DISSERTATION FROM THE
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Øyvind Skattebo

The importance of oxygen extraction and blood volume for maximal oxygen uptake

NIH NORWEGIAN SCHOOL OF SPORT SCIENCES

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Summary

This thesis includes four research papers, based on four separate studies aiming to elucidate the importance of O_2 extraction and blood volume (BV) for maximal O_2 uptake ($\dot{V}O_{2max}$).

In study I, twelve untrained subjects ($\dot{V}O_{2max}$: 44 ml · kg^{-1} · min^{-1}) completed ten weeks of supervised endurance training (three sessions per week). $\dot{V}O_{2max}$ and maximal cardiac output ($\dot{Q}_{max}$) were measured during upright and supine cycling before and after training, as well as immediately after the training-induced gain in BV was reversed by blood withdrawal. The supine position increases venous return to the heart and may thus counteract potential adverse effects of blood withdrawal. The BV increased by 4% (~2 dl) with training. After reversing BV to the pre-training level, $\dot{V}O_{2max}$ and $\dot{Q}_{max}$ remained 11% and 9% higher than before training, respectively, regardless of exercise position. By using the Fick principle ($\dot{V}O_2 = \dot{Q} \times a\text{-}\bar{v}O_2diff$), it was calculated that 30% and 70% of the increase in $\dot{V}O_{2max}$ was attributed to increased O_2 content difference between arterial and mixed venous blood (a-$\bar{v}O_2$diff) and increased $\dot{Q}_{max}$, respectively. These improvements coincided with increased protein content of mitochondrial enzymes, a small increase in the capillary-to-fibre ratio (m. vastus lateralis) and an increased left ventricular mass (echocardiography). Thus, $\dot{V}O_{2max}$ may increase with endurance training independent of BV expansion, caused by combined central and peripheral adaptations.

In study II, thirteen subjects ($\dot{V}O_{2max}$: 63 ml · kg^{-1} · min^{-1}) performed maximal exercise on a cycle ergometer in three experimental conditions: with normal BV and immediately after acute BV reductions of 150 ml and 450 ml, representing 2.5% and 7.6% of the total BV (6.0 l), respectively. After the 150 ml reduction, $\dot{V}O_{2max}$ was preserved compared with the control test (non-significant reduction of 1%), likely caused by a rapid plasma volume (PV) restoration (calculated from changes in haematocrit and haemoglobin concentration). After the 450 ml BV reduction, $\dot{V}O_{2max}$ was reduced by 7% despite partial PV restoration, increased maximal heart rate and increased leg O_2 extraction as indicated by near-infrared spectroscopy. The reduction in $\dot{V}O_{2max}$ was 2.5-fold larger after withdrawing 450 compared with 150 ml blood after normalising to the BV removed. Therefore, the body may cope with small but not moderate blood loss to preserve $\dot{V}O_{2max}$. These data may enhance our understanding regarding the impact of, e.g., acute BV manipulations, PV reduction following dehydration induced by prolonged exercise or hyperthermia, or daily oscillations of PV.

In study III, the muscle oxidative capacity in one leg was increased by six weeks of one-legged endurance training (3-4 sessions per week) in nine subjects ($\dot{V}O_{2max}$: 56 ml · kg^{-1} · min^{-1}). The impact on leg O_2 extraction fraction (arterial and femoral venous catheters) vs the untrained control leg was investigated during dynamic two-legged knee extension exercise with both legs performing the same power output. This exercise model involves a small muscle mass, does not tax $\dot{Q}_{max}$ and is thus not perfusion limited. Therefore, the muscle oxidative capacity may potentially be the principal limiting factor for O_2 extraction and $\dot{V}O_2$ before training. At low to moderate exercise intensities, O_2 extraction fraction was similar in both legs. At higher exercise intensities, which are associated with greater mitochondrial activation and lower time for haemoglobin-O_2 off-loading, the O_2 extraction fraction was increased in the trained leg. The between-leg difference in O_2 extraction correlated with the between-leg difference in mitochondrial protein content (m. vastus lateralis). Therefore, our data suggest that endurance training improves O_2 extraction in exercise models where the mitochondria do not possess an apparent excess oxidative capacity over O_2 delivery, particularly when the exercise intensity is close to maximal.

In study IV, the relationships between pulmonary $\dot{V}O_{2max}$ and systemic and leg O_2 extraction fractions were investigated by statistically analysing data from 43 previously published catheterisation studies, comprising 377 subjects. It was observed that a-$\bar{v}O_2$diff (mostly calculated by the Fick principle, and $\dot{Q}_{max}$ measured by the indicator-dilution method) increased curvilinearly and reached its maximum at ~4.5 l · min^{-1} in $\dot{V}O_{2max}$ (moderately trained subjects), and was, if anything, slightly lower in those subjects with the highest $\dot{V}O_{2max}$ (> 5 l · min^{-1}). However, after accounting for the hypoxemia-induced lowering of arterial O_2 content (CaO_2) with increasing $\dot{V}O_{2max}$, the calculated systemic O_2 extraction fraction (a-$\bar{v}O_2$diff / CaO_2) increased with $\dot{V}O_{2max}$ up to ~4.5-5.0 l · min^{-1} and approached a plateau at ~90%. This pattern was strengthened by the direct measurements using arterial and femoral venous catheters, with leg O_2 extraction fraction increasing progressively with $\dot{V}O_{2max}$ until reaching ~90-95%. These analyses emphasise that a-$\bar{v}O_2$diff and systemic O_2 extraction fraction cannot be used interchangeably, and that the systemic and peripheral O_2 extraction fractions improves with increasing $\dot{V}O_{2max}$ and training status. By using the theoretical model of Piiper and Scheid, it appeared that the limiting factors to $\dot{V}O_{2max}$ change with increasing $\dot{V}O_{2max}$: untrained, but healthy individuals display mixed perfusion-diffusion limitations, and this diffusion limitation reduces as $\dot{V}O_{2max}$ increase.

Sammendrag (summary in Norwegian)

Denne avhandlingen inkluderer fire forskningsartikler fra fire separate studier, og tar sikte på å belyse viktigheten av O_2-ekstraksjon og blodvolum (BV) for kroppens maksimale O_2-opptak ($\dot{V}O_{2maks}$).

I studie I gjennomførte tolv utrente deltakere ($\dot{V}O_{2maks}$: 44 ml · kg^{-1} · min^{-1}) ti uker med utholdenhetstrening (tre økter per uke). $\dot{V}O_{2maks}$ og hjertets maksimale minuttvolum ($\dot{Q}_{maks}$) ble målt før og etter treningsperioden, og i tillegg direkte etter at den treningsinduserte økningen i BV ble fjernet fra hver deltaker via blodtapping. $\dot{V}O_{2maks}$ ble målt mens deltakerne syklet på en ergometersykkel sittende, men også mens deltakerne lå på ryggen med beina hevet ~20 cm over hjertehøyde. Denne posisjonen øker den venøse tilbakestrømmingen og kan potensielt motvirke en negativ effekt av blodtapping. BV økte med 4% (~2 dl) over treningsperioden. Etter blodtappingen var $\dot{V}O_{2maks}$ og $\dot{Q}_{maks}$ fortsatt forhøyet med henholdsvis 11% og 9% i forhold til før trening, uavhengig av om det ble målt ved sittende eller liggende sykling. Ved å bruke Ficks prinsipp ($\dot{V}O_2 = \dot{Q} \times$ a-$\bar{v}O_2$diff) ble det regnet ut at henholdsvis 30% og 70% av økningen i $\dot{V}O_{2maks}$ kunne tilskrives økt differanse i O_2-innhold mellom arterielt og blandet venøst blod (a-$\bar{v}O_2$diff) og økt $\dot{Q}_{maks}$. Dette sammenfalt med økt proteininnhold av mitokondrielle enzymer og en liten økning i kapillær til fiber ratio i lårmuskulaturen (m. vastus lateralis), og økt mengde hjertemuskulatur målt i den venstre ventrikkelen (ekkokardiografi). Oppsummert kan økninger i $\dot{V}O_{2maks}$ forekomme uten BV ekspansjon, og kan tilskrives en kombinasjon av sentrale og perifere tilpasninger.

I studie II gjennomførte 13 deltakere ($\dot{V}O_{2maks}$: 63 ml · kg^{-1} · min^{-1}) maksimale tester på ergometersykkel i tre situasjoner: med normalt BV, og direkte etter BV-reduksjoner på 150 ml og 450 ml, noe som tilsvarte 2,5% og 7,6% av det totale BV (6,0 l). $\dot{V}O_{2maks}$ ble opprettholdt etter å ha tappet 150 ml blod (ikke-signifikant reduksjon på 1%), noe som skyldtes en rask regenerering av plasmavolumet (PV; utregnet fra endringer i hematokrit og hemoglobinkonsentrasjon). Etter å ha tappet 450 ml ble $\dot{V}O_{2maks}$ redusert med 7%, selv om kroppen kompenserte med delvis regenerering av PV, økt maksimal hjertefrekvens og økt O_2-ekstraksjon i beina indikert av nær-infrarød spektroskopi (NIRS). Selv etter å ha normalisert til det tappede volumet av blod var reduksjonen i $\dot{V}O_{2maks}$ 2,5 ganger større etter å ha tappet 450 ml sammenlignet med 150 ml blod. Dette tyder på at kroppen klarer å kompensere for små, men ikke moderate blodtap når det gjelder å opprettholde $\dot{V}O_{2maks}$. Funnene kan øke vår innsikt vedrørende effekten av f.eks. akutte BV-manipulasjoner, PV-reduksjoner som følge av langvarige treningsøkter og/eller hypertermi, og daglige svingninger i PV.

I studie III økte vi muskulaturens oksidative kapasitet i kun ett bein hos ni deltakere ($\dot{V}O_{2maks}$: 56 ml · kg^{-1} · min^{-1}), gjennom seks ukers ett-beins utholdenhetstrening (3-4 økter per uke). Etter treningsperioden undersøkte vi om forskjellen i treningsstatus påvirket muskulaturens evne til å trekke ut O_2 fra blodet (målt direkte via ett arteriekateter og femorale venekateter i begge bein). Begge bein

arbeidet samtidig ved dynamiske to-beins kne-ekstensjoner og utførte det samme arbeidet (power output). Denne øvelsen involverer en liten muskelmasse (~2,5 kg per bein) og begrenses ikke av hjertets evne til å pumpe blod og levere O_2 til vevet. Således er muskulaturens oksidative kapasitet potensielt avgjørende for O_2-ekstraksjon og $\dot{V}O_2$ ved denne arbeidsformen. O_2-ekstraksjonen var tilnærmet lik i begge bein ved lav til moderat arbeidsintensitet, men var betraktelig høyere i det trente beinet ved høy arbeidsintensitet. Forskjellen mellom beina i O_2-ekstraksjon hadde en sammenheng med forskjellen i proteininnhold av mitokondrielle enzymer i lårmuskulaturen (m. vastus lateralis). Derfor tyder dataene våre på at utholdenhetstrening kan øke O_2-ekstraksjonen når man arbeider med en liten muskelmasse, spesielt når arbeidsintensiteten er høy; altså i situasjoner hvor muskulaturens mitokondrier sannsynligvis ikke besitter en betydelig oksidativ overkapasitet over O_2-leveransen før trening.

I studie IV undersøkte vi sammenhengen mellom $\dot{V}O_{2maks}$ og O_2-ekstraksjon målt samlet for hele kroppen og lokalt i beina. Dette ble undersøkt ved å gjøre en statistisk analyse av 43 tidligere publiserte studier som benyttet seg av kateter-teknikker, og som inkluderte totalt 377 deltakere. Det ble observert at a-$\bar{v}O_2$diff økte kurvelineært og nådde et maksimum ved ~4.5 l · min^{-1} i $\dot{V}O_{2maks}$ (moderat trente individer), og ble deretter etterfulgt av en liten reduksjon for de med høyest $\dot{V}O_{2maks}$ (> 5 l · min^{-1}). Imidlertid, dersom man oppga O_2-ekstraksjonen i forhold til O_2-mengden i arterieblod (CaO_2), så økte den prosentvise systemiske O_2 ekstraksjonen (a-$\bar{v}O_2$diff / CaO_2) med økende $\dot{V}O_{2maks}$ opp til ~4.5-5.0 l · min^{-1} og nådde et platå på ~90%. Dette kan tilskrives at CaO_2 var redusert hos de med høyest $\dot{V}O_{2maks}$, noe som skyldes at blodstrømmen gjennom lungene er for høy til at blodet rekker å mettes fullstendig med O_2 (hypoksemi) hos disse individene som også besitter en høy $\dot{Q}_{maks}$. Dette mønsteret ble styrket av målingene direkte over beinet (arteriekateter og femoralt venekateter), hvor beinets prosentvise O_2-ekstraksjon økte progressivt med økende $\dot{V}O_{2maks}$ opp til ~90-95%. Disse funnene tyder på at O_2-ektraksjonen øker med økende $\dot{V}O_{2maks}$ og treningstilstand. Videre viser analysen at man ikke kan benytte begrepene a-$\bar{v}O_2$diff og prosentvis O_2-ektraksjon om hverandre, noe som ofte gjøres i forskningslitteraturen. Videre ble det funnet at begrensningene til $\dot{V}O_{2maks}$ endres med økende $\dot{V}O_{2maks}$: utrente friske individer har kombinerte gjennomblødnings- og diffusjonsbegrensninger, mens disse to begrensningene henholdsvis forsterkes og reduseres med økende $\dot{V}O_{2maks}$.

