ORIGINAL ARTICLE

WILEY

Increased oxygen extraction and mitochondrial protein expression after small muscle mass endurance training

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When exercising with a small muscle mass, the mass-specific O_2 delivery exceeds the muscle oxidative capacity resulting in a lower O_2 extraction compared with whole-body exercise. We elevated the muscle oxidative capacity and tested its impact on O_2 extraction during small muscle mass exercise. Nine individuals conducted six weeks of one-legged knee extension (1L-KE) endurance training. After training, the trained leg (TL) displayed 45% higher citrate synthase and COX-IV protein content in *vastus lateralis* and 15%-22% higher pulmonary oxygen uptake ($\dot{V}O_{2peak}$) and peak power output ($\dot{W}_{peak}$) during 1L-KE than the control leg (CON; all $P < .05$). Leg O_2 extraction (catheters) and blood flow (ultrasound Doppler) were measured while both legs exercised simultaneously during 2L-KE at the same submaximal power outputs (real-time feedback-controlled). TL displayed higher O_2 extraction than CON (main effect: $1.7 \pm 1.6\%$ points; $P = .010$; 40%-83% of $\dot{W}_{peak}$) with the largest between-leg difference at 83% of $\dot{W}_{peak}$ (O_2 extraction: $3.2 \pm 2.2\%$ points; arteriovenous O_2 difference: 7.1 ± 4.8 mL·L^{-1}; $P < .001$). At 83% of $\dot{W}_{peak}$, muscle O_2 conductance (D_MO_2; Fick law of diffusion) and the equilibration index Y were higher in TL ($P < .01$), indicating reduced diffusion limitations. The between-leg difference in O_2 extraction correlated with the between-leg ratio of citrate synthase and COX-IV ($r = .72\text{-}.73$; $P = .03$), but not with the difference in the capillary-to-fiber ratio ($P = .965$). In conclusion, endurance training improves O_2 extraction during small muscle mass exercise by elevating the muscle oxidative capacity and the recruitment of D_MO_2, especially evident during high-intensity exercise exploiting a larger fraction of the muscle oxidative capacity.

KEYWORDS
arteriovenous oxygen difference, blood flow, endurance training, fick method, limitations, maximal oxygen uptake, muscle oxygen diffusion, peripheral adaptations

1 | INTRODUCTION

During dynamic exercise involving a large muscle mass (>15 kg; ie, in cycling or running), maximal oxygen uptake ($\dot{V}O_{2max}$) is primarily limited by O_2 delivery to the recruited muscles.[1] However, during dynamic exercise involving a small muscle mass, such as dynamic one-legged knee extension (1L-KE), two-legged KE (2L-KE), and arm cycling, engaging only ~2.5-6.0 kg active skeletal muscle mass, maximal cardiac output ($\dot{Q}_{max}$) is not attained, and O_2 delivery does not

limit muscle $\dot{V}O_2$ per se.[2-4] Therefore, a more than twofold larger O_2 delivery per unit muscle mass is observed during 1L-KE compared with cycling (500 vs 240 mL·min^{-1}·kg$^{-1}_{muscle}$, respectively).[5] Despite this, the mass-specific $\dot{V}O_2$ is only ~70% higher due to a substantially lower leg O_2 extraction during 1L-KE.[5]

The decreased O_2 extraction may be caused by fully activated mitochondria respiring near their maximal rate,[5,6] a condition that may restrict further O_2 extraction and O_2 diffusion from the blood to cytochrome-c-oxidase. This is substantiated by a close to 1:1 relationship between the maximal activity of the Krebs cycle enzyme oxoglutarate dehydrogenase and the maximal flux rate within the Krebs cycle during 1L-KE.[7] Consequently, leg $\dot{V}O_2$ is not enhanced by breathing hyperoxic gas during maximal 1L-KE exercise,[8-11] although this effect may depend on the training status.[12] The higher mass-specific leg blood flow (LBF) and lower O_2 extraction indicate that the $\dot{V}O_2$ is more diffusion-limited than perfusion-limited, in contrast to the situation when exercising with a large muscle mass.[5] Accordingly, when exercising with a small muscle mass, lower O_2 extraction is expected if muscle oxidative capacity and O_2 conductance (D_MO_2; the $\dot{V}O_2$ divided by the O_2 pressure gradient between the muscle capillaries and the mitochondria) are lowered, and *vice versa*. Indeed, leg immobilization decreases the muscle oxidative capacity resulting in impaired leg O_2 extraction during 1L-KE.[13] Furthermore, during arm cycling, engaging a small muscle mass (~6 kg) characterized by a substantially lower maximal mitochondrial respiratory capacity (OXPHOS; measured in permeabilized muscle fibers ex vivo) than its maximal O_2 delivery,[14] endurance training has proven to enhance O_2 extraction.[15] Although there is some controversy whether O_2 extraction improves after endurance training during exercise with a large muscle mass,[16-19] these data, in conjunction with animal data,[20] suggest that the potential for improvement is greater during exercise with a small muscle mass.

When conducting whole-body endurance training (eg, cycling and running), both central and peripheral adaptations occur.[19] Therefore, as O_2 delivery and O_2 extraction are interdependent, evaluation of the isolated effect of enhanced muscle oxidative capacity has proven to be difficult in humans. One way of avoiding this problem is to increase the oxidative capacity in only one leg by using one-legged endurance training. Subsequently, the trained leg and the contralateral control leg within the same subject can exercise simultaneously at the same power output.[17] This experimental setup ensures equal perfusion pressure and arterial O_2 content for both legs, meaning that (a) the O_2 delivery should be similar and (b) every improvement in O_2 extraction should originate from the changes in muscle oxidative capacity and/or capillarization. Similar experiments involving simultaneous exercise with one trained leg and one untrained leg have been

conducted in the past.[17,21,22] However, in two of these studies, care was not taken to balance the power output between the trained leg and the untrained leg (the trained leg performed more work),[21,22] and all three studies used two-legged cycling where the muscles possess a large oxidative reserve capacity, even before training.[14] In the study balancing the power output between legs, it was found a 3.5% points higher O_2 extraction in the trained leg at the highest power output (91% of cycling $\dot{V}O_{2\,max}$), but no significant difference at low-to-moderate power outputs.[17] During exercise with small muscle mass (ie, knee extension) involving a trained leg and an untrained leg, it is conceivable that a between-leg difference in O_2 extraction may be more prominent since the mitochondria are respiring closer to their maximal rate and the O_2 extraction is lower than during cycling[5,6]—that is, the muscle oxidative capacity may be more limiting in the untrained leg.

Based on the above premises, we aimed to increase the muscle oxidative capacity and test its impact on O_2 extraction during exercise involving a small muscle mass. After six weeks of 1L-KE endurance training, the trained leg (TL) and the control leg (CON) were exercised simultaneously at the same absolute power output during 2L-KE, a model engaging only ~5 kg active skeletal muscle mass that minimizes the perfusion limitations as it does not tax $\dot{Q}_{max}$.[3] During three low- to high-intensity workloads, equaling $40 \pm 3\%$, $62 \pm 4\%$, and $83 \pm 4\%$ of 2L-KE peak power output ($\dot{W}_{peak}$), leg O_2 extraction (femoral arterial and venous catheters) and LBF (ultrasound Doppler) were measured for each leg. Muscle biopsies were taken from both legs before and after the training period and were analyzed for mitochondrial enzymes and capillarization. We hypothesized that leg O_2 extraction would be higher in TL than in CON due to increased oxidative capacity and larger recruitment of D_MO_2 after training. Moreover, as the oxidative capacity would be gradually more exploited by increasing exercise intensity, we hypothesized that a between-leg difference in O_2 extraction would be more evident at high compared with low power outputs.

2 | MATERIALS AND METHODS

2.1 | Subjects and ethical approval

Nine moderately trained subjects (age: 27.5 ± 5.2 years; height: 1.79 ± 0.07 m; weight: 79.0 ± 10.6 kg; lean body mass: 59.1 ± 4.9 kg; $\dot{V}O_{2\,max}$: 55.5 ± 9.5 mL·kg^{-1}·min^{-1}) were recruited for study participation. The study was approved by the Ethics Committee of the Norwegian School of Sport Sciences (03-020517) and the Norwegian Centre for Research Data (53 552). Before study initiation, oral and written informed consents were obtained from all participants, and the study was conducted according to the Declaration of Helsinki.

2.2 | Experimental design

After six weeks of 1L-KE endurance training, the participants performed 2L-KE exercise at three different power outputs: $40 \pm 3\%$, $62 \pm 4\%$, and $83 \pm 4\%$ of 2L-KE $\dot{W}_{peak}$. Submaximal and near-maximal power outputs were chosen to ensure identical absolute power output produced by TL and the non-trained CON, although having a substantial difference in their training status and $\dot{W}_{peak}$ during 1L-KE. This was ensured using a real-time feedback system displaying the balance in power output produced by the two legs. LBF was measured, and blood samples were drawn simultaneously from catheters indwelling the femoral veins of both legs and from a femoral artery to measure leg O_2 extraction and to calculate leg $\dot{V}O_2$ and D_MO_2 (main experiment).

Before and after the training intervention, measurements of pulmonary $\dot{V}O_{2\,max}$ during cycling and pulmonary peak $\dot{V}O_2$ ($\dot{V}O_{2peak}$) during 1L-KE (both legs) and 2L-KE were conducted. Body composition was measured after overnight fasting (dual-energy X-ray absorptiometry; Lunar iDXA; GE Healthcare), a muscle biopsy from each thigh was sampled, and the quadriceps femoris muscle mass was estimated anthropometrically[23] and adjusted as proposed by Radegran.[24]

Before testing, several short familiarization sessions were conducted to ensure optimal technique during 1L-KE and 2L-KE. The subjects were asked to refrain from physical activity the day before testing and sampling of muscle biopsies.

