males to display greater capillary contacts ($P = 0.06$, $\eta_p^2 = 0.14$) and capillary-to-fiber ratio ($P = 0.06$, $\eta_p^2 = 0.15$) compared to females. Our sample size may have precluded our ability to detect this difference and thus may reflect a type II error. However, the lack of statistical difference between sexes is consistent with the majority of investigations at the mixed-fiber level (8, 40–42) but conflicts with that of Roepstorff and colleagues (12), who observed higher capillary density in females compared to males. Differences between our results and those of Roepstorff et al. (12) may be explained by the lower training status of participants in the present study, differences in antibodies used to measure capillarization, and/or quantification of capillary density using fiber-specific (vs. mixed fiber) capillary contacts and CSA.

While there were no apparent sex differences in capillarization within type I or type II fibers, the difference in capillarization between fiber types varied by sex. Classic literature suggests that type II fibers have lower capillary density compared to type I fibers (27, 29). However, in the present study this finding was only observed in males and not females. The only other study to our knowledge to investigate sex differences in fiber-specific indices of capillarization came to similar conclusions (43). For example, healthy recreationally active males had lower capillary density in type II compared to type I fibers (43), whereas females displayed the reverse, whereby the capillary density of type I fibers was lower than that of type II fibers (43). Given that capillary contacts did not differ between type I and II fibers in males or females in our study or Barnouin et al. (43), sex differences in capillary density of type I versus type II fibers were mediated by differences in CSA of the fiber types. Indeed, in the present study, the CSA of type II fibers was 15% larger than that of type I fibers in males, consistent with classic literature (24, 27), which mathematically resulted in lower capillary density of type II muscle fibers. In contrast, there was no difference in CSA between type I and II fibers among females, consistent with others (12, 25), resulting in no difference in capillary density between fiber types. In Barnouin et al. (43), the authors observed greater CSA of type I relative to type II fibers among females, which explained the higher capillary density among type II fibers in that investigation. Smaller muscle fiber CSA, in addition to fewer "large" type IIa fibers, also seems to have driven the greater capillary density among females at the mixed-fiber level reported previously (12). Collectively, the classically reported increase in capillary density among type I versus type II fibers is mediated by increased CSA of type II fibers, which our results suggest is sex specific and observed in males only.

Our analysis also demonstrated sex differences in muscle fiber CSA that were fiber type specific. The CSAs of type IIa and IIx fibers were 18% and 35% larger in males compared to females, respectively, whereas there was a <1% difference among type I fibers between sexes. Our results are consistent with others that have demonstrated greater muscle fiber CSA of type IIa and IIx fibers in both sedentary (25) and moderately trained (40, 44) males compared to females (11, 25, 39). Although the difference in type IIa fiber CSA between sexes was not statistically significant in the present study, the difference is of comparable magnitude to previous research (~18%) (24, 25) and the effect size ($g = 0.67$) is consistent with a recent meta-analysis on sex differences in skeletal muscle fiber composition ($g = 0.81$) (39). It is likely that we were underpowered to detect a significant difference between sexes, most likely because of an observed higher standard error than classically reported for type IIa CSA (45). Regarding type I muscle fibers, we observed no sex difference in CSA, which is consistent with many earlier reports (15, 40, 43, 46–48). Although some studies with larger sample sizes have observed significantly greater type I fiber muscle CSA among males compared to females (24, 25), the magnitude of difference is typically smaller than that observed in type II fibers.

Limited research has investigated sex differences in indices of skeletal muscle mitochondrial dynamics. Montero and

colleagues (8) observed no difference in protein content of the mitochondrial fusion protein MFN2 among aerobically trained males and females. Consistent with these observations, we observed no sex difference in MFN2 or mitochondrial fission protein DRP1 among untrained adults, suggesting no difference in mitochondrial dynamics between males and females. Furthermore, we also observed no difference between sexes in the protein content of β-HAD, consistent with others (13, 23, 47). Despite the lack of sex difference in mitochondrial dynamics and fat metabolism potential assessed via protein content, mRNA content or activity may differ between sexes given previously observed differences in the global mRNA of genes related to lipid metabolism and mitochondrial function (23). Indeed, females have been demonstrated to have greater mixed-muscle fiber β-HAD mRNA content and β-HAD activity at rest compared to males despite no sex difference in β-HAD protein content (23).