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Oslo, March 2020

Øyvind Skattebo

List of papers

This dissertation is based on the following research papers, which are referred to in the text by their Roman numerals:

I. **Skattebo, Ø.**, Bjerring, A.W., Auensen, M., Sarvari, S.I., Cumming, K.T., Capelli, C., & Hallén, J. Blood volume expansion does not explain the increase in peak oxygen uptake induced by 10 weeks of endurance training. *European Journal of Applied Physiology*. Accepted 24.02.20. DOI: 10.1007/s00421-020-04336-2

II. **Skattebo, Ø.**, Johansen, E.S., Capelli, C., & Hallén, J. Effects of 150 and 450 ml acute blood losses on plasma volume shift and oxygen uptake during maximal exercise. Manuscript

III. **Skattebo Ø.**, Capelli C., Rud B., Auensen, M., Calbet J.A.L., & Hallén J. Increased oxygen extraction and mitochondrial protein expression after small muscle mass endurance training. Submitted to *Scandinavian Journal of Medicine & Science in Sports*

IV. **Skattebo Ø.**, Calbet J.A.L., Rud, B., Capelli C., & Hallén J. Contribution of oxygen extraction fraction to maximal oxygen uptake in healthy young men. Submitted to *Acta Physiologica*

Abbreviations

a-$v_fO_2$diff	arterial to femoral venous oxygen difference
a-$\bar{v}O_2$diff	arterial to mixed venous oxygen difference
BV	blood volume
CaO_2	arterial oxygen content
CO	carbon monoxide
COX-IV	cytochrome c oxidase subunit 4
CS	citrate synthase
Cv_fO_2	femoral venous oxygen content
$C\bar{v}O_2$	mixed venous oxygen content
CVP	central venous pressure
D_MO_2	muscle diffusional O_2 conductance
EDV	end-diastolic volume
ESV	end-systolic volume
ET	endurance training
HAD	hydroxyacyl-CoA dehydrogenase
[Hb]	haemoglobin concentration
Hb_{mass}	haemoglobin mass
HR	heart rate
HR_{peak}	peak heart rate
[La]	blood lactate concentration
LBF	leg blood flow
LV	left ventricle
MAP	mean arterial blood pressure
MCHC	mean corpuscular haemoglobin concentration
MTT	erythrocyte capillary mean transit time
O_2	Oxygen
$\bar{O}_2$ extraction	systemic oxygen extraction fraction
O_2 extraction	peripheral (leg) oxygen extraction fraction
OXPHOS	maximal mitochondrial respiratory capacity (*ex vivo*)
PO_2	partial pressure of oxygen
PV	plasma volume

$P_{50}O_2$	partial pressure of oxygen at 50% SO_2
$\dot{Q}$	cardiac output
$\dot{Q}_{max}$	maximal cardiac output
RBC	red blood cell
RBCV	red blood cell volume
RER_{peak}	peak respiratory exchange ratio
RPE	rating of perceived exertion
SD	standard deviation
SO_2	oxygen saturation of haemoglobin
SV	stroke volume
$\dot{V}E_{peak}$	peak ventilation
$\dot{V}O_2$	oxygen uptake
$\dot{V}O_{2max}$	maximal oxygen uptake (exercise with large active muscle mass, e.g., cycling, running and cross-country skiing)
$\dot{V}O_{2peak}$	peak oxygen uptake (exercise with small active muscle mass, e.g., 1L-KE, 2L-KE and arm-cycling)
$\dot{W}_{peak}$	peak power output
1L-KE	One-legged knee extension (dynamic)
2L-KE	Two-legged knee extension (dynamic)

1. Introduction

Oxygen (O_2) enables mitochondrial oxidative phosphorylation, which provides the largest fraction of the adenosine triphosphate (ATP) resynthesis in humans. Under resting conditions, O_2 consumption ($\dot{V}O_2$) is 3-5 ml · kg^{-1} · min^{-1}, and only a small fraction is consumed within the skeletal muscles (Joyner & Casey, 2015). However, during incremental exercise, the muscles' ATP turnover and metabolic rate increases and may facilitate a dramatic rise in pulmonary $\dot{V}O_2$. The highest rate at which O_2 can be taken up and utilised in the body per unit of time is defined as the maximal O_2 uptake ($\dot{V}O_{2max}$) and varies greatly depending on sex, age, body weight, genetics, training status, exercise mode and health (Bouchard et al., 2011; Joyner & Casey, 2015; Saltin & Åstrand, 1967). For instance, highly trained male endurance athletes often have a $\dot{V}O_{2max}$ of 80-85 ml · kg^{-1} · min^{-1} (Saltin & Åstrand, 1967) and occasionally ~90 ml · kg^{-1} · min^{-1} or above (Skattebo et al., 2019); twice that measured in sedentary, healthy male subjects.

Due to the size of the human body, diffusion per se cannot provide a sufficiently rapid supply of O_2 from the ambient air to the peripheral tissues. Rather, convective O_2 transport via blood flowing conduits facilitates the longest section of the O_2 pathway from its point of intake, the lungs, to the peripheral tissues. Diffusion down O_2 pressure (PO_2) gradients occurs only where very short distances are involved, mainly through the alveolar-capillary barrier and the capillary-to-cell barrier. Therefore, the cardiovascular system is of principal importance in supplying O_2 to exercising muscles and serves a vital role in determining $\dot{V}O_{2max}$. This is also emphasised by the Fick principle ($\dot{V}O_2 = \dot{Q} \times a\text{-}\bar{v}O_2diff$), which states that every change in $\dot{V}O_{2max}$ can be explained by a concomitant change in maximal cardiac output ($\dot{Q}_{max}$) and/or the O_2 content difference between the arterial and mixed venous blood ($a\text{-}\bar{v}O_2diff$) (Joyner & Casey, 2015; Wagner, 1992).

The limiting factors to $\dot{V}O_{2max}$ are often separated into central (the pulmonary diffusing capacity, $\dot{Q}_{max}$ and O_2-carrying capacity of the blood) and peripheral factors (skeletal muscle characteristics), i.e., factors regulating the systemic O_2 delivery and its extraction, respectively (Bassett & Howley, 2000). It is well established that the circulating blood volume (BV) and the total haemoglobin mass (Hb_{mass}) are of principal importance for $\dot{V}O_{2max}$ and thereby, the maximal exercise capacity (Ekblom et al., 1972; Martino et al., 2002; Schmidt & Prommer, 2010). A standard blood donation of 400-500 ml reduces $\dot{V}O_{2max}$ by ~5-

10% (Balke et al., 1954; Ekblom et al., 1972; Gordon et al., 2014; Panebianco et al., 1995) and the reinfusion of 360-450 ml of freeze-preserved packed red blood cells (RBC) increases $\dot{V}O_{2max}$ by ~2-6% (Buick et al., 1980; Ekblom et al., 1976; Spriet et al., 1986). The underlying mechanisms responsible are the accompanying changes in stroke volume (SV), $\dot{Q}_{max}$ and the O_2-carrying capacity of the blood, thus causing a modification of the systemic O_2 delivery (Ekblom et al., 1976; Spriet et al., 1986).

BV expansion can occur rapidly at the commencement of endurance training (ET) and is proposed to play a significant role in the enhancement of $\dot{V}O_{2max}$ and maximal exercise capacity (Montero et al., 2017). However, not all investigations detect a change in BV after ET despite robust increases in $\dot{V}O_{2max}$ (Helgerud et al., 2007; Jacobs et al., 2013; Shoemaker et al., 1996). The role of BV can be investigated experimentally by reversing BV to the pre-ET level by phlebotomy, immediately followed by an assessment of $\dot{V}O_{2max}$ and maximal exercise capacity. Using this experimental design, the 7-10% improvements in $\dot{V}O_{2max}$ and $\dot{Q}_{max}$ after six weeks of ET were completely reversed to pre-ET levels (Bonne et al., 2014; Montero et al., 2015a). Concomitantly, no change in cardiac morphology (Bonne et al., 2014) nor in the a-$\bar{v}O_2$diff were observed, despite the mitochondrial volume density and capillary-to-fibre ratio increased by ~40% and ~20%, respectively (Montero et al., 2015a). Therefore, the authors suggested that the improvement in $\dot{V}O_{2max}$ was explained by increased $\dot{Q}_{max}$ attributed to BV expansion alone.

Acute hypovolaemia due to phlebotomy may increase the sympathetic tone, leading to arteriolar and venous constriction (Fortrat et al., 1998; Zollei et al., 2004) and consequently offset the ET-induced drop in peripheral vascular resistance in the above studies. In this context, reduced BV and impaired haemodynamic control after prolonged bed rest (3-5 weeks) can lead to a reduction in $\dot{Q}_{max}$ and $\dot{V}O_{2max}$ during upright cycling (Saltin et al., 1968a), which is reversed when measured during supine cycling (Bringard et al., 2010) due to gravitational effects on central BV, preload and thus SV (Warburton et al., 2002). The supine position increases central venous pressure and baroreflex loading compared to the upright position (Ray et al., 1993), thus potentially avoiding increases in sympathetic tone and total peripheral resistance in the face of reduced BV. **Therefore, it may be that ET-induced improvements are maintained during supine cycling, in contrast to upright cycling, after manipulating BV to pre-ET levels immediately followed by an assessment of $\dot{V}O_{2max}$ and maximal exercise capacity,** similar to the effects seen after bed rest.

Indications of maintained venous return despite small BV reductions have been observed in studies examining fluid loss after heat stress and prolonged exercise (Saltin, 1964; Saltin & Stenberg, 1964). In those studies, small to moderate reductions in plasma volume (PV) were not sufficient to decrease $\dot{Q}_{max}$, assessed in normothermic conditions. Thus, it was argued that an effective contribution from the muscle pump and increased vasomotor activity redistributed venous blood volumes and thus enabled a maintained SV. Therefore, a certain threshold might exist, at which small BV reductions are not detrimental to $\dot{Q}_{max}$, $\dot{V}O_{2max}$ and maximal exercise capacity, acting as a mechanism for the circulation to cope with small BV losses and PV reductions. **However, no study has investigated the acute effects of a small blood loss (e.g., 150 ml) on $\dot{V}O_{2max}$ and maximal exercise capacity**, and previous studies employing either blood withdrawals or transfusions have included a minimum BV manipulation of ~400 ml, corresponding to a change of 50-60 g in Hb_{mass} (Fig. 1.1).

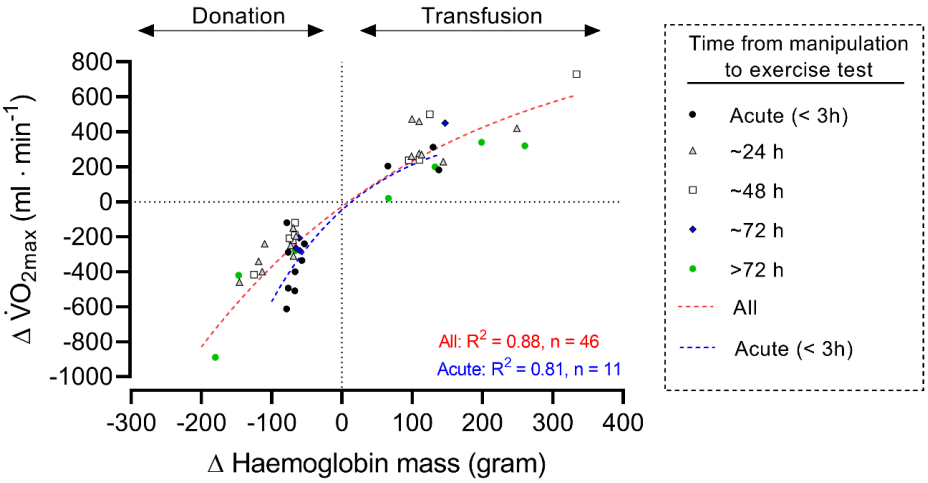

Fig. 1.1 The magnitude of blood volume withdrawal and transfusion presented as a change in haemoglobin mass on the x-axis and its effect on maximal oxygen uptake ($\dot{V}O_{2max}$). The studies are organised according to the time from blood withdrawal or transfusion to the initiation of exercise (Balke et al., 1954; Bejder et al., 2019; Bennett-Guerrero et al., 2017; Birnbaum et al., 2006; Bonne et al., 2014; Buick et al., 1980; Burnley et al., 2006; Celsing et al., 1986; Celsing et al., 1987; Christensen & Christensen, 1978; Ekblom et al., 1972; Ekblom et al., 1976; Freedson, 1981; Goforth et al., 1982; Gordon et al., 2014; Hill et al., 2013; Judd et al., 2011; Kanstrup & Ekblom, 1982, 1984; Krip et al., 1997; Malm et al., 2016; McDonagh et al., 2016; Meurrens et al., 2016; Montero et al., 2015a; Panebianco et al., 1995; Prommer et al., 2007; Robertson et al., 1984; Robertson et al., 1982; Sawka et al., 1987; Spriet et al., 1986; Stangerup et al., 2017; Taylor et al., 1997; Thomson et al., 1982; Turner et al., 1993; Woodson et al., 1978; Ziegler et al., 2015).