2.3 | Exercise training

The subjects conducted supervised 1L-KE training 3-4 times per week over six weeks (21 sessions, 100% adherence). Training legs were counterbalanced between right and left legs among subjects. One session consisted of continuous exercise at 70% of $\dot{W}_{peak}$ and progressed from 35 to 65 minutes during the training period. Another session consisted of 10-min warm-up, 20 minutes "all-out" at the highest possible power output, and a cooldown of 5 minutes. The third session consisted of a 10-min warm-up, and 4-6 repetitions of 5 minutes at 85% of $\dot{W}_{peak}$, which were interspersed with recovery periods of 2 minutes and followed by a 5-min cooldown. From weeks 1 to 6, the total work performed increased from 197 ± 23 to 493 ± 54 kJ per week. The subjects were asked to maintain their regular training in addition to the supervised 1L-KE training, which was 2.9 ± 2.4 hours of endurance training and 1.0 ± 0.7 hours of strength training per week during the intervention period. Each subject recorded their training by reporting the training mode, duration, and intensity in a custom-made Excel spreadsheet provided by the investigators.

2.4 | Non-invasive physical tests

Pulmonary $\dot{V}O_{2\,max}$ was measured during cycling (Excalibur Sport; Lode BV, Groningen, The Netherlands) with step increments of 25 W $\cdot min^{-1}$ after 15-minutes warm-up. On a separate day, pulmonary $\dot{V}O_{2peak}$ and $\dot{W}_{peak}$ were measured during 1L-KE (both legs, counterbalanced order) and 2L-KE. Rest periods between legs and exercise modes were 15 and 45 minutes, respectively. The incremental tests began at 30 W or 60 W, after a 15-minutes warm-up, and were followed by step increments of 5 W·min^{-1} or 10 W·min^{-1} during 1L-KE and 2L-KE, respectively.

2.5 | Catheterization and preparation for the main experiment

The subjects reported to the laboratory at 8:00 or 14:00, and catheters were placed percutaneously under local anesthesia (2% lidocaine) using the Seldinger technique. Briefly, a 20-gauge catheter (Arrow ref. # ES-04150; Teleflex Medical) was placed into the right femoral artery, 2-5 cm below the inguinal ligament and advanced 8 cm in the proximal direction. Another two 20-gauge catheters were placed in the femoral veins, 2 cm below the inguinal ligament and advanced 8 cm in the proximal direction. Catheter tip placement was confirmed using ultrasound B mode, and all catheters were sutured to the skin. The catheters were used for arterial and venous blood sampling and were connected to blood pressure transducers (TruWave ref. # T450217A; Edward Lifesciences) positioned at the height of the parasternal fourth intercostal space. Via an amplifier (Gould Instrument Systems), blood pressures were sampled at 100 Hz using a PC with LabVIEW software (National Instruments).

2.6 | Blood sampling and analytic procedures

Blood was sampled anaerobically using heparinized syringes (safePICO; Radiometer) and immediately analyzed for hemoglobin concentration ([Hb]), hemoglobin O_2 saturation (SO_2), the tension of O_2 (PO_2) and CO_2 (PCO_2), pH, and lactate using an ABL90 FLEX (Radiometer). Blood O_2 content (ctO_2) was calculated as follows: $(1.34 \times [Hb] \times SO_2) + (0.003 \times PO_2)$. The in vivo $P_{50}O_2$ (the PO_2 at 50% SO_2 of Hb) was calculated from blood gas measurements

correcting for PCO_2 and pH according to Kelman.[25] Blood samples were taken simultaneously from both veins and the artery.

2.7 | Femoral arterial blood flow

LBF was measured in the femoral artery of both legs using ultrasound Doppler (Vivid E95; GE Vingmed Ultrasound AS) equipped with a linear transducer (9L-D; GE Vingmed Ultrasound AS) operating with an image frequency of 10 MHz and a Doppler frequency of 3.7-4.0 MHz. Blood velocity was measured in the common femoral artery distal to the inguinal ligament, but above the bifurcation into the superficial and profound femoral branches to avoid turbulence. The sample volume was maximized according to the vessel width but kept clear of the vessel walls. The insonation angle was minimized and always below 60°. A low-velocity filter was applied to reject noise created from turbulence at the vessel wall. The arterial diameter was determined during systole (mean of three heart cycles) on each exercise bout from B-mode images with the transducer parallel to the vascular walls. For all subjects, measurements were first conducted on the left leg, followed by the right leg: B-mode left, Doppler left, Doppler right, and B-mode right. Blood velocity was measured continuously and averaged over ~45 seconds. LBF was measured ~1.0-1.5 min earlier in the left leg compared with the right leg, but with no difference between the mean of TL and CON due to counterbalancing (see "Exercise protocol and timing of measurements" for more details). Recordings were anonymized before LBF was analyzed using software incorporated in the Vivid E95.

2.8 | Calculations

Arteriovenous O_2 difference (a-vO_2diff) was computed as the difference between femoral arterial and femoral venous ctO_2 for each leg. The a-vO_2diff divided by the arterial ctO_2 gave O_2 extraction. Leg O_2 delivery was calculated as the product of LBF and arterial ctO_2. Leg $\dot{V}O_2$ was the product of LBF and a-vO_2diff. Leg vascular conductance was calculated as LBF divided by the mean arterial pressure minus femoral venous pressure. Mean capillary PO_2 and the muscle O_2 conductance (D_MO_2) were calculated as previously described,[26,27] using the measured arterial and femoral venous PO_2. D_MO_2 [$\dot{V}O_2$/(mean capillary PO_2 – mitochondrial PO_2)] is recognized as a compound variable integrating several steps in the O_2 cascade, including the chemical dissociation of O_2 from Hb, and diffusion through the erythrocyte membrane, plasma, capillary wall, interstitial space, sarcolemma, cytoplasm (myoglobin-facilitated or by diffusion), and into the mitochondria for utilization by the cytochromes. The calculated D_MO_2 depends on the recruited exercise muscle mass,[5] F_IO_2, and exercise intensity.[2,28] Since the observed D_MO_2 is higher during cycling than 1L-KE (reversed if standardizing to the active muscle mass),[5,12] and also higher in hypoxia than normoxia;[2,28] we describe potential between-leg differences in D_MO_2 as differences in the "recruitment" of D_MO_2 rather than a difference in the maximal capacity.[28] The equilibration index Y [D_MO_2/(LBF·β)], which quantitatively describes perfusion vs diffusion limitations to $\dot{V}O_2$, was calculated according to Piiper and Scheid.[29] In this calculation, β is the mean slope of the O_2-Hb dissociation curve (ODC) and was calculated as $\dot{V}O_2$/[LBF·(arterial PO_2 – femoral venous PO_2)].[29]