Although the findings of the present study provide novel insight into fiber-specific sex differences in skeletal muscle oxidative capacity, we acknowledge limitations within our study design. This study was conducted retrospectively on previously collected resting muscle biopsy samples from two studies (30, 31) that did not measure participant $\dot{V}O_{2max}$ or substrate metabolism during exercise. As a result, we were unable to match sexes for fitness or examine relationships between skeletal muscle measurements and exercise fuel utilization. Future studies are needed that investigate the relationship between fiber-specific characteristics and exercise metabolism and/or performance between sexes to elucidate the functional significance of findings reported here. Regarding our analytical methods, we did not observe the classically higher mitochondrial content among type I versus type II fibers with the COX IV immunofluorescent stain despite using similar methodology as others (32). We have previously observed fiber-specific differences of 13–18% in untrained individuals using this methodology (32). Discrepancies between studies may be a result of small sample sizes, interindividual variability, and/or the sensitivity of the semiquantitative methodology employed. Additionally, we did not distinguish between type IIa and IIx fibers when assessing fiber-specific mitochondrial content and capillarization, owing to methodological constraints. It is possible that sex differences in mitochondrial content and capillarization in type IIa or IIx fibers may have gone unnoticed; however, the analytical methods available at the time did not allow for this analysis. Because of limited sample availability, we were also unable to perform fiber-specific SDS-PAGE or mixed-muscle mitochondrial enzyme activity measurements. Finally, hybrid fibers identified as fluorescing for two MHC isoforms were excluded from analyses of all fiber type outcomes, as there were insufficient hybrid fibers to be appropriately analyzed. As suggested by others (24, 49), it is possible that sex differences may exist in the characteristics of hybrid fibers, which remains a fruitful area for future investigation.

To conclude, we observed no sex differences in indices of mitochondrial content or capillarization in type I or type II fibers among healthy untrained males and eumenorrheic females. However, reduced capillary density of type II versus type I fibers was sex specific and observed in males only, a finding that was attributed to increased size of type II fibers in male but not female skeletal muscle. Consistent with previous literature, we also observed increased proportionate area of type I fibers among females compared to males. Collectively, these results demonstrate that although sex influences skeletal muscle fiber type proportionate area and size, there are limited differences in fiber-specific indices of mitochondrial content and capillarization among healthy untrained adults.

GLOSSARY

ANOVA	Analysis of variance
β-HAD	3-Beta-hydroxyacyl-CoA dehydrogenase
BMI	Body mass index
BSA	Bovine serum albumin
CD31	Endothelial cell adhesion molecule 1
CFPE index	Capillary-to-fiber exchange index
C/F_i ratio	Capillary-to-fiber ratio
COX	Cytochrome *c* oxidase
CSA	Cross-sectional area
DSHB	Developmental Studies Hybridoma Bank
FFM	Fat-free mass
IMCL	Intramyocellular lipid
MFN2	Mitofusin-2
MHCI	Myosin heavy chain 1
MHCIIa	Myosin heavy chain IIa
MHCIIx	Myosin heavy chain IIx
MitoVD	Mitochondrial volume density
NGS	Normal goat serum
OXPHOS	Oxidative phosphorylation
PBS	Phosphate-buffered saline solution
PBST	Phosphate-buffered saline solution with a low-concentration detergent solution
PFA	Paraformaldehyde
SD	Standard deviation
TBST	Tris-buffered saline with a low-concentration detergent solution
RER	Respiratory exchange ratio
$\dot{V}O_{2max}$	Maximal oxygen consumption

DATA AVAILABILITY

The data supporting the findings of this study are included within text, figures, and tables of the present article.

SUPPLEMENTAL MATERIAL

Supplemental Material: https://doi.org/10.6084/m9.figshare.28924034.v1.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

C.B., N.H., and J.B.G. conceived and designed research; C.B., N.H., S.A.S., D.R.M., and J.B.G. performed experiments; C.B. and N.H. analyzed data; C.B., N.H., and J.B.G. interpreted results of experiments; C.B. prepared figures; C.B. and N.H. drafted manuscript; C.B., N.H., S.A.S., D.K., D.R.M., and J.B.G. edited and revised manuscript; C.B., N.H., S.A.S., D.K., D.R.M., and J.B.G. approved final version of manuscript.

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