Several experiments have addressed whether $\dot{V}O_{2max}$ is limited by convective O_2 delivery or the muscles' ability to extract and utilise the provided O_2. Some of the most striking findings come from comparing exercises using a large muscle mass (such as running, cycling and cross-country skiing; 15-30 kg active skeletal muscle mass) vs a small muscle mass such as dynamic one-legged knee-extension (1L-KE), isolating the exercise to the quadriceps femoris muscles (~2.5 kg). $\dot{Q}_{max}$ is not taxed and does not restrict leg blood flow (LBF) during 1L-KE (Mortensen et al., 2008), the maximum LBF is in the range of 5-9 l · min^{-1}, thus representing a perfusion of 2.5-3.5 l · min^{-1} per kg active muscle mass (Andersen & Saltin, 1985; Richardson et al., 1993). This is ~2-3 times higher than during cycling and diagonal cross-country skiing (Boushel & Saltin, 2013; Calbet et al., 2004; Gonzalez-Alonso & Calbet, 2003; Mortensen et al., 2005), implying that the pumping capacity of the heart restricts blood flow and O_2 delivery when exercising with large muscle masses. A ~1.5-2 times higher mass-specific $\dot{V}O_2$ accompanies the larger LBF during 1L-KE compared to cycling exercise (Boushel & Saltin, 2013; Cardinale et al., 2019), indicating that the muscles possess a large oxidative reserve capacity when exercising with a large muscle mass.

Although the mitochondria possess an apparent excess oxidative capacity over O_2 delivery during whole-body exercise, ET induces a remarkable increase in mitochondrial density, commonly improving by ~40% after a few months of ET (Granata et al., 2018). Combined with increased capillarisation (Klausen et al., 1981), these adaptations are likely more important for endurance performance than $\dot{V}O_{2max}$ (Coyle et al., 1988; Henriksson, 1977), but despite a long-standing debate (Bassett & Howley, 2000; Saltin & Calbet, 2006; Wagner, 2006), it remains uncertain whether $\dot{V}O_{2max}$ is limited by central or by combined central-peripheral factors. By applying short term ET, it is typical to observe an increased $\dot{Q}_{max}$, whereas more heterogeneous findings exist for a-$\bar{v}O_2$diff (Montero & Diaz-Canestro, 2016). **Therefore, the contribution of mitochondrial biogenesis and angiogenesis after ET to a-$\bar{v}O_2$diff and improvements in whole-body $\dot{V}O_{2max}$ is still unclear.**

Despite the mass-specific LBF and O_2 delivery being ~2-3 times higher during 1L-KE than during cycling, the mass-specific $\dot{V}O_2$ is only ~70% higher due to a substantially lower leg O_2 extraction fraction (Cardinale et al., 2019). The decreased O_2 extraction fraction may be caused by completely activated mitochondria respiring close to their maximal rate (Cardinale et al., 2019), a condition that may restrict O_2 diffusion and further O_2 extraction from the muscle capillaries and utilisation by the cytochrome-c-oxidases. 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 (Blomstrand et al., 1997). Consequently, leg $\dot{V}O_2$ is not enhanced by breathing hyperoxic gas during maximal 1L-KE exercise in healthy individuals (Esposito et al., 2011; Gonzalez-Alonso et al., 2002; Mourtzakis et al., 2004; Pedersen et al., 1999) although this effect may depend on the training status (Cardinale et al., 2019; Richardson et al., 1999). The higher mass-specific LBF and lower O_2 extraction fraction indicate that the $\dot{V}O_2$ is more diffusion- than perfusion-limited, in contrast to the situation when exercising with a large muscle mass (Cardinale et al., 2019). 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 PO_2 gradient between the muscle capillaries and the mitochondria) are lowered, and *vice versa*. Indeed, leg immobilisation decreases the muscle oxidative capacity, resulting in impaired leg O_2 extraction fraction during 1L-KE (Mortensen et al., 2012). Furthermore, during arm-cycling, engaging a small muscle mass (~6 kg) characterised by a substantially lower maximal mitochondrial respiratory capacity (OXPHOS; measured in permeabilised muscle fibres *ex vivo*) than its maximal O_2 delivery (Boushel et al., 2011), ET has proven to enhance O_2 extraction (Boushel et al., 2014). Although the controversy persists over whether the a-$\bar{v}O_2$diff improves after short-term ET during exercise with a large muscle mass (Montero et al., 2015b; Roca et al., 1992), these data suggest that the potential for improvement is greater during exercise with a small muscle mass.

When conducting whole-body ET (e.g., cycling and running), both central and peripheral adaptations occur. 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, using one-legged training. Subsequently, the trained leg and the contralateral control leg within the same subject can exercise simultaneously at the same power output (Rud et al., 2012). This experimental setup ensures equal perfusion pressure and CaO_2 for both legs, meaning that: 1) the O_2 delivery should be similar and; 2) any improvement in O_2 extraction should originate from the changes in muscle oxidative capacity and/or capillarisation. Experiments involving simultaneous exercise with one trained and one untrained leg have been conducted in the past (Henriksson, 1977; Rud et al., 2012; Saltin et al., 1976). However, only Rud et al. (2012) balanced the power output between the trained and the untrained leg (the trained leg performed more work in the two other studies), and all

three studies used two-legged cycling, in which the muscles possess a large oxidative reserve capacity, even before training. **Therefore, it remains to be investigated whether muscles within the same subject with distinct differences in muscle oxidative capacity affect O_2 extraction and D_MO_2 when O_2 delivery is not limited (i.e., one trained and one untrained leg simultaneously exercising during 2L-KE).**

Despite extensive research since the 1950s on the factors limiting whole-body $\dot{V}O_{2max}$, **no study has aimed to statistically analyse all the existing data on the association between $\dot{V}O_{2max}$ and its limiting factors**. This kind of analysis is warranted, as the original studies often used homogenous groups with a small number of subjects (< 10) since they applied costly and invasive techniques involving catheterisations to determine $\dot{Q}_{max}$ (indicator-dilution techniques or the direct Fick method), regional blood flows (thermodilution or indicator-dilution techniques) and O_2 extraction fraction (calculated by the Fick principle or directly measured through arterial and venous catheters). Consequently, the statistical power is often too low to detect small but meaningful differences between subjects, groups with different training status, and before and after training, thus precluding a definite conclusion.

It is documented that the a-$\bar{v}O_2$diff at $\dot{V}O_{2max}$ is only slightly different between untrained and endurance-trained individuals (Ekblom & Hermansen, 1968; Ekblom et al., 1968), suggesting that peripheral adaptations to ET have a rather small impact on $\dot{V}O_{2max}$. However, the a-$\bar{v}O_2$diff is determined not only by the peripheries' ability to extract O_2, reflected in the mixed venous O_2 content ($C\bar{v}O_2$), but also by the CaO_2, which sets the upper limit for the a-$\bar{v}O_2$diff during maximal exercise. The CaO_2 is set by the Hb concentration ([Hb]) and the O_2 saturation of Hb (SO_2), which may change with training. For instance, ET causes PV expansion (Sawka et al., 2000), which can lead to haemodilution and a lower O_2-carrying capacity of the blood (Ekblom & Hermansen, 1968). A high $\dot{Q}_{max}$ shortens the time for alveolar/capillary gas equilibration at the lung, causing exercise-induced arterial hypoxemia that further reduces the CaO_2 (Nielsen, 2003; Powers et al., 1989). **Therefore, it is conceivable that despite the a-$\bar{v}O_2$diff does not increase substantially with increasing $\dot{V}O_{2max}$ and training status, systemic O_2 extraction fraction may still improve ($\bar{O}_2$ extraction fraction: a-$\bar{v}O_2$diff / CaO_2), due to a concurrent training-induced lowering of CaO_2.**

Most studies, even those employing catheterisation techniques, have not measured a-$\bar{v}O_2$diff directly but calculated it using the Fick principle (Ekblom & Hermansen, 1968;

Ekblom et al., 1968; Gleser, 1973; Hermansen et al., 1970; Mitchell et al., 1958; Åstrand et al., 1964). The reason why so few studies have measured a-$\bar{v}O_2$diff directly during maximal exercise is due to the need for right heart catheterisation, preferably with the catheter advanced to the pulmonary artery. **Therefore, studies measuring the arterial to femoral venous O_2 content difference (a-$v_fO_2$diff) and leg O_2 extraction fraction directly using peripheral catheters may be more sensitive in evaluating whether the O_2 extraction capacity changes with ET.**

2. Research aims and hypotheses

The overall aim of this thesis was to investigate the role of O_2 extraction fraction and blood volume for $\dot{V}O_{2max}$, with particular emphasis on the research gaps highlighted in the introduction. Four separate studies were conducted: two interventional ET studies (studies I and III), an acute study (study II) and statistical analysis of previously published research data (study IV).

The specific aims of the studies were:

I. To investigate the importance of ET-induced increases in Hb_{mass} and BV in changes in $\dot{V}O_{2max}$ and $\dot{Q}_{max}$. Maximal exercise tests in upright and supine cycling were conducted before and after removing an amount of blood corresponding to the measured individual increase in Hb_{mass} induced by ET. Mechanisms behind potential changes in a-$\bar{v}O_2$diff and $\dot{Q}_{max}$ were studied in muscle biopsies (mitochondrial enzymes and capillarisation) and by echocardiography, respectively.

II. To investigate the effects of small (150 ml) and moderate (450 ml) blood losses on $\dot{V}O_{2max}$ and maximal exercise capacity. Potential transcapillary fluid shifts after blood withdrawal and during exercise were monitored by measuring [Hb] and haematocrit and by calculating fluctuations of intravascular volumes (BV, PV and RBCV).

III. To increase the muscle oxidative capacity in one leg using one-legged ET and test its impact on leg O_2 extraction fraction (arterial and femoral venous catheters) vs the untrained control leg during 2L-KE; an exercise model that is not perfusion limited by not taxing $\dot{Q}_{max}$ (Koskolou et al., 1997) and with the muscle oxidative capacity potentially being one of the principal limitations before training. Mechanisms behind potential between-leg differences in O_2 extraction fraction were studied in muscle biopsies (mitochondrial enzymes and capillarisation).

IV. To statistically analyse previously published data on the association between $\dot{V}O_{2max}$ and O_2 extraction fraction, using only catheterisation studies. Two approaches were used: 1) research papers containing individual data on pulmonary $\dot{V}O_{2max}$, $\dot{Q}_{max}$, a-$\bar{v}O_2$diff and $\bar{O}_2$ extraction fraction measured during whole-body maximal exercise were included; 2) the relationship between pulmonary $\dot{V}O_{2max}$ and peripheral O_2 extraction fraction was analysed from group-mean data from studies reporting LBF, a-$v_fO_2$diff and leg O_2 extraction fraction measured during whole-body maximal exercise. To investigate whether the limiting factors vary with $\dot{V}O_{2max}$, the Fick law of diffusion was used to calculate D_MO_2, and the Piiper and Scheid model was subsequently used to calculate the relative roles of perfusion vs diffusion limitations to $\dot{V}O_{2max}$ (Piiper, 2000).

The hypotheses were:

A. The increases in $\dot{V}O_{2max}$ and $\dot{Q}_{max}$ will return to pre-ET levels after counteracting the BV expansion during upright cycling but remain elevated during supine cycling owing to improved venous return (study I).
B. The change in $\dot{V}O_{2max}$ after ET is mostly facilitated by elevated $\dot{Q}_{max}$, but also by a widened a-$\bar{v}O_2$diff (study I).

C. Compensatory mechanisms will preserve $\dot{V}O_{2max}$ and peak power output ($\dot{W}_{peak}$) during incremental exercise to exhaustion after the acute withdrawal of 150 ml blood, but will be insufficient after withdrawing 450 ml (study II).

D. Leg O_2 extraction fraction will be higher in the trained leg compared with the untrained leg while exercising simultaneously at the same power output (study III).
E. A higher leg O_2 extraction fraction will be facilitated by increased muscle oxidative capacity and larger recruitment of D_MO_2 in the trained leg (study III).
F. As the oxidative capacity will be gradually more exploited by increasing exercise intensity, a potential between-leg difference in O_2 extraction fraction will be more evident at high compared with low power outputs (study III).

Study IV was an exploratory study and had no prior research hypothesis.

3. Methods

This thesis presents data from four research papers originating from three separate experimental studies conducted by our research group between 2017-2019 (study I-III), and from a statistical analysis of 43 previously published research papers (study IV).

3.1. Subjects

A total of 34 subjects gave their written, informed consent to participate in studies I-III and completed the experiments (Table 3.1). An additional two subjects in each of study I and II were recruited but dropped out due to factors unrelated to the studies. Based on their regular training and $\dot{V}O_{2max}$, the subjects in study I, II and III are described as untrained (≤ 1 ET session per week), moderately to well-trained (range: 1-7 ET sessions per week) and moderately trained (range: 1-5 ET sessions per week), respectively. The subjects were instructed to abstain from strenuous exercise, alcohol consumption and caffeine consumption during the 24 h, 24 h and 12 h before lab visits, respectively.