2.9 | Pulmonary gas exchange

Pulmonary $\dot{V}O_2$ was measured using a metabolic cart with a mixing chamber (Oxycon Pro; Jaeger Instrument). Before each test, the gas analyzers and flow transducer (Triple V; Erich Jaeger GmbH) were calibrated according to the instruction manual.
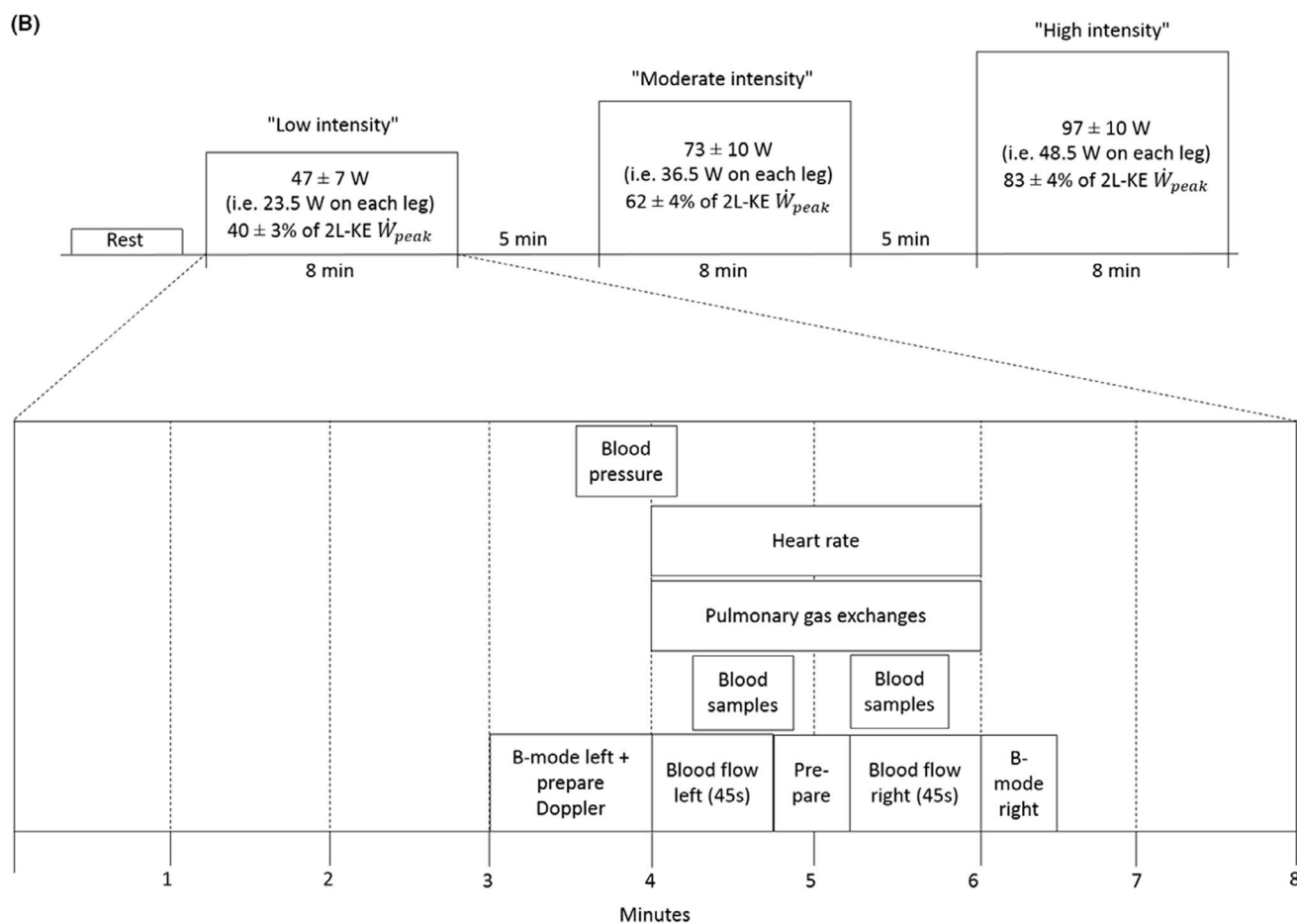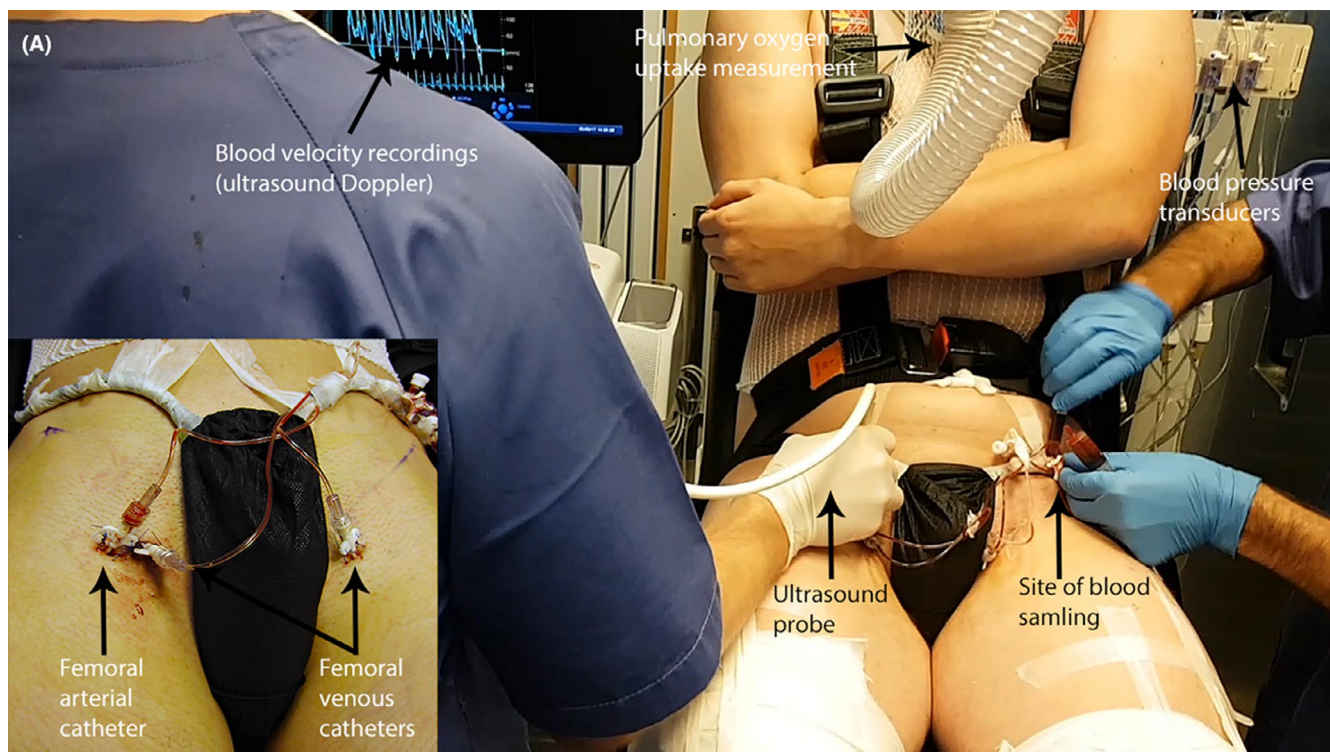
2.10 | Knee extension ergometer

Knee extension exercises were performed on an electromagnetically braked ergometer modified after Hallén.[30] Briefly, the ergometer isolates leg muscle contractions to quadriceps femoris during knee extensions.[31] The hip angle was ~128°, and the upper body was strapped using a four-point seat belt to minimize the engagement of muscle mass to stabilize the body. By adding a steel bar to the pedal arm on either one or both sides of the flywheel, both 1L-KE and 2L-KE could be conducted. The steel bars have an integrated telescope function and are shortened if the subject tries to produce any force on the flywheel in the recovery/knee-flexion phase, guaranteeing no hamstring contribution to the power output. Strain gauges were incorporated in the steel bars, and the angles of the pedal arms were continuously monitored. The work performed on the flywheel was calculated and recorded by custom-made software. During 2L-KE, the work expressed as the balance between the legs was displayed in real time on a monitor in front of the subjects to ensure equal involvement of both legs. In addition, the kicking frequency was displayed on the same monitor and was maintained at 60 rpm.

2.11 | Exercise protocol and timing of measurements (main experiment)

First, a baseline measurement was conducted after 10-minutes rest seated in the KE ergometer. Thereafter, three 8-minutes exercise bouts at low (47 ± 7 W; ie, 23.5 W by each

FIGURE 1 The full setup of a subject seated in the knee extension ergometer (A); and the exercise protocol and timing of measurements (B). These measurements were conducted after training only

leg), moderate (73 ± 10 W; ie, 36.5 W by each leg), and high (97 ± 10 W; ie, 48.5 W by each leg) intensity (40 ± 3%, 62 ± 4% and 83 ± 4% of 2L-KE $\dot{W}_{peak}$, respectively) were conducted, interspersed by 5-min passive rest periods (see Figure 1 for the full setup of a subject and the design and timing of measurements). Post-hoc analysis showed equal involvement of both legs during experiments (TL accounted for 50.3 ± 0.8%, 50.6 ± 0.8%, and 50.8 ± 1.3% of the power output at low, moderate, and high intensity, respectively). During each bout, the subjects used the first 1-2 minute(s) to fine-tune equal involvements of legs and to reach "steady state." After 3 minutes, B-mode imaging of the left femoral artery began, followed by Doppler measurements from precisely 4 minutes on the left leg and approximately from 5 minutes on the right leg. Two blood samples from each vessel were drawn during the Doppler measurements (~4.5 and ~5.5 minutes), and the duplicates were averaged. Blood pressures were calculated as ~30-seconds averages preceding the first blood sample. Heart rate was averaged from ECGs during Doppler measurements. At the end of each exercise bout, the subjects were asked to rate the perceived intensity for each leg separately using the CR-10 scale.[32] Exercising at low intensity elicited equal perceived intensity (2.3 ± 1.0 vs 2.3 ± 1.5; $P = 1.00$), while moderate (4.6 ± 1.8 vs 3.8 ± 2.1; $P = .008$) and high (8.6 ± 1.7 vs 6.9 ± 2.3; $P < .001$) intensity exercise elicited significantly higher perceived intensity in CON than in TL.

2.12 | Skeletal muscle biopsy

Muscle biopsies (~100-200 mg) were collected from the mid-portion of *vastus lateralis* after local anesthesia, using the Bergström technique with manual suction. The tissue was immediately dissected free from visible fat and connective tissue. An appropriate sample for immunohistochemistry was embedded in OCT embedding matrix (CellPath) and quickly frozen in isopentane cooled on liquid nitrogen to freezing point (approx. −120°C). Tissue allocated for Western blotting was immediately snap-frozen in liquid nitrogen. All tissue samples were stored at −80°C until further analysis.

2.13 | Immunohistochemistry

Serial 8-μm transverse cross sections were cut at −20°C (Leica CM1860 UV; Leica Biosystems), mounted on microscope slides (Superfrost Plus; Thermo Fisher Scientific), air-dried, and stored at −80°C until further analysis. The sections were blocked for 60 min with 10% goat serum (ab7481; Abcam) and 1% bovine serum albumin (Sigma Life Science) in a phosphate-buffered saline (Sigma Life Science) and 0.05% Tween-20 (VWR) solution (PBS-t). Primary

antibodies against (a) myosin heavy-chain type 1 (1:500 dilution; BA-D5, developed by Schiaffino, S., obtained from DSHB) and dystrophin (1:500; ab15277; Abcam), or (b) the endothelial marker CD31 (1:100; M0823; Dako A/S) and dystrophin were diluted in the blocking solution and incubated overnight at 4°C. The sections were then washed 3 × 10 minutes in PBS-t, incubated with secondary antibodies (1:200; Alexa Fluor 488, A11001 and A11012; Invitrogen Molecular Probes) for 60 minutes, and again washed 3 × 10 minutes in PBS-t before being mounted with Prolong Gold Antifade Reagent with DAPI (Life Technologies Corp.) and covered with a cover glass. The sections were visualized under a 10×/0.30 NA air objective (UplanFL N; Olympus corp.) and micrographed using a high-resolution digital camera (DP72; Olympus corp.) attached to a microscope (BX61; Olympus corp.) with a fluorescence light source (X-Cite 120 PC Q; EXFO Photonic Solution Inc). Fiber cross-sectional areas, fiber types, and manual identification of capillaries were conducted and analyzed using TEMA software (CheckVision). The investigator was blinded for the subject and leg identity. A mean of 189 ± 41 (range: 118-251) fibers was analyzed. Capillarization was expressed as the capillary-to-fiber ratio, capillaries in contact with each fiber, and capillary density (capillaries per mm^2).