The studies were approved by the Ethics Committee of the Norwegian School of Sport Sciences (appendix I-III) and The Norwegian Centre for Research Data (appendix IV-VI) and were carried out in accordance with the Declaration of Helsinki.

Table 3.1 Subject characteristics (mean ± SD)

Study	n (♂ / ♀)	Age (yrs)	Height (cm)	Body mass (kg)	$\dot{V}O_{2max}$ (ml · kg^{-1} · min^{-1})	Blood volume (l)	Haemoglobin mass (g)
I	7 / 5	29 ± 6	175 ± 11	72 ± 13	44 ± 6	5.1 ± 0.9	795 ± 196
II	13 / 0	27 ± 4	179 ± 7	75 ± 7	63 ± 9	6.0 ± 0.7	891 ± 127
III	9 / 0	28 ± 5	179 ± 7	79 ± 11	56 ± 10	-	-

3.2. Study designs (studies I-III)

3.2.1. Study I

Before and after 10 weeks of ET, subjects' Hb_{mass}, BV, body composition (dual-energy X-ray absorptiometry in the fasted state; Lunar iDXA; GE Healthcare, WI, USA) and cardiac dimensions were assessed, and a muscle biopsy from the thigh was obtained under resting conditions. Maximal exercise testing was performed for determination of $\dot{W}_{peak}$ and $\dot{V}O_{2max}$ during upright and supine cycling before and after ET as well as directly after removing the BV necessary to counteract the individual increase in Hb_{mass} elicited by the ET (Fig. 3.1).

Before pre-ET measurements, all subjects were familiarised with supine cycling (two sessions) and maximal exercise during upright cycling.

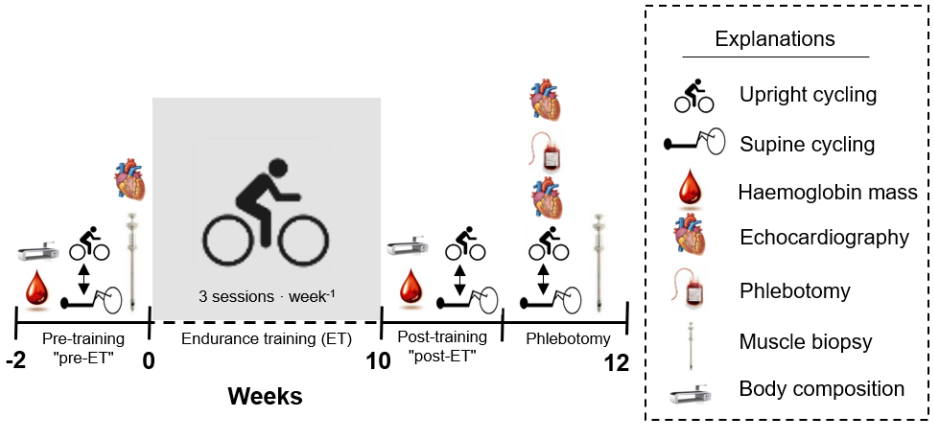

Fig. 3.1 The experimental design of study I. During the phlebotomy trial, echocardiography was conducted first (the post-ET echocardiography), following which the subjects were phlebotomised. This was followed by a second echocardiography. The first of two cycling exercises was initiated precisely 45 min after phlebotomy

3.2.2. Study II

The subjects visited the laboratory on three occasions at the same time of day (08:00 or 14:00): on a familiarisation day, a control day and an experimental day, each separated by 1 week (Fig. 3.2). Exercise testing on the cycle ergometer was repeated twice on each day, separated by 90 min rest. Each trial included three submaximal workloads followed by a maximal test with step-increments every minute until exhaustion for determination of $\dot{W}_{peak}$ and $\dot{V}O_{2max}$. The two control trials were conducted in euvolaemia (normal BV), whereas the 1st and the 2nd experimental trial were preceded by the removal of 150 ml blood (referred to as BVR_{150ml}) and 450 ml blood (150 ml + 300 ml; BVR_{450ml}), respectively. In this way, the 1st and the 2nd control trial served as controls for BVR_{150ml} and BVR_{450ml}, respectively. Hb_{mass} was measured on the familiarisation day for calculations of intravascular volumes.

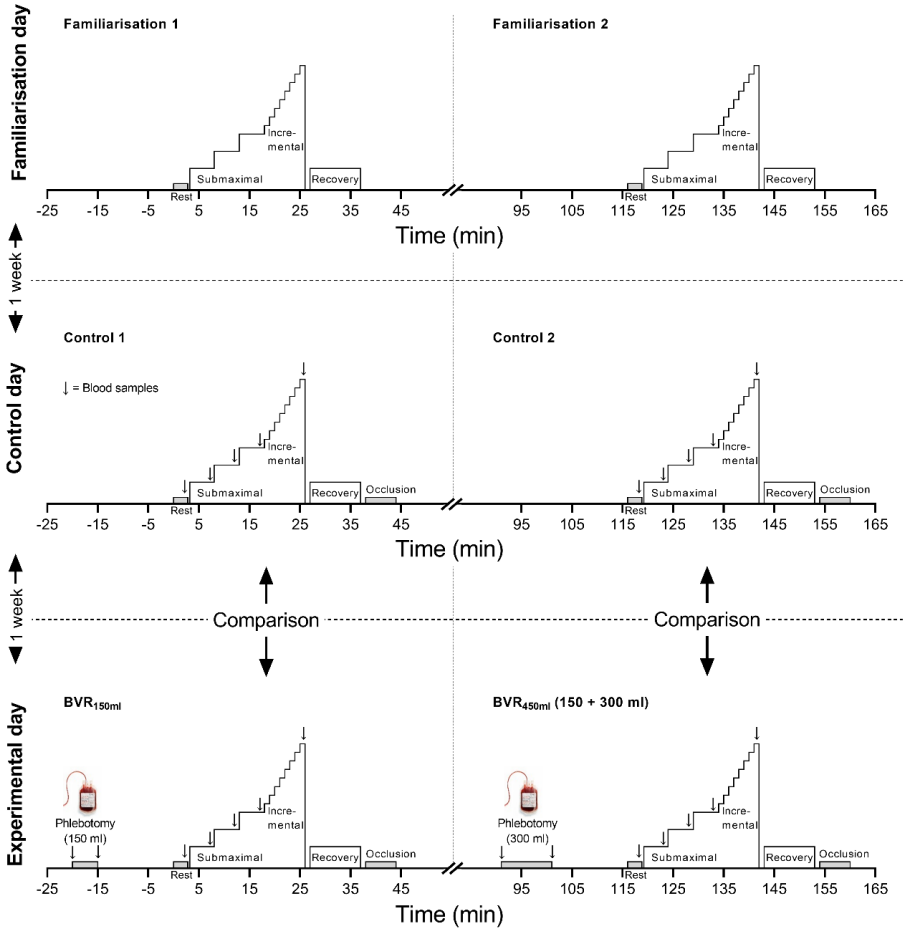

Fig. 3.2 The experimental design of study II. The three submaximal workloads (90 ± 32, 138 ± 36 and 185 ± 42 W) were followed by step-increments of 25 W every min until exhaustion. After reaching exhaustion, the subjects cycled at 50-100 W for 10 min to speed up recovery.

3.2.3. Study III

After six weeks of 1L-KE ET (Fig. 3.3), the participants performed 2L-KE exercise at three different power outputs: 40 ± 3%, 62 ± 4% and 83 ± 4% of 2L-KE $\dot{W}_{peak}$. Submaximal and near-maximal power outputs were chosen to ensure identical absolute power output produced by the trained leg (TL) and the non-trained control leg (CON), despite 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 fraction and to calculate leg $\dot{V}O_2$ and D_MO_2.

Before and after the training intervention, measurements of pulmonary $\dot{V}O_{2max}$ during cycling and pulmonary peak $\dot{V}O_2$ ($\dot{V}O_{2peak}$) during 1L-KE (both legs, separately) and 2L-KE were conducted. Body composition was measured after overnight fasting (dual-energy X-ray absorptiometry), a muscle biopsy from each thigh was sampled, and the quadriceps femoris muscle mass was estimated anthropometrically (Andersen & Saltin, 1985) and adjusted as proposed by Rådegran et al. (1999). Before testing, several short familiarisation sessions were conducted to ensure optimal technique during 1L-KE and 2L-KE.

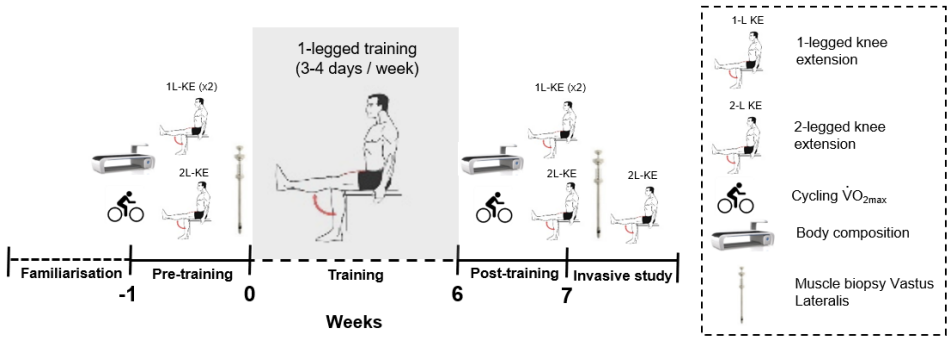

Fig. 3.3 The experimental design of study III.

3.3. Exercise training

3.3.1. Study I

During the 10-week ET period, the subjects underwent three supervised training sessions per week. Session 1 consisted of 60 min of continuous exercise at 70-80% of peak heart rate (HR_{peak}) (Fig. 3.4 a). Session 2 included 4 × 8 min intervals with a target intensity between 85-90% of HR_{peak} (Fig. 3.4 b). Session 3 consisted of 4-6 × 4 min intervals with a target intensity ≥ 90% of HR_{peak} (4 repetitions in weeks 1-3, 5 repetitions in weeks 4-6, and 6 repetitions in weeks 7-9; Fig. 3.4 c). The intervals were interspersed with 3 or 2 min active recovery at ~70% of HR_{peak}, respectively. A short tapering was performed in week 10 to maximise performance during the post-ET testing. The continuous session was shortened to 40 min, and the number of repetitions during the 8 and 4 min intervals were reduced to 3 and 4 repetitions, respectively. All subjects conducted 27-30 ET sessions (compliance: 94.4 ± 3.6%).

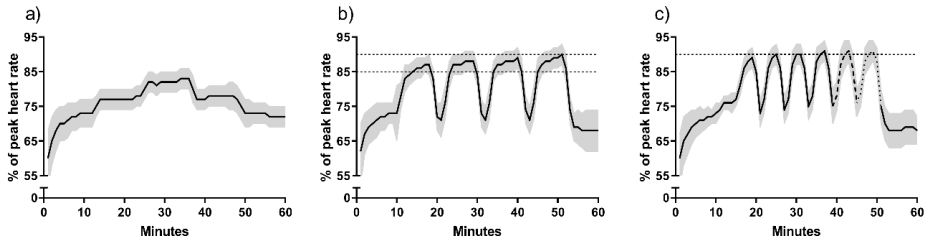

Fig. 3.4 Percentage of peak heart rate during continuous moderate-intensity (a); 8-min interval (b); and 4-min interval (c) sessions conducted during the training period in study I. The black lines and the grey-shaded areas denote their mean values and standard deviations, respectively.

3.3.2. Study III

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 35-65 min during the training period. The second session consisted of a 10 min warm-up, 20 min "all-out" at the highest possible power output and a cool-down of 5 min. The third session consisted of a 10 min warm-up, and 4-6 repetitions of 5 min at 85% of $\dot{W}_{peak}$, which were interspersed with recovery periods of 2 min and followed by a 5-min cooldown. From weeks 1 to 6, the total work performed increased from 197 ± 23 kJ to 493 ± 54 kJ per week. The subjects were asked to maintain their regular training in addition to the 1L-KE training, which was 2.9 ± 2.4 h of ET and 1.0 ± 0.7 h of strength training per week during the intervention period.

3.4. Measurements and procedures

3.4.1. Exercise testing (study I)

Upright and supine cycling were conducted in counterbalanced order on the same day, separated by 45 min of passive rest. Food intake was standardised, and *ad libitum* water intake was recorded during the pre-ET test day and was subsequently repeated. During upright cycling (Excalibur Sport; Lode B.V., Groningen, The Netherlands), the test started with a 3-min resting measurement while seated on the cycle ergometer. Thereafter, three 5-min submaximal workloads (♂ 50-150 W, ♀ 50-100 W) were conducted, directly followed by a maximal test with step-increments of 25 W every minute until exhaustion (the same protocol as depicted in Fig. 3.2). The mean workload during the last 60 s was defined as the

$\dot{W}_{peak}$. During supine cycling (Angio 2000; Lode B.V.), the subjects were secured with a 4-point harness while lying on a bench. The axis of rotation and thus the legs were raised ~20 cm above the heart. The submaximal workloads (♂ 50-100 W, ♀ 50-75 W) were followed by step-increments of 20 W every minute. After reaching exhaustion, the subjects cycled at 40-50 W for 5 min to speed up their recovery. $\dot{V}O_2$ was measured over the last 2.5 min at each submaximal stage and continuously during the resting measurements and incremental tests, using open-circuit indirect calorimetry with a mixing chamber (Oxycon Pro; Jaeger Instrument, Friedberg, Germany) (Foss & Hallén, 2005).