2.14 | Protein immunoblot

For Western blotting analyses, ~60 mg of muscle tissue was homogenized in 1 mL T-PER (Tissue Protein Extraction Reagent, 78 510; Thermo Fisher Scientific) and 20 μL Halt Protease and Phosphatase Inhibitor Cocktail (78 440; Thermo Fisher Scientific). The tissue lysate was extracted, aliquoted, and stored at −80°C until further analyses. The protein concentration was measured using a commercial kit (Bio-Rad DC Protein Assay, 5 000 116; Bio-Rad Laboratories) and a FLUOstar Omega microplate reader (BMG Labtech). Standard Western blotting procedures were applied for quantification of citrate synthase, cytochrome-c-oxidase subunit 4 (COX-IV), and hydroxyacyl-CoA dehydrogenase (HAD) as surrogates indices of mitochondrial volume density.[33] 20μg of protein was separated by 4%-12% gradient Bis-Tris gels (Invitrogen, Life Technologies) for ~ 45 min at 200 volts in cold buffer (NuPage MES SDS Running Buffer; Invitrogen, Life Technologies). Proteins were subsequently transferred onto a PVDF membrane (Bio-Rad Laboratories) at 30 volts for 90 min in cold buffer (NuPage Transfer Buffer; Invitrogen, Life Technologies). Membranes were blocked at room temperature for 2 hours in a 5% fat-free skimmed milk (Merck) and 0.1% TBS-t solution (TBS: Bio-Rad Laboratories; Tween-20: VWR). Thereafter, the membranes were divided into pieces based on molecular weight (Protein Ladder 310 005; GeneON) and then incubated

overnight (4°C) with primary antibodies against citrate synthase (1:4000; ab96600; Abcam), COX-IV (1:2000; ab16056; Abcam), or HAD (1:8000; ab154088; Abcam). An anti-rabbit IgG (1:3000; 7074S; Cell Signaling Technology) secondary antibody was applied for 1 hour at room temperature followed by visualization using an HRP detection system (Super Signal West Dura Extended Duration Substrate; Thermo Fisher Scientific). An antibody against the loading control GAPDH (1:3000; ab9484; Abcam) was applied as a secondary probe after using Restore Western Blot Stripping Buffer (21 059; Thermo Fisher Scientific). All antibodies were diluted in a 1% fat-free skimmed milk and 0.1% TBS-t solution. Between steps, membranes were washed in 0.1% TBS-t and TBS solutions. Chemiluminescence was detected using the ChemiDoc MP system with band intensities quantified using Image Lab 5.1 software (Bio-Rad Laboratories). Pre-samples and post-samples of both legs were loaded on the same gel in duplicates using a counterbalanced order, and mean values were used for statistical analysis. In addition, a human control sample (a pool of all biopsies in the present project) was loaded in duplicate on each gel, and the average intensity of this sample was used for normalization to allow for semi-quantitative comparisons across gels/subjects.[34]

2.15 | Statistical analyses

Data in text and tables are presented as mean ± standard deviation (SD) and in graphs as mean ± standard error of the mean (SEM) if not otherwise stated. The data were initially assessed for normal distribution using the D'Agostino-Pearson test. Differences between legs during exercise and differences in protein content between pre-training and post-training were analyzed using two-way repeated-measures ANOVA (leg × power output and leg × time point, respectively). If a significant main effect or interaction effect was found, the ANOVA was followed by Bonferroni's multiple comparison post-hoc test. Differences between legs during rest and one-level changes from pre- to post-training were analyzed with a paired sample t test. The alpha level was set to <0.05, and values between ≥0.05 and ≤0.10 were considered to indicate trends. GraphPad Prism (v. 8.0.1; GraphPad Software) and Microsoft Office Excel 2013 (Microsoft Corporation) were used for statistical analysis.

3 | RESULTS

3.1 | Pulmonary gas exchange and muscle mass

Post-training, $\dot{W}_{peak}$ during 1L-KE was 15 ± 8% higher during incremental exercise to exhaustion with TL compared to CON ($P < .001$; Table 1). This was accompanied by 20 ± 11% longer time to exhaustion ($P < .001$), 22 ± 17% higher $\dot{V}O_{2peak}$ ($P = .004$), 31 ± 21% higher peak ventilation ($P = .002$), 7 ± 8% higher peak heart rate ($P = .026$), and 28 ± 12% higher post-exercise blood lactate concentration ($P < .001$). $\dot{V}O_{2peak}$ during 2L-KE tended to increase (4 ± 6%; $P = .059$), but $\dot{V}O_{2max}$ during cycling was unchanged from pre- to post-training ($P = .890$; Table 1).

Post-training, TL tended to have a larger thigh lean mass than CON (7.55 ± 0.81 kg vs 7.37 ± 0.70 kg; difference: 2.4 ± 3.3%; $P = .053$), but the quadriceps femoris muscle

TABLE 1 Peak values (mean ± SD) achieved during incremental exercise to exhaustion during one-legged knee extension (KE), two-legged KE, and cycling (n = 9)

Exercise	Pulmonary $\dot{V}O_{2peak}$ (L·min^{-1})	Peak power (watt)	Time to exhaustion (mm:ss)	VE$_{peak}$ (L·min^{-1})	HR$_{peak}$ (beats·min^{-1})	Lactate (mmol·L^{-1})
One-legged KE						
Control leg (post)	1.27 ± 0.15	56 ± 6	9:36 ± 1:16	50 ± 4	151 ± 17	4.8 ± 0.7
Trained leg (post)	1.54 ± 0.21*	64 ± 5*	11:24 ± 1:00*	65 ± 10*	160 ± 13*	6.0 ± 0.7*
Two-legged KE						
Pre-training	2.31 ± 0.38	113 ± 11	9:57 ± 1:10	93 ± 13	171 ± 9	7.2 ± 1.0
Post-training	2.40 ± 0.34#	116 ± 11*	10:55 ± 1:14*	100 ± 18	170 ± 9	7.8 ± 1.1
Cycling						
Pre-training	4.30 ± 0.37	354 ± 40	8:10 ± 0:53	182 ± 13	193 ± 10	14.8 ± 1.5
Post-training	4.31 ± 0.32	371 ± 35*	8:51 ± 0:43*	180 ± 11	194 ± 8	14.7 ± 1.4

Note: HR$_{peak}$, peak heart rate; VE$_{peak}$, peak ventilation; $\dot{V}O_{2peak}$, peak oxygen uptake (pulmonary); * significantly ($P < .05$) or # trend toward ($0.05 \leq P \leq .10$) difference between legs (one-legged KE) or between pre-training and post-training values (cycling and two-legged KE). $\dot{V}O_{2peak}$, peak power, and VE$_{peak}$ are 60-sec averages, and HR$_{peak}$ is 5-sec average.

FIGURE 2 Mass-specific leg blood flow (A); mass-specific O_2 delivery (B); arteriovenous O_2 difference (C); and mass-specific O_2 uptake (D) in the trained leg and the untrained control leg after six weeks of one-legged endurance training. Note that the trained leg and the control leg exercised simultaneously at equal absolute power output (PO). Embedded in the graphs are the ANOVA P-values indicating whether it was a main effect of the leg or an interaction effect between leg and PO. The data are means, and error bars denote SEM (n = 9). *Significant ($P < .05$) and #trend toward ($.05 \leq P \leq .10$) difference between legs (post-hoc comparisons)


mass did not differ (2.48 ± 0.38 kg vs 2.43 ± 0.37 kg; difference: $2.0 \pm 3.6\%$; $P = .138$).