Stroke volume (SV), HR and $\dot{Q}$ were continuously monitored by impedance cardiography and an integrated electrocardiogram (ECG) using a PhysioFlow Q-link device (Manatec Biomedical, Paris, France). This method is evaluated against the direct Fick method (Charloux et al., 2000; Richard et al., 2001; Siebenmann et al., 2015) and uses the cyclic variations in transthoracic impedance during the cardiac cycle to estimate SV, as these pulsatile variations represent the changes in volume and velocity of the aortic BV (Charloux et al., 2000; Richard et al., 2001). Six electrodes (PF-50; Manatec Biomedical) were placed on each subject's neck, chest and back after the skin was cleaned with alcohol and rubbed with an abrasive ECG preparation gel (Custo prep; Custo med, Ottobrunn, Germany). Equal placement of electrodes in all tests was ensured by tracking their positions on transparent plastic sheets according to skin and anatomical landmarks. The subjects wore a tight mesh t-shirt to avoid displacement of the electrodes and their attached leads. A fan was placed in front of the subjects for heat dissipation and to counteract the accumulation of sweat during exercise, to ensure that the electrodes maintained their adhesiveness throughout the test. After instrumentation, the subjects rested on the ergometer for 5 min before autocalibration of the software (version 2.7.4). Immediately after the autocalibration, blood pressure was measured in duplicate (ProBP 3400 series; Welch Allyn, Skaneateles, NY, USA) and the mean values were fed to the software.

$\dot{V}O_2$, SV, HR and $\dot{Q}$ were recorded using 10-s averages. For the submaximal workloads, the averages of the last 2 min served as the steady-state values. During the incremental tests to exhaustion, the highest 30-s average was taken as the maximal value. The a-$\bar{v}O_2$diff was calculated as the ratio between $\dot{V}O_2$ and $\dot{Q}$ according to the Fick principle. The peak capillary blood lactate concentration ($[La]_{peak}$) was measured 1 min after exhaustion (Biosen C-line; EKF Diagnostic, Cardiff, UK). The coefficients of variation for $\dot{W}_{peak}$ and $\dot{V}O_{2max}$ measured during upright cycling, expressed as the percent typical error (standard

deviation of the difference scores / $\sqrt{2}$), were 3.0% (intraclass correlation: 0.98) and 2.9% (intraclass correlation: 0.99), respectively (familiarisation vs pre-ET) (Hopkins, 2015). The PhysioFlow was not used on the familiarisation test. However, based on another subject group (n = 17) conducting a total of 58 incremental exercise tests to exhaustion (2-4 tests per subject), the typical error was 7.0% for $\dot{Q}_{max}$ measured using PhysioFlow (intraclass correlation: 0.86). Based on 27 subjects conducting 115 tests including a total of 575 resting, submaximal and maximal measurements using PhysioFlow and Oxycon Pro (research studies and pilot testing), the regression equation between $\dot{V}O_2$ (l ·min^{-1}) and $\dot{Q}$ (l ·min^{-1}) was y = 5.17 + 5.08 (R^2 = 0.90).

3.4.2. Exercise testing (study II)

Three 5-min submaximal workloads (Excalibur Sport) were individually set to induce a capillary [La] ≤ 2.5 mmol · l^{-1} on the third workload (protocol: Fig. 3.2). The starting workload on the maximal test was set to induce a time to exhaustion of ~8 min (based on the familiarisation day). After reaching exhaustion, the subjects cycled at 50-100 W for 10 min to speed up their recovery. Between exercise trials, recovery and food intake were standardised. *Ad libitum* water intake was recorded on the control day and was repeated on the experimental day. $\dot{V}O_2$ and [La] were measured as described in study I. HR was measured using ECG. The average 30-s HR during $\dot{V}O_{2max}$ was used to calculate the O_2 pulse ($\dot{V}O_2$ / HR) as an indicator of SV (O_2 pulse = SV · a-$\bar{v}O_2$diff). The typical errors for $\dot{W}_{peak}$ and $\dot{V}O_{2max}$, calculated across the four exercise trials in euvolemia (familiarisation and control trials), were 1.7% (intraclass correlation: 0.99) and 1.9% (intraclass correlation: 0.99), respectively.

As a substitute measure of arterial Hb O_2 saturation (SO_2), peripheral capillary O_2 saturation (SpO_2) was measured using a pulse oximeter placed on the pre-heated right index finger (901-M; Masimo, Irvine, CA, USA). CaO_2 at $\dot{W}_{peak}$ was estimated as: (1.34 × [Hb] × SpO_2) + 3 ml dissolved in plasma. The tissue oxygenation index (TOI) was obtained from the Vastus Lateralis muscle as the ratio of oxygenated to total tissue Hb and myoglobin via spatially resolved near-infrared spectroscopy (NIRS; PortaMon; Artinis Medical Systems, Elst, The Netherlands). NIRS reflects primarily capillary oxygenation but is also affected by the arteriolar and venular blood and muscle myoglobin. The Vastus Lateralis TOI correlates well with the femoral venous SO_2 and may thus serve as a substitute measure of leg O_2 extraction fraction (Boushel et al., 2001; Esaki et al., 2005). The oxygenation parameters

were obtained using three light-emitting diodes (wavelengths: 760 and 850 nm) spaced 30, 35 and 40 mm from the detector. The NIRS optode was covered with Saran wrap, placed midway between the trochanter major and the lateral epicondyle and secured with elastic bandages around the thigh. Equivalent positioning across days was ensured by using skin and anatomical landmarks. The data was recorded at 10 Hz and analysed using the Oxysoft software (v. 3.0.95; Aritnis). One minute after the termination of exercise, an 8.5 cm-wide cuff (Zimmer Biomet, Warsaw, IN, USA) was manually inflated (VBM Medizintechnik, Sulz am Neckar, Germany) to 300 mmHg to occlude the arterial blood flow for 6 min (prolonged up to 8 min if necessary) until the minimum TOI was reached. After cuff-release, the maximum TOI was recorded during hyperaemia. The subjects had a skin and subcutaneous tissue thickness of 5.3 ± 1.8 mm (range: 3.1–9.7) below the optode, assessed using an ultrasound device (Vivid E95; GE Vingmed Ultrasound AS, Horten, Norway).

3.4.3. Exercise testing (study III)

Non-invasive

Before and after the 1L-KE training, 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 min and 45 min, respectively. The incremental tests began at 30 W or 60 W, after a 15 min 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. On a separate day, $\dot{V}O_{2max}$ was measured during cycling (Excalibur Sport).

Exercise protocol and timing of measurements (main experiment)

First, a baseline measurement was conducted after 10 min rest seated in the KE ergometer. Thereafter, three 8-min exercise bouts at low (47 ± 7 W; i.e., 23.5 W by each leg), moderate (73 ± 10 W; i.e., 36.5 W by each leg) and high (97 ± 10 W; i.e., 48.5 W by each leg) intensity (40 ± 3%, 62 ± 4% and 83 ± 4% of 2L-KE $\dot{W}_{peak}$, respectively) were conducted, separated by 5-min passive rest periods (see Fig. 3.5). During each bout, the subjects used the first 1-2 minute(s) to fine-tune equal involvements of legs and to reach “steady state”. LBF was measured between 4 and 6 min. Two blood samples from each vessel were drawn during the LBF measurements (~4.5 min and ~5.5 min), and the duplicates were averaged. Blood pressures were calculated as ~30-s averages preceding the first blood sample. HR was

averaged from ECGs during Doppler measurements. At the end of each exercise bout, the subjects were asked to rate the perceived exertion for each leg separately using the CR-10 scale (Borg, 1990). 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 = 0.008) and high (8.6 ± 1.7 vs 6.9 ± 2.3; P < 0.001) intensity exercise elicited significantly higher perceived intensity in CON than TL.

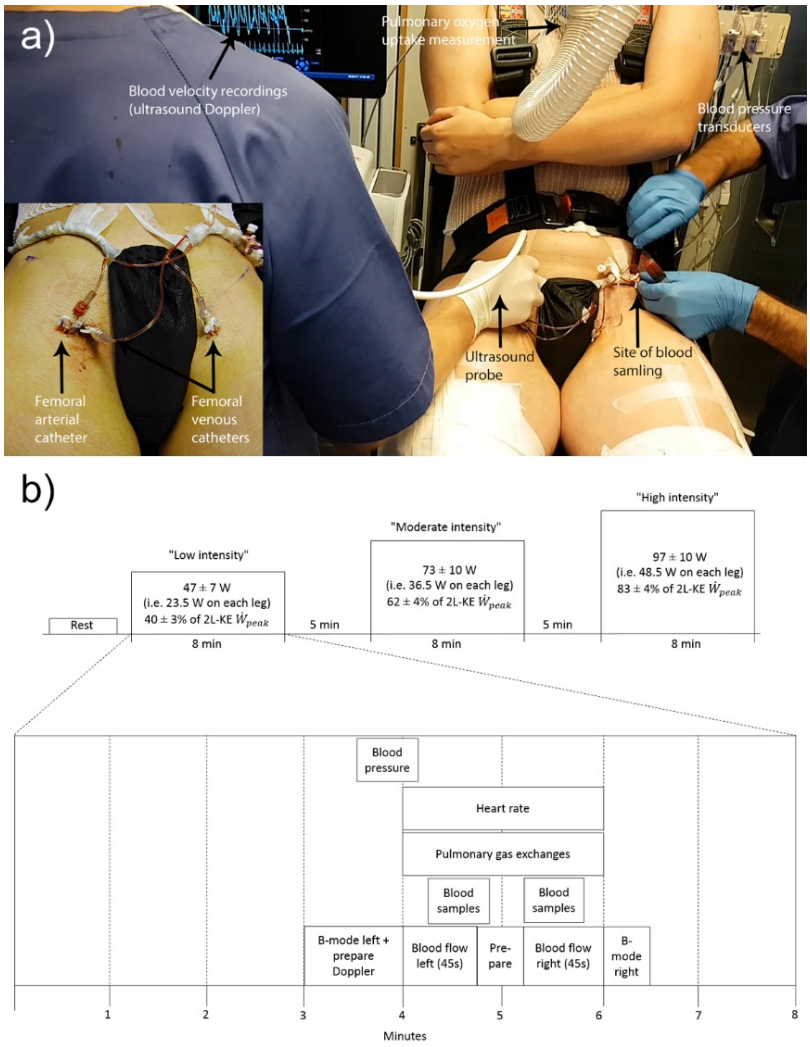

Fig. 3.5 The full setup of a subject seated in the knee extension ergometer (a), and the exercise protocol and timing of measurements (b).

3.4.4. Haematology and blood withdrawal (studies I & II)

Hb_{mass} was measured in duplicate on separate days before and after ET (study I), or once (study II), using a carbon monoxide (CO) rebreathing method (Prommer & Schmidt, 2007; Schmidt & Prommer, 2005). First, the subjects rested seated for 10 min, followed by capillary blood sampling into two 125-µl pre-heparinised tubes (Clinitubes; Radiometer, Copenhagen, Denmark) from a pre-heated fingertip. The subjects then inhaled a bolus of 1.0 (♀) or 1.2 (♂) ml per kg body weight of 99.97% chemically pure CO (AGA Norge, Oslo, Norway) administered via a 100-ml plastic syringe (Omnifix; Braun, Kronberg im Taunus, Germany) to a spirometer (Blood tec GmbH, Germany). In this closed circuit, the CO was rebreathed for 2 min together with 3 l of pure O_2 (AGA Norge) while checking for leakages using a CO analyser (Draeger, Lübeck, Germany). Two capillary blood samples were collected, 6 and 8 min after the administration of CO. All blood samples were immediately analysed in duplicate for percent carboxyhaemoglobin using an ABL80 CO-OX FLEX (Radiometer, Copenhagen, Denmark). After rebreathing, the CO not absorbed by the body was calculated by multiplying the CO concentration of the rebreathing bag by the bag volume and the subject's estimated residual lung volume (Miller et al., 1998). The CO exhaled between the time-point of disconnecting from the spirometer to the blood sampling was estimated by multiplying the difference in end-tidal CO concentration before and after rebreathing by the estimated alveolar ventilation (West, 2008). The Hb_{mass} was calculated by dilution of CO in blood (Schmidt & Prommer, 2005) with correction for loss of CO to myoglobin (0.3% of the administered CO per minute) (Prommer & Schmidt, 2007). The typical error in the duplicate Hb_{mass} determinations was 1.10% (error in absolute values: 8.4 g; intraclass correlation: 0.99), and a Bland-Altman plot is presented in Fig. 3.6.

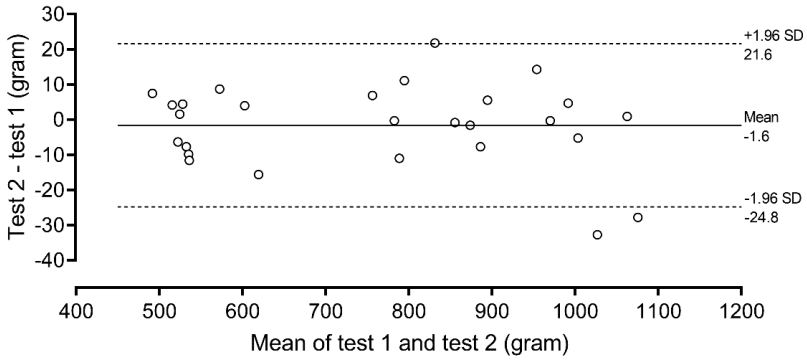

Fig. 3.6 Bland-Altman plot of duplicate haemoglobin mass measurements (study I). Lines represent mean bias and mean bias ± 1.96 standard deviations (SD) of the difference scores.

Before and after ET in study I, blood from an antecubital vein was collected in the morning under standardised, seated conditions. Haematological variables were analysed for EDTA blood (3 ml; BD, Franklin Lakes, NJ, USA) using a Sysmex XN-9000 (Sysmex, Kobe, Japan). Serum samples (SST II Advance 5 ml; BD) were placed at room temperature for coagulation (30 min), centrifuged at 1500 G for 10 min and stored at 4°C until analysed for ferritin concentration (Advia Chemistry XPT; Siemens Healthineers, Erlangen, Germany). To derive intravascular volumes (BV, RBCV, PV), the formulae given by Siebenmann et al. (2017) were used with no correction for venous to whole-body haematocrit. The phlebotomy trial was conducted 3.9 ± 2.2 days after the post-ET trial. A BV equal to each individual's ET-induced increase in Hb_{mass} (ΔHb_{mass} / [Hb]) was removed by phlebotomy via an 18 G catheter (BD) indwelling in an antecubital vein.

In study II, during both the control and the experimental days, blood samples (3 ml, EDTA; BD) were obtained via an 18 G catheter (BD) indwelling in an antecubital vein that was regularly flushed with normal saline (0.9% NaCl) to maintain patency. [Hb] was measured in duplicate (triplicate if large deviations occurred) on a hemoximeter (ABL80 CO-OX FLEX) and haematocrit was measured in triplicate using the micro-centrifugation method (6 min at 12,800 rpm; Hettich, Tuttlingen, Germany) and adjusted for trapped plasma (3%). The intra-assay typical error was 1.6% (error in absolute values: 0.23 $g \cdot dl^{-1}$; intraclass correlation: 0.95) and 0.5% (intraclass correlation: 0.99) for [Hb] and haematocrit, respectively (n = 296-309). Sysmex XN-9000 and the micro-centrifugation method resulted in practically identical haematocrit after adjusting for trapped plasma (bias: 0.14 ± 0.72 %-points; Bland-Altman ± 95% limits of agreement: 1.4 %-points; Pearson r = 0.98; n = 16).

The same catheter was used for phlebotomy of 150 ml blood before BVR_{150ml}, and an additional 300 ml blood before BVR_{450ml} (450 ml in total). The blood was drawn slowly over 5 min (BVR_{150ml}) or 10 min (BVR_{450ml}) during which the blood pressure was monitored. The subjects rested seated for 10 min after phlebotomy before moving to the cycle ergometer. The blood removed equalled 2.5 ± 0.3% (range: 2.1-3.2) and 7.6 ± 1.0% (range: 6.4-9.6) of the participants' total BV.

In study II, the RBCV, PV and BV during exercise trials were calculated assuming a constant Hb_{mass} over the experimental period (Eastwood et al., 2008; Prommer et al., 2008). The reductions in intravascular volumes induced by phlebotomy and blood sampling were included in these calculations, and it was assumed that all changes in [Hb] and haematocrit could be accounted for by fluctuations of PV occurring due to factors such as sweating,

transcapillary fluid shifts, swelling/shrinking of RBCs, intestinal fluid uptake and urine production (Dill & Costill, 1974; Stewart et al., 2003).

3.4.5. Transthoracic echocardiography (study I)

All subjects underwent three echocardiographic studies (pre-ET, post-ET and directly after phlebotomy) (Vivid E95) using a 2.5 MHz (M5Sc) and an active matrix 4D volume phased array transducer. Echocardiographic views were obtained using greyscale harmonic imaging according to the recommendations of the European Association of Cardiovascular Imaging (Lang et al., 2015). Recordings were digitally stored for offline post-hoc analysis (EchoPac; GE Vingmed Ultrasound AS), carried out by a blinded observer. From 2D echocardiography, left ventricular (LV) dimensions and LV diastolic function parameters were assessed. Tissue Doppler was used to assess wall motion velocities at the mitral annulus level and mean values from the septal and lateral walls are reported. LV mass was calculated using the Devereux' formula (Devereux et al., 1986). 3D data sets, including LV volumes and ejection fraction, were obtained using a dedicated semi-automated algorithm.

Strain analysis was performed using 2D speckle tracking echocardiography with automatic tracking of acoustic markers on a frame-by-frame basis throughout the cardiac cycle. The endocardial borders were traced in the end-systolic frame of the 2D images from the apical 4-, 2-chamber, and apical long-axis views for the assessment of longitudinal strain. The operator manually adjusted segments where the automatic tracking failed. Peak systolic LV global longitudinal strain was averaged from 16 LV segments. The frame rate was 61 ± 5 Hz.

3.4.6. Catheterisation and blood sampling (study III)

The subjects reported to the laboratory at 8:00 or 14:00 and catheters were placed percutaneously under local anaesthesia (2% lidocaine) using the Seldinger technique. Briefly, a 20-gauge catheter (Arrow ref. # ES-04150; Teleflex Medical, Wayne, PA, USA) 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, Irvine, CA, USA) positioned at the height of the parasternal fourth intercostal space. Via an amplifier

(Gould instrument systems, Cleveland, OH, USA), blood pressures were sampled at 100 Hz using a PC with LabVIEW software (National Instruments, Austin, TX, USA).
Blood was sampled anaerobically using heparinised syringes (safePICO; Radiometer, Copenhagen, Denmark) and immediately analysed for [Hb], SO_2, O_2 tension (PO_2) and CO_2 tension (PCO_2), pH and [La] using an ABL90 FLEX (Radiometer, Denmark). CaO_2 and femoral venous O_2 content (Cv_fO_2) were calculated as: $(1.34 \times [Hb] \times SO_2) + (0.003 \times PO_2)$. 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 (1966). Blood samples were taken simultaneously from both veins and the artery.

3.4.7. Femoral arterial blood flow (study III)

LBF was measured in the femoral artery of both legs using ultrasound Doppler (Vivid E95) 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 maximised according to the vessel width but kept clear of the vessel walls. The insonation angle was minimised 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) in 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 before the right leg in the following order: B-mode left, Doppler left, Doppler right and B-mode right (Fig. 3.5). Blood velocity was measured continuously and averaged over ~45 s. LBF was measured ~1.0-1.5 min earlier in the left leg compared to the right leg, but with no difference between the mean of TL and CON due to counterbalancing. Recordings were anonymised before LBF was analysed using the Vivid E95 software.

3.4.8. Calculations (study III)

Leg a-$v_fO_2$diff was computed as the difference between CaO_2 and Cv_fO_2 for each leg. The a-$v_fO_2$diff divided by CaO_2 gave the O_2 extraction fraction. Leg O_2 delivery was calculated as the product of LBF and CaO_2. Leg $\dot{V}O_2$ was the product of LBF and a-$v_fO_2$diff. Leg vascular conductance was calculated as LBF divided by the mean arterial pressure minus femoral venous pressure. Mean capillary PO_2 and muscle O_2 conductance (D_MO_2) were calculated as

previously described (Calbet et al., 2005; Roca et al., 1989), using the measured arterial and femoral venous PO_2. D_MO_2 [$\dot{V}O_2$ / (mean capillary PO_2 – mitochondrial PO_2)] is recognised 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 utilisation by the cytochromes. The calculated D_MO_2 depends on the recruited exercise muscle mass (Cardinale et al., 2019), F_IO_2 and exercise intensity (Calbet et al., 2015b; Calbet et al., 2009). Therefore, between-leg differences in D_MO_2 are described as differences in the "recruitment" of D_MO_2 rather than a difference in the maximal capacity (Calbet et al., 2015b). 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 (2000). 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)] (Piiper, 2000).

3.4.9. Knee extension ergometer (study III)

Knee extension exercises were performed on an electromagnetically braked ergometer modified after Hallén et al. (1996). The ergometer isolates leg muscle contractions to the quadriceps femoris (Andersen et al., 1985). The hip angle was ~128º, and the upper body was strapped using a four-point seat belt to minimise the engagement of muscle mass to stabilise 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 using custom-made software (Labview). 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. Post-hoc analysis (Fig. 3.7), showed equal involvement of both legs during the invasive 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).

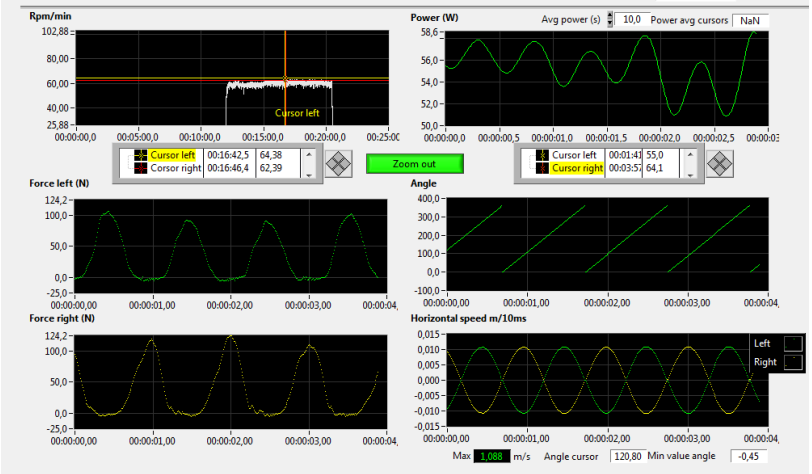

Fig. 3.7 Custom-made software used for post-hoc analysis. In this example, the cursor is zoomed in on three and four duty cycles for the right and left leg, respectively. Note the minimal negative force during the recovery phase.

3.4.10. Muscle biopsy analysis (study I & III)

Skeletal muscle biopsy

Muscle biopsies (~100-200 mg) were collected from the mid-portion of Vastus Lateralis after local anaesthesia (xylocaine), 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, Newtown, UK) and quickly frozen in isopentane cooled on liquid nitrogen to freezing point (approx. -120^{o}C). Tissue allocated for Western Blotting was immediately snap-frozen in liquid nitrogen. All tissue samples were stored at -80^{o}C until further analysis.

Immunohistochemistry

Serial 8 µm transverse cross sections were cut at -20^{o}C (Leica CM1860 UV; Leica Biosystems, Danvers, MA, USA), mounted on microscope slides (Superfrost Plus; Thermo Fischer Scientific, Waltham, MA, USA), air-dried and stored at -80^{o}C until further analysis. The sections were blocked for 60 min with 1% bovine serum albumin (Sigma Life Science, St Louis, MO, USA) (study I) or with 10% goat serum (ab7481; Abcam, Cambridge, UK) and 1% bovine serum albumin (study III) in a phosphate-buffered saline (Sigma Life Science) and 0.05% Tween-20 (VWR, West Chester, PA, USA) solution (PBS-t). Primary antibodies against 1) myosin heavy chain type 1 (1:500 dilution; BA-D5, developed by

Schiaffino, S., obtained from DSHB, Iowa City, IA, USA) and dystrophin (1:500; ab15277; Abcam, Cambridge, UK), or 2) the endothelial marker CD31 (1:100; M0823; Dako A/S, Glostrup, Denmark) and dystrophin were diluted in the blocking solution and incubated overnight at 4°C. The sections were then washed 3×10 min in PBS-t, incubated with secondary antibodies (1:200; Alexa Fluor 488, A11001 and A11012; Invitrogen Molecular Probes, Carlsbad, CA, USA) for 60 min and again washed 3×10 min in PBS-t before being mounted with Prolong Gold antifade reagent with DAPI (Life Technologies Corp., Carlsbad, CA, USA) and covered with a cover glass. The sections were visualised under a 10× / 0.30 NA air objective (UplanFL N; Olympus corp.; Tokyo, Japan) 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., Mississauga, ON, Canada). Fibre cross-sectional areas, fibre types and manual identification of capillaries were conducted and analysed using TEMA software (CheckVision, Denmark). Capillarisation was expressed as the capillary-to-fibre ratio, capillaries in contact with each fibre, and capillary density (capillaries per mm^2). The investigator was blinded for the subject, time point and leg identity. A mean of 198 ± 56 (range: 96-345) and 189 ± 41 (range: 118-251) fibres were analysed for each cross section in study I and III, respectively.