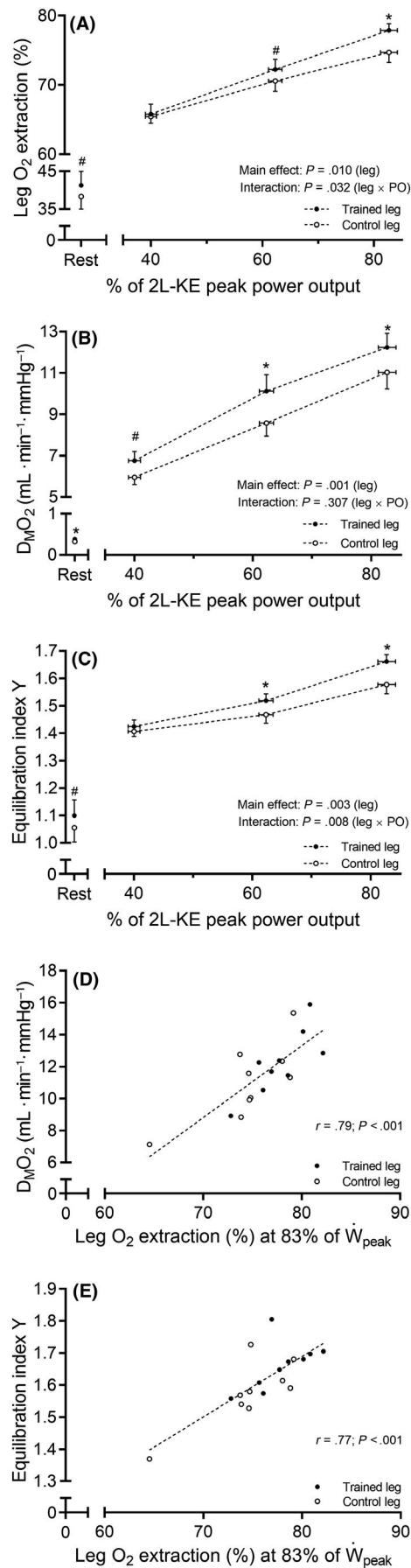## 3.2 | O_2 extraction in the trained leg and the control leg

At rest, O_2 extraction (difference: $2.9 \pm 4.7\%$ points; $P = .099$) and $\dot{V}O_2$ (difference: $18.7 \pm 23.0\%$; $P = .080$) tended to be higher in TL compared with CON (Figures 2-3), while LBF was similar (difference: $8.7 \pm 24.1\%$; $P = .611$). During 2L-KE with equal absolute power output produced by both legs, O_2 extraction was higher (difference: $1.7 \pm 1.6\%$ points; $P = .010$; Figure 3A), mass-specific LBF tended to be higher (difference: $7.1 \pm 9.5\%$; $P = .051$; Figure 2A), and mass-specific $\dot{V}O_2$ was slightly higher (difference: $9.8 \pm 9.3\%$; $P = .016$; Figure 2D) in TL compared with CON (the ANOVA main effects are given above; see Figures 2-3 for post-hoc tests). Significant interactions (leg × power output) were found for O_2 extraction ($P = .032$) and arterio-venous O_2 difference (a-vO_2diff; $P = .029$), with the largest training-induced between-leg difference at the highest power output ($3.2 \pm 2.2\%$ points and 7.1 ± 4.8 mL·L^{-1}, respectively; both $P < .001$). Moreover, the subjects with the lowest O_2 extraction in CON were those who had the largest between-leg difference at the highest power output ($r = -.81$; n = 9; $P = .008$), that is, the largest training effect. The increased O_2 extraction was not caused by a right-shifted ODC, since the $P_{50}O_2$ was slightly higher in CON ($P = .005$) due to a lower femoral venous pH (Table 2; $P = .007$).

3.3 | Muscle O_2 conductance

The larger O_2 extraction resulted in lower femoral venous PO_2 ($P = .004$) and calculated mean capillary PO_2 ($P = .003$) in TL than in CON (Table 2). According to the Fick law of diffusion, it can be estimated that the recruitment of D_MO_2 was $13.9 \pm 8.3\%$ larger in TL than in CON ($P = .001$; Figure 3B). The equilibration index Y, which quantitatively describes diffusion vs perfusion limitations (where Y < 0.1 indicates pure diffusion limitation, 0.1 < Y < 3 indicates
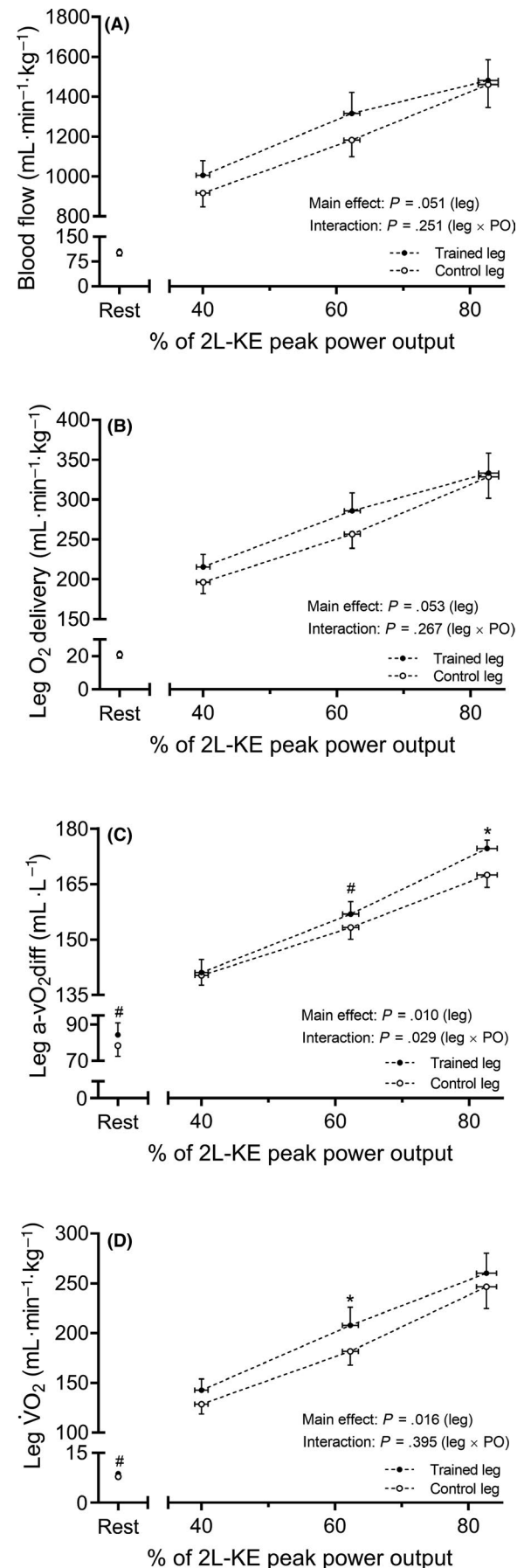
FIGURE 3 Leg O_2 extraction (A); recruitment of muscle O_2 conductance (D_MO_2) (B); and the equilibration index Y (C) in the trained leg and the untrained control leg after 6 weeks of one-legged endurance training. The equilibration index Y quantitatively describes diffusion vs perfusion limitations (where Y < 0.1 indicates pure diffusion limitation, 0.1 < Y < 3 indicates mixed perfusion-diffusion limitation, and Y > 3 indicates pure perfusion limitation), that is, the diffusion limitation is lower in the trained leg. The data are means, and error bars denote SEM (n = 9). *Significant ($P < .05$) and #trend toward ($0.05 \leq P \leq .10$) difference between legs (post-hoc comparisons). In graphs D and E, the relationships between leg O_2 extraction and the recruitment of D_MO_2 and the equilibration index Y are displayed, respectively

mixed perfusion-diffusion limitation, and Y > 3 indicates pure perfusion limitation),[29] was higher in TL (mean values for 40%-83% of $\dot{W}_{peak}$: 1.53 ± 0.07 vs 1.48 ± 0.08; $P = .003$; Figure 3C) and increased more with power output than in CON (values at 83% of $\dot{W}_{peak}$: 1.66 ± 0.08 vs 1.58 ± 0.10; interaction: $P = .008$; Figure 3C). Therefore, the resistance to O_2 diffusion from the capillaries to cytochrome-c-oxidase was lower in TL than in CON, and the between-leg difference increased with power output. Furthermore, at the highest power output, leg O_2 extraction correlated with the recruitment of D_MO_2 and the equilibration index Y (both $P < .001$; Figure 3D-E).

3.4 | Skeletal muscle adaptations

Before training, none of the analyzed mitochondrial enzymes differed between legs ($P \geq .56$). After training, TL expressed a higher protein content of citrate synthase ($45 \pm 29\%$; $P < .001$), COX-IV ($44 \pm 46\%$; $P = .044$), and HAD ($35 \pm 28\%$; $P = .007$; Figure 4). After training, the capillary-to-fiber ratio was $18 \pm 23\%$ ($P = .031$) higher in TL than in CON and the capillary density tended to be higher ($12 \pm 17\%$; $P = .067$; Table 3).

3.5 | Relationships between muscle morphology and in vivo measurements

At the highest power output (83% of $\dot{W}_{peak}$), the between-leg difference in O_2 extraction correlated with the between-leg ratio of citrate synthase ($r = .72$; n = 9; $P = .028$) and COX-IV ($r = .73$; n = 9; $P = .024$), as well did the two enzymes combined into an average ($P = .010$; n = 9; Figure 5A). When the mitochondrial protein contents were standardized to a human control sample, to allow for comparisons across subjects, COX-IV ($r = .57$; n = 18; $P = .013$), citrate synthase ($r = .47$; n = 18; $P = .049$), and their average correlated with leg O_2 extraction ($P = .011$; n = 18; Figure 5C). The average of citrate synthase and COX-IV standardized to a human control

sample also showed a close relationship with the recruitment of $D_M O_2$ at 83% of $\dot{W}_{peak}$ ($r = .74$; n = 18; $P < .001$). Leg O_2 extraction correlated with the capillary-to-fiber ratio ($P = .029$; n = 18; Figure 5D). However, no relationship was evident when the between-leg difference in capillary-to-fiber ratio was plotted against the between-leg difference in oxygen extraction ($P = .965$; n = 9; Figure 5B), potentially indicating a more important role of the increased citrate synthase and COX-IV protein content than increased capillary-to-fiber ratio on the improved O_2 extraction capacity.

4 | DISCUSSION

In the present study, we show that the primary mechanism by which endurance training increases O_2 extraction during small muscle mass exercise is by elevating the muscle oxidative capacity, which facilitates a more efficient O_2 diffusion from the capillaries to the mitochondria. This was especially evident during high-intensity exercise that exploits a large fraction of the muscle oxidative capacity. In support, significant between-leg differences in mitochondrial enzyme contents (35%-45%) were observed after the training period, which was associated with the between-leg difference in O_2 extraction during high-intensity exercise. The recruitment of $D_M O_2$ and the equilibration index Y were higher in the trained leg than in the control leg, indicating reduced resistance to O_2 diffusion, with these variables also displaying strong relationships with leg O_2 extraction.