Protein immunoblot

For Western blotting analyses, ~60 mg of muscle tissue was homogenised in 1 ml T-PER (Tissue Protein Extraction Reagent, 78510; Thermo Fischer Scientific) and 20 µL Halt Protease & Phosphatase Inhibitor Cocktail (78440; Thermo Fischer Scientific). The tissue lysate was extracted, aliquoted and stored at -80°C until further analysis. The protein concentration was measured using a commercial kit (BioRad DC Protein Assay, 5000116; Bio-Rad Laboratories, Hercules, CA, USA) and a FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany). Standard Western blotting procedures were applied for quantification of citrate synthase, cytochrome-c-oxidase subunit 4 (COX-IV) and hydroxyacyl-CoA dehydrogenase (HAD) as surrogate indices of mitochondrial volume density (Larsen et al., 2012). 20µg of protein were separated using 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 h in a 5% fat-free skimmed milk (Merck, Darmstadt, Germany) 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 310005; GeneON, Ludwigshafen am Rhein, Germany) and then incubated overnight (4^{o}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, Danvers, MA, USA) secondary antibody was applied for 1 h at room temperature followed by visualisation using an HRP detection system (Super Signal West Dura Extended Duration Substrate; Thermo Fischer 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 (21059; Thermo Fischer Scientific) in study III. 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 and post samples (from both legs in study III), were loaded on the same gel in duplicate in counterbalanced order, and mean values were used for statistical analysis. In study III, a human control sample (a pool of all biopsies) was loaded in duplicate on each gel, and the average intensity of this sample was used for normalisation to allow for semi-quantitative comparisons across gels/subjects (Thomassen et al., 2018).

3.5. Study IV: Data extraction and processing

The data used in study IV were collected using two approaches:

1) Papers containing individual data on pulmonary $\dot{V}O_{2max}$, $\dot{Q}_{max}$, a-$\bar{v}O_2$diff and systemic O_2 extraction ($\bar{O}_2$ extraction) fraction measured during whole-body maximal exercise were included (Part 1).
2) To investigate the relationship between $\dot{V}O_{2max}$ and peripheral O_2 extraction fraction, mean data from studies reporting LBF, a-$v_fO_2$diff and leg O_2 extraction fraction measured during whole-body maximal exercise were included (Part 2).

This strategy was chosen since a large amount of individual data has been published on systemic responses, whereas we were unable to identify other than mean values in studies investigating peripheral haemodynamics and O_2 extraction fraction. The data were identified through searches conducted in the PubMed database using several combinations of the

following search terms: circulation, circulatory, hemodynamic(s), cardiac output, leg blood flow, arteriovenous oxygen difference, oxygen extraction and exercise. Cross-reference checks were also conducted, in addition to separate searches on authors with research papers already included in the database. Only exercise modes engaging a large muscle mass that could elicit $\dot{V}O_{2max}$ were included (cycling, running and cross-country skiing using the diagonal technique). Data from cross-sectional studies or before and after training interventions that were collected in normoxia on young (< 40 years old) and healthy individuals were included. Data collected in hypoxia, after acclimatisation to altitude, in altitude natives, in hyperthermia, with atrial pacing, after bed rest and after BV manipulations were excluded. The control condition was used when the above forms of manipulation of the cardiovascular system were conducted. Only catheterisation studies that used invasive methods to measure $\dot{Q}_{max}$ (indicator-dilution techniques or the Fick method) and LBF (bolus or continuous infusion thermodilution and indicator-dilution techniques) were included. Only individual data from men were used (Part 1). In Part 2, studies that had a sample with a majority of men were used (≥ 50%). When several papers reported data from the same data collection, only one of the papers was included. If a paper used some of the same subjects as previously reported, but with supplementation with new subjects, the data were included. The included papers are presented in Tables 3.2 and 3.3 for Parts 1 and 2, respectively.

Table 3.2 Research papers reporting individual values of maximal O_2 uptake ($\dot{V}O_{2max}$), maximal cardiac output ($\dot{Q}_{max}$) and arterial to mixed venous O_2 difference (a-$\bar{v}O_2$diff). In studies reporting arterial O_2 content (or was possible to calculate), the systemic O_2 extraction fraction ($\bar{O}_2$ extraction) was calculated.

				Methods used to measure:			$\bar{O}_2$
Research paper	n	Exercise	Age	$\dot{Q}_{max}$	$\dot{V}O_{2max}$	a-$\bar{v}O_2$diff	extraction
Blomqvist et al. (1970)	4	Cycling	23-33	ID	DB		-
Ekblom & Hermansen (1968)	14	Running	22-34	ID	DB	Calculated using the Fick principle	Yes
Ekblom et al. (1968)	8	Cycling	19-27	ID	DB		Yes
Ekblom (1970)	7	Cycling	22-26	ID	DB		Yes
Epstein et al. (1965)	2	Running	21	ID	Custom		-
Epstein et al. (1967)	4	Running	18-30	Fick	Custom	Measured	-
Gleser (1973)	6	Cycling	20-23	ID	DB	Calculated using the Fick principle	Yes
Hermansen et al. (1970)	13	C / R	19-34	ID	DB		Yes
Mitchell et al. (1958)	6	Running	-	ID	DB		-
Robinson et al. (1966)	5	Running	19-31	ID	Custom		-
Saltin (1964)	4	Cycling	23-26	ID	DB		-
Saltin & Stenberg (1964)	4	Cycling	23-25	ID	DB		-
Saltin et al. (1968a)	5	Cycling	19-21	ID	DB		Yes
Saltin et al. (1968b)	4	Cycling	20-21	ID	DB		-
Stenberg et al. (1966)	6	Cycling	20-36	ID	DB		Yes
Stenberg et al. (1967)	5	Cycling	20-39	ID	DB		-
Åstrand et al. (1964)	12	Cycling	21-30	ID	DB		Yes

n: number of subjects meeting the inclusion criteria. Note that some subjects were investigated on more than one occasion (before/after training, running/cycling); ID: indicator-dilution method using indocyanine green or Evans blue dye (only used in Mitchell et al., 1958); DB: Douglas bag technique

Table 3.3 Research papers reporting mean values of leg O_2 uptake ($\dot{V}O_2$), leg blood flow (LBF) and arterial to femoral venous O_2 difference (a-$v_fO_2$diff) during maximal exercise (cycling and cross-country skiing).

Research paper	n	Age ($\bar{x}$)	Method used to measure: Pulmonary $\dot{V}O_2$	LBF	a-$v_fO_2$diff	O_2 extraction reported / may be calculated
Bender et al. (1988)	7♂	22	Custom	TD-B	Measured via arterial and femoral venous blood sampling	Yes
Calbet et al. (2003)	4♂3♀	24	Med. Graph. CPX	TD-C		Yes
Calbet et al. (2005); Calbet et al. (2004)	3♂	24	Amis 2001	TD-C		Yes
Calbet et al. (2006)	10♂	24	Quark b2	TD-C		Yes
Calbet et al. (2007)	9♂	33	Quark b2	TD-C		Yes
Calbet et al. (2015a)	9♂	31	Quark b2	TD-C		Yes
Calbet et al. (2015b)	11♂	22	Vmax 29	TD-C		Yes
Cardinale et al. (2019)	4♂3♀	33	Oxycon Pro	TD-C		Yes
Cardus et al. (1998)	13♂5♀	23	Custom	TD-C		Yes
Gonzalez-Alonso & Calbet (2003)	8♂	24	OCM-2	TD-C		Yes
Harms et al. (1997)	7♂	29	Custom	TD-C		Yes
Klausen et al. (1982)	6♂	23	Douglas bag	ID-B		-
Knight et al. (1992)	7♂	29	Custom	TD-C		Yes
Knight et al. (1993)	12♂	29	Custom	TD-C		Yes
Lundby et al. (2008a)	8♂	26	Quark b2	TD-C		Yes
Lundby et al. (2008b)	8♂	27	Quark b2	TD-C		Yes
Lundby et al. (2004); Lundby et al. (2006)	6♂	26	Custom	TD-C		Yes
Mortensen et al. (2008)	13♂	28	Quark b2	TD-C		Yes
Mortensen et al. (2005)	10♂	27	Quark b2	TD-C		Yes
Munch et al. (2014)	10♂	27	Quark CPET	TD-C		Yes
Poole et al. (1991)	6♂	26	Custom	TD-C		Yes
Roca et al. (1989)	6♂	24	Custom	-		Yes
Roca et al. (1992)	8♂4♀	22	Custom	TD-C		Yes
Rud et al. (2012)	4♂4♀	23	Douglas bag	TD-C		Yes
Trangmar et al. (2017)	9♂	26	Not reported	TD-C		Yes
van Hall et al. (2001)	5♂1♀	26	Med. Graph. CPX	TD-C		Yes

n: number of subjects; TD-B: bolus-infusion thermodilution method; TD-C: continuous-infusion TD; ID-B; bolus indicator-dilution method (I-labelled human albumin).

3.5.1. Calculations

When the data were published in graphs and not in tables or text, ImageJ (v1.50b; National Institutes of Health, USA) was used for data extraction. If not all variables were reported in the research paper, the reported data were used to derive the missing values via the following formulas or combination of formulas if possible:

$$\text{Pulmonary } \dot{V}O_{2max} = \dot{Q}_{max} \times \text{a-}\bar{v}O_2\text{diff} \quad (1)$$

$$\text{Leg } \dot{V}O_2 = \text{LBF} \times \text{a-}v_fO_2\text{diff} \quad (2)$$

$$SV = \dot{Q}_{max} / HR \quad (3)$$

$$\text{Blood } O_2 \text{ content (e.g., } CaO_2) = 1.39 \times [Hb] \times SO_2 + 0.003 \times PO_2 \quad (4)$$

$$\text{Leg } O_2 \text{ delivery} = \text{LBF} \times CaO_2 \quad (5)$$

$\overline{O}_2$ extraction fraction = a-$\bar{v}O_2$diff / CaO_2 (6)

O_2 extraction fraction = a-$v_fO_2$diff / CaO_2 (7)

Systemic vascular conductance = $\dot{Q}_{max}$ / (MAP-CVP) (8)

If no arterial PO_2 was reported, 100 mmHg was assumed for the calculation of CaO_2 (i.e., 3 ml O_2 freely dissolved in blood plasma per 1 L of blood). Central venous pressure (CVP) at $\dot{V}O_{2max}$ was taken as 5 mmHg (Mortensen et al., 2005).

3.6. Statistical analysis

Data in text and tables are presented as mean ± standard deviation (SD). In graphs, error bars denote 95% confidence limits or standard error of the mean, which is specified in each figure text. The data were initially assessed for normal distribution using the D'Agostino-Pearson test. The alpha-level was set to ≤ 0.05 and P-values between > 0.05 and ≤ 0.10 were considered to indicate trends. GraphPad Prism (v. 8.0.1; GraphPad Software, CA, USA) and Microsoft Office Excel 2013 (Microsoft Corporation, WA, USA) were used for statistical analysis.

3.6.1. Study I

For variables only measured pre-ET and post-ET, group changes were analysed with a paired sample t-test. Maximal and submaximal responses measured on three time points were analysed using repeated measures ANOVA and two-way repeated measures ANOVA (workload x time point), respectively. If a significant main effect or interaction effect was found, ANOVA was followed by Dunnett's multiple comparisons test, comparing the control situation (pre-ET) with the post-ET and the phlebotomy trial.

3.6.2. Study II

Maximal responses and resting measurements were analysed using a paired sample t-test and only planned comparisons were conducted: control 1 vs BVR_{150ml} and control 2 vs BVR_{450ml}. A paired sample t-test was used to analyse whether the change in $\dot{V}O_{2max}$ from control 1 to BVR_{150ml} was different from the change from control 2 to BVR_{150ml}. Submaximal responses were analysed using a two-way repeated measures ANOVA (trial x workload) followed by

the Bonferroni's multiple comparisons test. Control 1 vs BVR_{150ml} and control 2 vs BVR_{450ml} were analysed separately.

3.6.3. Study III

Differences between legs during exercise and in protein content between pre- and post-test were analysed using two-way repeated measures ANOVA (leg × power output and leg × time point, respectively). If a significant main effect or interaction effect was found, Bonferroni's multiple comparison post-hoc test was applied. Differences between legs during rest and one-level changes from pre- to post-test were analysed with a paired sample t-test.

3.6.4. Study IV

Regression analysis was conducted using simple linear regression, second-order polynomials and exponential decay models ($y = a \cdot e^{-K \cdot X} + plateau$), all using least squares as the fitting method. Regression lines/curves are presented with 95% confidence bands representing the likely location of the true curve.

4. Results and discussion

In this chapter, the main findings of the four studies are presented and discussed according to the aims. Detailed results for each specific study are presented in the manuscripts at the end of this thesis.

4.1. Increased $\dot{V}O_{2max}$ after endurance training despite blunted blood volume expansion

In study I, we tested the hypothesis that the ET-induced increases in $\dot{V}O_{2max}$ and $\dot{Q}_{max}$ would return to pre-ET levels after counteracting the BV expansion during upright cycling but remain elevated during supine cycling owing to improved venous return. Contrary to hypothesis A, $\dot{V}O_{2max}$ and $\dot{Q}_{max}$ were increased by 11% ($P < 0.005$) and 9% ($P = 0.02$-0.08), respectively after phlebotomy, compared with before training, regardless of whether the testing was conducted during upright or supine cycling (Fig. 4.1).