4.1 | Endurance training increases O_2 extraction

During maximal exercise with a small muscle mass (eg, 1L-KE), the mass-specific blood flow and $\dot{V}O_2$ are high. Consequently, the oxidative capacity of the exercising muscles is close to being fully exploited,[14] which may restrict O_2 diffusion from the capillaries to cytochrome-c-oxidase.[5] Therefore, we hypothesized that elevating the muscle oxidative capacity with endurance training would enhance the recruitment of $D_M O_2$ and increase O_2 extraction when exercising with a small muscle mass. In support of this hypothesis, the O_2 extraction was significantly higher in TL than in CON during 2L-KE exercise at high, but not at low power outputs. Importantly, the two legs were exercising with the same power outputs, indicating that the between-leg difference was exclusively caused by the difference in training status. A difference in leg O_2 extraction at high but not at low power outputs has been reported after one-legged training before[17] and indicates that the effect of training on O_2 extraction is intensity-dependent and most evident when most of the muscle oxidative capacity is exploited.

Previous studies assessing the trained and non-trained legs separately during 1L-KE have reported increased[9,10,35,36] or unchanged[13,37,38] leg O_2 extraction after endurance training. Most of the studies reporting no improvement of O_2 extraction employed exercise protocols designed to assess vascular function at low-to-moderate intensities.[13,38] This agrees with our findings since no significant enhancement of O_2 extraction was observed at low and moderate exercise intensities also in the present study. Similarly, Klausen and coworkers found an unchanged leg a-vO$_2$diff during submaximal exercise but found a 7 mL·L^{-1} increase during maximal one-legged cycling after 8 weeks of one-legged endurance training.[39] The latter concurs with the study by Boushel and coworkers in which maximal exercise arm O_2 extraction was raised from 62% to 68% after low-intensity training.[15] During arm cycling, the active muscle mass is small (~6 kg) and the mass-specific blood flow is large.[40] Thus, our results combined with previous studies strongly suggest that O_2 extraction during small muscle mass exercise is improved after endurance training, particularly when the exercise intensity is close to maximal.

We expected a larger between-leg difference in O_2 extraction when TL and CON were assessed simultaneously during 2L-KE compared with during two-legged cycling because of the more limited muscle oxidative capacity and lower O_2 extraction in this exercise model.[5] However, a similar pattern was observed in the present study and Rud et al (2012), with no difference at low-intensity exercise, a tendency to difference at moderate-intensity exercise, and a significant difference at high-intensity exercise.[17] The between-leg differences in O_2 extraction (3.2% vs 3.5% points) and a-vO$_2$diff (7.1 vs 6.6 mL·L^{-1}) were also almost identical in the two studies, indicating no amplifying effect when comparing trained and untrained muscles simultaneously during small vs large muscle mass exercise. However, the total training volume was larger in Rud et al (2012) than in the present study (4 sessions·week^{-1} over 7 weeks, with the session duration progressively increasing from 40 to 100 minutes), and without an assessment within the same subjects after similar training, no definite conclusion can be given.

The effect of endurance training on O_2 extraction during large muscle mass exercise remains controversial[14,17,18,41] since the muscle oxidative capacity is far from being fully utilized and is therefore not considered a limiting factor for muscle O_2 extraction and $\dot{V}O_{2\,max}$.[14] This agrees with a recent meta-analysis reporting no significant change in systemic a-vO$_2$diff after endurance training, as calculated using the Fick equation from the measured $\dot{V}O_{2\,max}$ and $\dot{Q}_{max}$ (mostly estimated from non-invasive methods).[16] In contrast, some experimental data indicate otherwise when O_2 extraction is calculated directly from a-vO$_2$diff in the exercising limb (arterial and venous catheters). For example,

TABLE 2 Blood variables during two-legged knee extension exercise with the trained (TL) and the control (CON) leg working at the same absolute power output (mean ± SD)

| Variable	Rest	Low (40 ± 3% of 2L-KE $\dot{W}_{peak}$)	Moderate (62 ± 4% of 2L-KE $\dot{W}_{peak}$)	High (83 ± 4% of 2L-KE $\dot{W}_{peak}$)	ANOVA main (leg) and interaction effects
Hb (g·dL^{-1})					
Femoral artery	15.5 ± 0.5	16.2 ± 0.5	16.4 ± 0.5	16.8 ± 0.6	
Femoral vein CON	15.8 ± 0.4	16.1 ± 0.5	16.4 ± 0.6	17.0 ± 0.7	Main: $P = .719$
Femoral vein TL	15.7 ± 0.5	16.1 ± 0.6	16.4 ± 0.6	16.9 ± 0.6	Interaction: $P = .829$
SO$_2$ (%)					
Femoral artery	97.5 ± 0.7	97.6 ± 0.7	97.7 ± 0.7	98.2 ± 0.5	
Femoral vein CON	59.7 ± 10.2	33.9 ± 3.0	28.8 ± 4.8	24.7 ± 4.3	Main: $P = .018$
Femoral vein TL	57.3 ± 11.7	33.6 ± 4.1	27.2 ± 4.4#	21.6 ± 2.8*	Interaction: $P = .029$
PO$_2$ (mmHg)					
Femoral artery	92.8 ± 4.1	96.2 ± 3.1	97.1 ± 2.8	105.7 ± 3.9	
Femoral vein CON	32.6 ± 4.3	23.6 ± 1.6	22.4 ± 2.1	21.9 ± 2.2	Main: $P = .004$
Femoral vein TL	31.2 ± 4.1	23.2 ± 2.0	21.3 ± 1.7*	20.1 ± 1.5*	Interaction: $P = .013$
ctO$_2$ (ml·L^{-1})					
Femoral artery	205.8 ± 8.1	214.4 ± 7.9	217.3 ± 7.3	224.4 ± 7.9	
Femoral vein CON	127.4 ± 23.4	74.1 ± 7.0	64.0 ± 10.5	56.9 ± 10.4	Main: $P = .010$
Femoral vein TL	121.5 ± 25.7#	73.3 ± 9.5	60.4 ± 10.2#	49.7 ± 7.4*	Interaction: $P = .030$
P$_{50}$O$_2$ (mmHg)					
Femoral vein CON	27.5 ± 0.4	29.6 ± 0.7	30.8 ± 1.2	32.8 ± 1.7	Main: $P = .005$
Femoral vein TL	27.5 ± 0.5	29.4 ± 0.7*	30.3 ± 1.1*	32.5 ± 1.6*	Interaction: $P = .251$
Mean cap. PO$_2$ (mmHg)					
CON	57.4 ± 3.3	51.7 ± 2.2	50.9 ± 2.1	53.2 ± 2.5	Main: $P = .003$
TL	56.1 ± 3.0#	51.3 ± 2.5	49.9 ± 2.0*	51.6 ± 2.1*	Interaction: $P = .008$
pH					
Femoral artery	7.42 ± 0.01	7.40 ± 0.02	7.40 ± 0.02	7.39 ± 0.03	
Femoral vein CON	7.38 ± 0.01	7.31 ± 0.02	7.28 ± 0.04	7.21 ± 0.05	Main: $P = .007$
Femoral vein TL	7.38 ± 0.02	7.32 ± 0.02*	7.29 ± 0.03*	7.22 ± 0.05*	Interaction: $P = .208$
Lactate (mmol·L^{-1})					
Femoral artery	0.6 ± 0.2	1.5 ± 0.6	3.2 ± 1.2	8.0 ± 1.8	
Femoral vein CON	0.8 ± 0.2	2.0 ± 0.8	4.5 ± 1.7	11.0 ± 2.6	Main: $P = .005$
Femoral vein TL	0.9 ± 0.2	1.7 ± 0.7*	3.9 ± 1.6*	10.4 ± 2.2*	Interaction: $P = .242$

Note: n = 9; the main effect indicates the difference between legs, and the interaction effect is between legs and power output (two-way repeated-measures ANOVA). Post-hoc tests identify significant (*$P < .05$) or trend toward (#$0.05 \leq P \leq .10$) differences between legs within each workload. A paired sample t test analyzed the difference between legs during rest.

Roca and coworkers[18] found that leg O$_2$ extraction increased from 72% to 82% during maximal cycling after sedentary subjects underwent nine weeks of intense endurance training ($\dot{V}O_{2\,max}$ rose from 37 to 51 mL·kg^{-1}·min^{-1}). After several years of training, leg O$_2$ extraction is reported to be as high as 91%-93% in competitive cyclists and cross-country skiers ($\dot{V}O_{2\,max}$: 65-72 mL·kg^{-1}·min^{-1}).[27,42] Thus, even though conflicting evidence exists on whether systemic a-vO$_2$diff increases after short-term endurance training during large muscle mass exercise, compelling evidence suggests that the O$_2$ extraction increases when measured

directly with arterial and venous blood sampling in the exercising limbs.[15,17,18,21,39,43]

4.1.1 | Oxidative capacity of the leg

In a previous study,[17] increased leg O$_2$ extraction coincided with increased muscle oxidative capacity (citrate synthase activity) after endurance training. Likewise, muscle oxidative capacity is shown to be important for O$_2$ extraction in the isolated rat hind limb, especially when pump-perfused
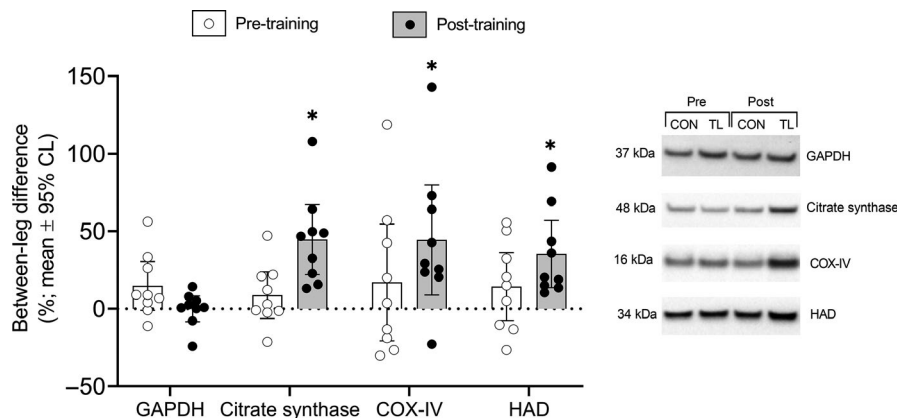
FIGURE 4 The protein content of the mitochondrial enzymes citrate synthase, cytochrome-c-oxidase subunit 4 (COX-IV), and hydroxyacyl-CoA dehydrogenase (HAD) along with the loading control glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The individual and mean values are presented as percentage between-leg differences (trained leg – control leg) at pre-training and post-training (n = 9). Error bars denote 95% confidence limits (CL). *Significant difference ($P < .05$) between legs at post-training, that is, at the time of the invasive experiment. Representative blots of the four proteins along with their observed molecular weights are presented

TABLE 3 *Vastus lateralis* muscle composition in the trained leg and the control leg post-training (mean ± SD)

| Variable	Control leg	Trained leg	P-value
Fiber size (µm²)			
All fibers	4954 ± 1063	5187 ± 1167	.396
Type I	4510 ± 1156	4870 ± 1050	.315
Type II	5245 ± 1321	5487 ± 1134	.394
Fiber type (%)			
Type I	41 ± 7	45 ± 10	.212
Type II	59 ± 7	55 ± 10	.212
Capillaries around a fiber			
All fibers	3.1 ± 0.5	3.5 ± 0.8	.122
Type I	3.3 ± 0.5	3.6 ± 0.7	.170
Type II	3.1 ± 0.5	3.5 ± 0.8	.138
Capillaries/mm²	280 ± 59	311 ± 75	.067
Capillary-to-fiber ratio	1.35 ± 0.26	1.58 ± 0.33	.031

Note: n = 9.

with high muscle blood flow and O_2 delivery[20,44,45] that mimic the situation observed during small muscle mass exercise in humans. In the present study, the between-leg difference in mitochondrial enzymes (citrate synthase, COX-IV, and the average of these two markers) significantly correlated with the difference in O_2 extraction. Therefore, it is likely that the enhanced muscle oxidative capacity in TL was the primary contributing factor to the improved O_2 extraction, particularly since the between-leg difference in capillary density was similar to that of LBF,

indicating that the erythrocyte capillary mean transit time (MTT) was similar in both legs.

It has been suggested that the surface area between the capillaries and the myocytes is of particular importance for D_MO_2 and O_2 extraction.[46] Therefore, the increased capillary-to-fiber ratio may have played a role in increasing the O_2 extraction. However, new evidence suggests that the muscle possesses a functional reserve in D_MO_2 that is utilized when breathing hypoxic gas during incremental exercise to exhaustion on the cycle ergometer[28] and the 1L-KE ergometer.[2] These studies indicate that greater D_MO_2 can be achieved with the same capillary-to-fiber ratio during exercise in hypoxia, meaning that the capillary-to-fiber ratio is not the predominant factor limiting D_MO_2 in healthy men. This interpretation is further supported by our current findings since the between-leg difference in the capillary-to-fiber ratio was not associated with the between-leg difference in O_2 extraction (Figure 5B). Most likely, it is the interplay between muscle oxidative capacity, mitochondrial p50, the tissue O_2 consumption (and by extension, the mitochondrial activation), MTT, perfusion-metabolism matching, and O_2 delivery that establishes the ceiling for O_2 extraction.[5,27,28,47] Moreover, the subsarcolemmal mitochondrial population increases relatively more than the intermyofibrillar population after endurance training.[48,49] Although this population is assumed to be more important for the ATP demands of membrane ion and substrate transporters than for the myofibrils, increased subsarcolemmal mitochondrial clusters may amplify the O_2 concentration gradient and thus O_2 diffusion across the sarcolemma.[50,51] It cannot be excluded that such an adaptation may have contributed to the reduced resistance to O_2 diffusion and increased O_2 extraction in TL.

In contrast, a few studies have challenged the idea that the oxidative capacity of the muscles is important for O_2 extraction in humans. For example, after bed rest, systemic O_2 extraction
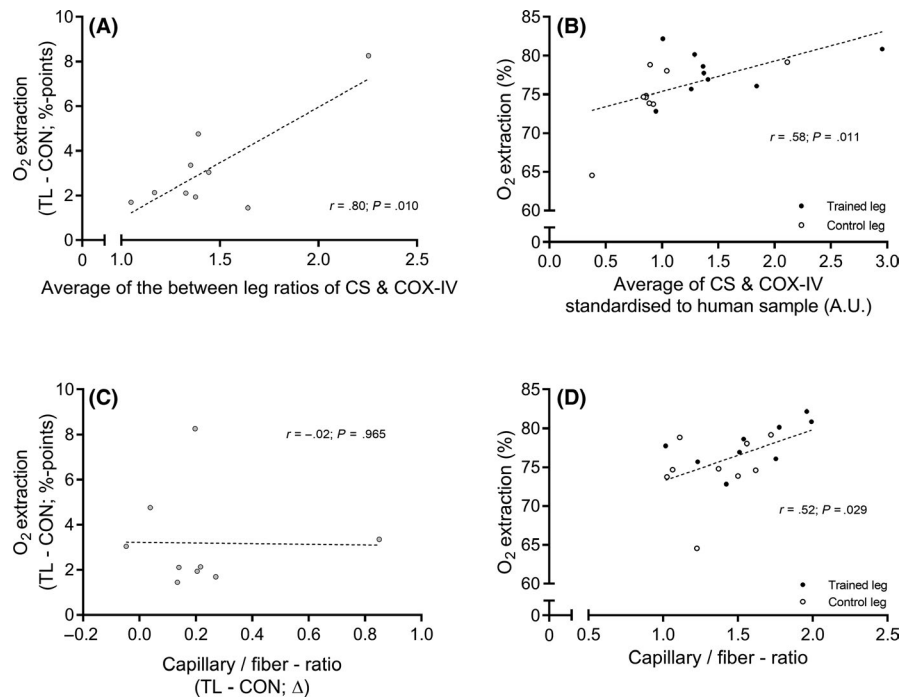
FIGURE 5 Data are from the highest power output. The relationship between the between-leg difference in O_2 extraction and the between-leg ratio of mitochondrial protein content (presented as an average of the between-leg ratios of CS and COX-IV: $[(CS_{TL}/CS_{CON}) + (COX\text{-}IV_{TL}/COX\text{-}IV_{CON})]/2$; Figure 5A) or the between-leg difference in the capillary-to-fiber ratio (Figure 5B). In Figure 5C, the mitochondrial protein content was standardized to a human control sample, which was loaded on each gel to allow for between-subject comparisons, and the individual values (presented as arbitrary units; AU) were plotted against O_2 extraction. In Figure 5D, the capillary-to-fiber ratio is plotted against O_2 extraction. CS, citrate synthase; COX-IV, cytochrome-c-oxidase subunit 4; TL, trained leg; CON, control leg

is maintained during whole-body exercise even though the muscle oxidative capacity and $\dot{V}O_{2\,max}$ are substantially reduced.[52,53] Therefore, it is argued that the muscles have a substantial reserve of oxidative capacity and that $\dot{V}O_{2\,max}$ is almost exclusively limited by O_2 delivery.[14] This might be true during large muscle mass exercise but is not directly transferable to small muscle mass exercise, where this oxidative reserve capacity is exploited.[7] Nevertheless, even during large muscle mass exercise, muscle $\dot{V}O_2$ is multifactorial.[54,55] In this context, by decreasing $\dot{Q}_{max}$ as a result of bed rest, the decreased blood flow to the exercising muscles would prolong capillary MTT substantially since bed rest only causes a minor change in the capillary density,[52,53] ultimately favoring Hb-O_2 off-loading that potentially compensates for the detrimental effects of reduced mitochondrial volume density. The same response can be seen when decreasing $\dot{Q}_{max}$ and LBF during maximal exercise with β-adrenergic blockade, which increases systemic and leg a-vO_2diff through increased MTT.[56,57] Therefore, training-induced improvements in whole-body $\dot{V}O_{2\,max}$ in parallel with increased $\dot{Q}_{max}$ and muscle oxidative capacity may not always result in improved O_2 extraction if MTT is substantially reduced caused by elevated muscle blood flow. This is substantiated by the positive relationship between the ratio of OXPHOS/ O_2 delivery and the leg O_2 extraction,[5] meaning that the balance between muscle oxidative capacity and blood flow (ie, oxidative

capacity and MTT) is more critical for O_2 extraction than any of these factors alone.

4.1.2 | Kinetics of O_2 off-loading from Hb

The off-loading of O_2 from Hb at the tissue level is facilitated by increased temperature, decreased pH, and increased PCO_2, which causes a right shift in the ODC and lowers the Hb-O_2 affinity.[58] Hence, a close relationship between O_2 extraction and $P_{50}O_2$ has been found in humans during exercise.[27] Moreover, increased O_2 extraction and $\dot{V}O_{2\,max}$ were found in pump-perfused dog muscle when the ODC was right-shifted by artificially elevating $P_{50}O_2$ from its normal value 32 to 53 mmHg.[59] In the present study, $P_{50}O_2$ increased alongside with O_2 extraction when increasing the power output in both legs, indicating that elevated $P_{50}O_2$ contributed to more efficient O_2 off-loading and O_2 extraction during high-intensity exercise. However, the right-shifted ODC was less pronounced in TL than in CON, excluding $P_{50}O_2$ as an explanatory factor for the improvement of O_2 extraction. In this context, for a given $P_{50}O_2$, TL was, however, able to extract more O_2 (Figure 6), likely because of the enhanced muscle oxidative capacity and larger recruitment of D_MO_2.
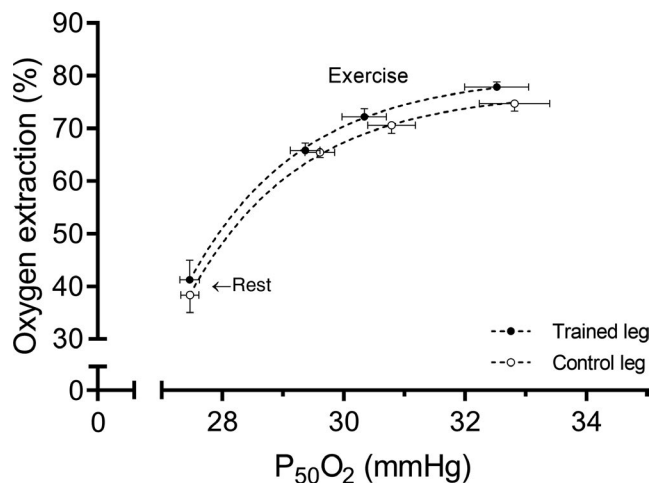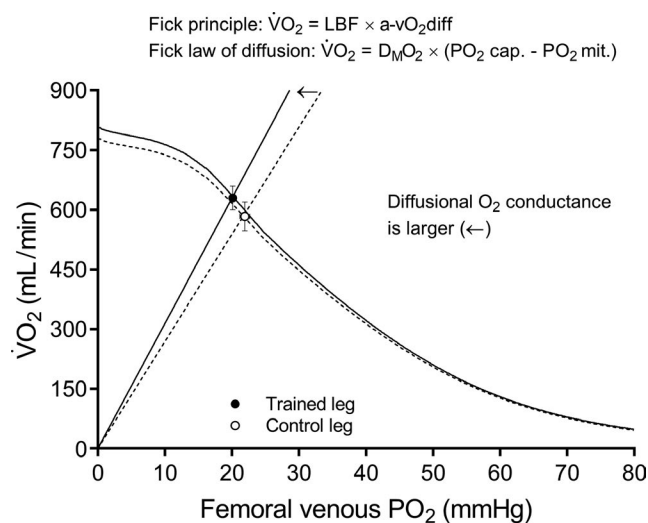
FIGURE 6 O_2 extraction during rest and exercise as a function of the partial pressure of O_2 that causes hemoglobin to be 50% saturated ($P_{50}O_2$). Note that a high $P_{50}O_2$ is beneficial for O_2 off-loading and that the trained leg had the highest O_2 extraction despite having a lower $P_{50}O_2$ than the control leg

FIGURE 7 The $\dot{V}O_2$ in the trained leg and the control leg is plotted as a function of femoral venous PO_2 at 83% of peak power output (filled symbols). The sigmoid curves represent the $\dot{V}O_2$ (the trained and the control legs are illustrated by a whole line or a dotted line, respectively) determined by the Fick principle (LBF × a-vO_2diff). The lines from the origin through the data points represent the Fick law of diffusion. Note that a larger diffusional O_2 conductance ($\leftarrow$) is recruited in the trained leg compared with the control leg. $\dot{V}O_2$, oxygen uptake; LBF, leg blood flow; a-vO_2diff, arteriovenous O_2 difference; D_MO_2, muscle O_2 conductance; PO_2, the partial pressure of O_2; PO_2 cap., mean capillary PO_2; PO_2 mit., PO_2 of the mitochondria

4.1.3 | Oxygen diffusion was more efficient in the trained leg

The lower femoral venous PO_2 and mean capillary PO_2 were achieved through enhanced O_2 extraction and resulted in higher recruitment of D_MO_2 in TL (Figure 7). In this context, by applying the Piiper and Scheid model, we calculated the equilibration index Y, which quantitatively describes diffusion vs perfusion limitations.[29] The equilibration index Y increased with power output in both legs, indicating that perfusion and diffusion limitations were rising and decreasing, respectively, as power output was elevated. Interestingly, the equilibration index Y increased more with power output in TL (Figure 3C), indicating that the training-induced reduction in diffusion limitations was more marked during high-intensity exercise. Thus, it seems that the benefit from increasing the muscle oxidative capacity is more apparent when the mitochondria operate closer to their maximal oxidative capacity. In agreement, a significant correlation between the protein content of mitochondrial enzymes (the average of citrate synthase and COX-IV) and the recruitment of D_MO_2 ($r = .74$; $P < .001$) and the equilibration index Y ($r = .50$; $n = 18$; $P = .034$) was observed at the highest power output, potentially allowing for the maintenance of an efficient O_2 diffusion and O_2 extraction even in the most distal capillary regions with the lowest PO_2 gradients.

4.2 | Blood flow and O_2 delivery

LBF and O_2 delivery were slightly higher in TL than in CON, and although this effect was not statistically significant, it deserves some attention. The release of ATP from

erythrocytes acts as a potent vasodilator via purinergic receptors in vascular endothelial cells, ultimately leading to nitric oxide and prostacyclin formation, followed by smooth muscle cell relaxation.[60] It has been demonstrated that erythrocyte ATP release is inhibited in the presence of high lactate concentration[61] and accelerated by low PO_2.[62] Thus, the lower femoral venous PO_2 and lower lactate concentration during exercise in TL may have facilitated larger ATP-mediated vasodilation compared with CON, which could have facilitated the slightly higher LBF. The fact that LBF was very similar at the highest exercise intensity may question these speculations. However, sympathetic vasoconstrictor activity is higher at high-intensity exercise and may dominate over ATP-mediated vasodilation, even if exercise training also has been shown to improve functional sympatholysis.[13,63] However, to study the mechanisms regulating skeletal muscle blood flow was beyond the scope of the present investigation.

Other researchers have reported no change or a small reduction in submaximal LBF after one-legged training.[9,13,39] In most of these studies, the effect of training has been assessed separately in the trained leg and the non-trained control leg, using the 1L-KE or one-legged cycling models. At submaximal exercise intensities, decreased heart rate, mean arterial pressure, and, hence, perfusion pressure have

been found when exercising after training.[9,13,39] This is most likely caused by a reduced influence of the metaboreflex and therefore a reduced sympathetic activation during submaximal exercise after training. Decreased perfusion pressure has been accompanied by increased vascular conductance.[37,39] In the present study, both legs were exercised simultaneously and therefore had the same perfusion pressure resulting in a slightly higher LBF in TL due to a larger vascular conductance (data not presented). To our knowledge, only three previous investigations have assessed the hemodynamic response while exercising with one trained leg and one control leg simultaneously,[17,21,22] of which only one of these investigations balanced the power output between legs.[17] In this study, no difference in LBF was found at low-to-moderate power outputs, but it was found a tendency to higher LBF and a significantly higher leg a-vO$_2$diff in TL at high exercise intensity, in agreement with our results.

In conclusion, the present study confirms that endurance training increases O$_2$ extraction during exercise involving a small muscle mass in humans. This was achieved primarily through increased muscle oxidative capacity and larger recruitment of D$_M$O$_2$ that reduced the restrictions on O$_2$ diffusion from the capillaries to the mitochondria. The increased capillary-to-fiber ratio may also have played a secondary role in increasing the O$_2$ extraction by increasing the surface area between the capillaries and the myocytes.

5 | PERSPECTIVE

A major aim of endurance training is to enhance exercise capacity by improving O$_2$ utilization, which can be achieved by acting on the principal limiting factors. The present findings indicate that a small muscle mass exercise (knee extension, in the present study) can efficiently improve exercise capacity and O$_2$ extraction with training by a mechanism tightly connected to enhancement of mitochondrial proteins (here used as a proxy of mitochondrial volume). This effect seems more important than the accompanying change in the capillary-to-fiber ratio. Of particular interest, increased muscle oxidative capacity did not translate into improved O$_2$ extraction during low-intensity exercise, that is, when the MTT is longer. At higher exercise intensities which are associated with greater mitochondrial activation and lower time for Hb-O$_2$ off-loading, the benefit from an endurance training-induced increase in muscle oxidative capacity on O$_2$ extraction is most prominent. Our results indicate that to enhance exercise capacity during small muscle mass exercise, increased mitochondrial oxidative capacity is likely mandatory in subjects with low-to-moderate fitness levels. This has important implications for people training while taking mitochondrion-toxic drugs such as metformin or statins, which may blunt the expected mitochondrial biogenesis.

CONFLICT OF INTEREST
The authors declare no conflict of interest, financial or otherwise.

AUTHOR CONTRIBUTIONS
Ø.S., JH, CC, and BR conceived and designed the experiment. Ø.S., JH, CC, JALC, and MA collected the data. Ø.S., BR, and MA analyzed the data. Ø.S., JH, CC, and JALC interpreted the data. Ø.S. wrote the first draft. All authors revised and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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