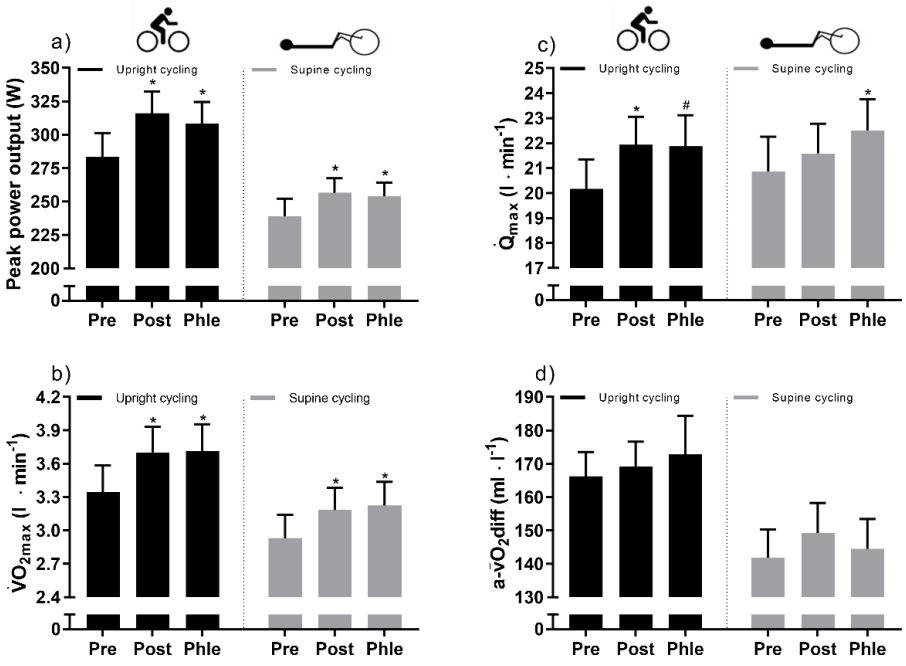

Fig. 4.1 Peak power output (a); maximal O_2 uptake ($\dot{V}O_{2max}$) (b); cardiac output ($\dot{Q}_{max}$) (c); and arteriovenous O_2 difference (a-$\bar{v}O_2$diff) (d) before training (Pre), after training (Post) and after training and phlebotomy (Phle). Error bars indicate the standard error of the mean. * Significant change from pre-training ($P \leq 0.05$). # Trend towards change from pre-training ($0.05 < P \leq 0.10$). n = 11

This contradicts previous studies suggesting that the ET-induced increases in $\dot{V}O_{2max}$ and $\dot{Q}_{max}$ are completely abolished after reversing the BV to the pre-ET level (Bonne et al., 2014; Montero et al., 2015a). However, our data falls in line with studies finding robust improvements in $\dot{V}O_{2max}$ and maximal exercise capacity despite absent BV expansion (Helgerud et al., 2007; Jacobs et al., 2013; Shoemaker et al., 1996). In this context, for Bonne et al. (2014) and Montero et al. (2015a), the ET-induced increases in BV were 382 ml (7%) and 310 ml (6%), respectively, compared to 181 ml (4%) in the present study (Table 4.1). Yet, improvements in $\dot{V}O_{2max}$ (9-11%) and $\dot{Q}_{max}$ (7-10%) during upright cycling were similar in the three studies. Thus, ET-induced increases in $\dot{V}O_{2max}$ and $\dot{Q}_{max}$ of this magnitude do not depend on BV expansion alone, may have been mediated by different mechanisms in the three studies, and suggests that the enhancement of $\dot{V}O_{2max}$ with ET is multifactorial (Saltin et al., 1968a). This notion is also strengthened by an ET-induced increased LV mass in the present study (13 ± 17 g; P = 0.027; Table 4.2) as opposed to no change as reported by Bonne et al. (2014).

Table 4.1 Haematological variables and body composition measured before and after 10 weeks of endurance training.

	Pre-training (mean ± SD)	Post-training (mean ± SD)	% Change (mean ± SD)
Body composition			
Body weight (kg)	74.2 ± 12.1	72.7 ± 11.3	-1.8 ± 2.6*
Lean mass (kg)	51.2 ± 9.0	51.3 ± 9.5	0.0 ± 2.6
2-leg lean mass (kg)	18.0 ± 3.9	18.0 ± 4.0	-0.2 ± 1.8
Fat mass (kg)	20.5 ± 4.3	18.8 ± 4.4	-9.0 ± 8.2*
Haematology			
Hb_{mass} (g)	795 ± 196	820 ± 196	3.3 ± 2.9*
BV (ml)	5098 ± 929	5279 ± 947	3.7 ± 5.6*
RBCV (ml)	2388 ± 548	2370 ± 528	-0.3 ± 5.0
PV (ml)	2712 ± 432	2909 ± 464	7.5 ± 9.2*
[Hb] ($g \cdot dl^{-1}$)	15.5 ± 1.5	15.4 ± 1.3	-0.2 ± 3.6
Haematocrit (%)	46.5 ± 3.7	44.6 ± 3.3	-3.8 ± 4.5*
MCHC ($g \cdot dl^{-1}$)	33.3 ± 1.8	34.5 ± 0.9	4.0 ± 4.8*
S-Ferritin ($\mu g \cdot l^{-1}$)	87.7 ± 58.8	86.9 ± 52.9	11.9 ± 41.7

n = 11. BV, blood volume; [Hb], haemoglobin concentration; Hb_{mass}, haemoglobin mass; MCHC, mean corpuscular haemoglobin concentration; PV, plasma volume; RBCV, red blood cell volume. * Significant change from pre- to post-training ($P \leq 0.05$).

Because of the small BV expansion and consequently small BV withdrawal, little hypovolemia-induced impairment on venous return was likely elicited in our subjects. This was supported by the absence of reductions in $\dot{Q}_{max}$, $\dot{V}O_{2max}$, and submaximal SV (data are shown in paper I) during upright cycling as well as there being no change in diastolic filling parameters from before to after phlebotomy. Consequently, we were unable to test one of our main hypotheses: that, when $\dot{V}O_{2max}$ and $\dot{Q}_{max}$ during upright cycling were reversed to pre-ET

levels after phlebotomy; the improvements would be preserved during supine cycling owing to the beneficial gravitational effects on venous return.

Table 4.2. Cardiac morphology and function measured by echocardiography at rest before and after 10 weeks of endurance training, as well as directly after phlebotomy.

Variable	Pre-training (mean ± SD)	Post-training (mean ± SD)	Phlebotomy (mean ± SD)
Left ventricular morphology			
LV mass (g)	123 ± 37	137 ± 37*	
IVSd (mm)	7.4 ± 0.8	8.1 ± 0.8	
LV PWd (mm)	7.3 ± 0.9	7.7 ± 0.9	
LV end-diastolic diameter (mm)	49.1 ± 6.5	50.1 ± 6.2	49.9 ± 6.6
3D end-diastolic volume (ml)	124 ± 35	124 ± 40	117 ± 39
3D end-systolic volume (ml)	49 ± 15	50 ± 19	46 ± 17
LV mass-to-volume ratio ($g \cdot ml^{-1}$)	1.02 ± 0.26	1.12 ± 0.20	1.19 ± 0.21*
Left ventricular systolic function			
3D ejection fraction (%)	61 ± 3	60 ± 3	61 ± 4
3D stroke volume (ml)	75 ± 21	74 ± 21	71 ± 23
Global longitudinal strain (%)	-22.5 ± 1.4	-22.1 ± 1.3	-21.1 ± 1.8#
Left ventricular diastolic function			
E ($cm \cdot s^{-1}$)	72.9 ± 14.5	73.2 ± 17.9	69.2 ± 13.3
A ($cm \cdot s^{-1}$)	47.2 ± 5.4	47.3 ± 5.0	46.2 ± 10.4
E / A ratio	1.6 ± 0.4	1.6 ± 0.4	1.6 ± 0.4
E' ($cm \cdot s^{-1}$)	12.5 ± 2.1	12.0 ± 2.6	11.6 ± 1.9
A' ($cm \cdot s^{-1}$)	7.8 ± 1.1	7.5 ± 1.4	7.2 ± 1.5
E / E' ratio	5.8 ± 0.6	6.1 ± 1.0	6.1 ± 1.2

n = 11, except for global longitudinal strain (n = 10). E and A, peak mitral inflow velocity during early diastole and during atrial systole, respectively; E' and A', myocardial peak velocity during early diastole and atrial systole, respectively; IVSd, interventricular septum thickness in end-diastole; LV, left ventricular; PWd, posterior wall thickness in end-diastole.* Significantly different from pre-training ($P \leq 0.05$). # Trend towards being different from pre-training ($0.05 < P \leq 0.10$).

It may be that the cardiovascular system can maintain venous return to the heart despite small BV-reductions by redistributing venous volumes through increased vasomotor activity acting on capacitance vessels. This is supported by the unchanged LV EDV, SV and peak mitral inflow velocity during early diastole from before to just after the phlebotomy procedure. There is, however, some uncertainty in extrapolating these responses measured during supine rest to upright maximal exercise, where LV filling times are shorter and challenge ventricular filling (Gledhill et al., 1994). However, in line with our findings, maintained $\dot{Q}_{max}$ despite small to moderate reductions in PV and thus BV have been observed in studies examining fluid loss after heat stress and prolonged exercise (Saltin, 1964; Saltin & Stenberg, 1964). Thus, it was argued that an effective contribution from the muscle pump and increased vasomotor activity enabled a maintained SV despite reduced BV. In contrast to small BV losses, phlebotomy of one unit of blood (450 ml) or more reduces $\dot{V}O_{2max}$ by lowering venous return (Kanstrup & Ekblom, 1984; Krip et al., 1997). Therefore, a certain threshold might exist at which small reductions in BV are not detrimental to venous return,

acting as a mechanism for the circulation to cope with small BV losses and PV reductions induced by factors such as dehydration.

4.2. The body can cope with small blood losses to maintain $\dot{V}O_{2max}$

The findings in study I resulted in hypothesis C: "Compensatory mechanisms will preserve $\dot{V}O_{2max}$ and $\dot{W}_{peak}$ during incremental exercise to exhaustion after the acute withdrawal of 150 ml blood, but will be insufficient after withdrawing 450 ml". This was experimentally tested in study II by comparing measurements obtained during maximal exercise conducted on a control day with normal BV to those obtained after BV removals of 150 ml (BVR_{150ml}) and 450 ml (BVR_{450ml}).

In line with our hypothesis, $\dot{V}O_{2max}$ was unchanged after BVR_{150ml} (-1.1 ± 2.3%; P = 0.124) but reduced by 6.9 ± 3.1% after BVR_{450ml} (P < 0.001; Fig. 4.2). The reduction in $\dot{V}O_{2max}$ per millilitre blood removed was larger after BVR_{450ml} than after BVR_{150ml} (-0.7 ± 0.3 vs -0.3 ± 0.6 ml · min^{-1} · ml^{-1}, respectively; P = 0.029) and per gram Hb_{mass} removed (-4.9 ± 1.9 vs -2.0 ± 4.3 ml · min^{-1} · g^{-1}, respectively; P = 0.024).

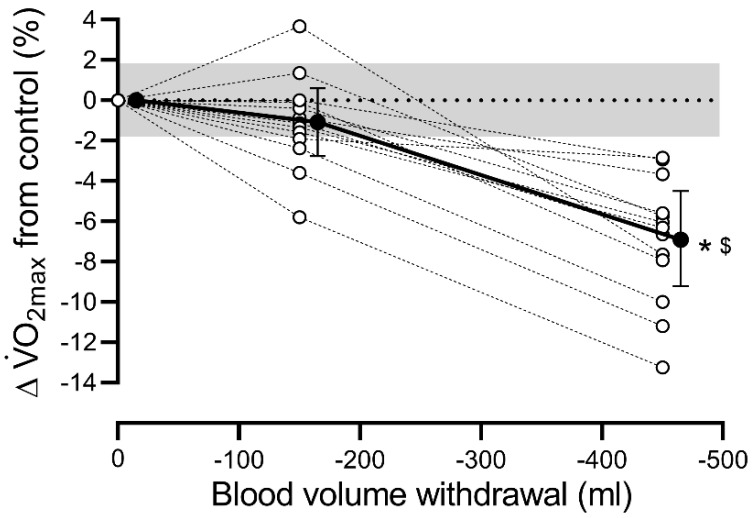

Fig. 4.2 The individual (white circles and dotted lines) and mean (black circles and solid line) differences in maximal O_2 uptake ($\dot{V}O_{2max}$) compared to control as a function of the blood volume removed. Error bars indicate 95% confidence limits. Grey-shaded area indicates the typical error of measurement for $\dot{V}O_{2max}$ in these subjects (± 1.9%). * Significant change from control (P < 0.001). $ Significantly larger decrease after removing 450 ml compared to 150 ml blood (P < 0.001). n = 13

Therefore, compensatory mechanisms enable the circulation to cope with small but not moderate blood loss; this confirms hypothesis C. These results comply with study I and also agree with the observation of unchanged $\dot{Q}_{max}$ and $\dot{V}O_{2max}$ after small to moderate PV reductions induced by heat stress and prolonged exercise (Saltin, 1964; Saltin & Stenberg, 1964). Our results also agree with previous studies investigating the acute effect of blood withdrawal on $\dot{V}O_{2max}$ when the exercise test is initiated within 3 h after phlebotomy (Fig. 4.3). A statistical analysis of these studies shows a linear decrease in $\dot{V}O_{2max}$ as a function of the BV removed with the x-intercept at ~150 ml (Balke et al., 1954; Bonne et al., 2014; Hill et al., 2013; Krip et al., 1997; Montero et al., 2015a; Panebianco et al., 1995; Rowell et al., 1964; Skattebo et al., 2020; Woodson et al., 1978). This relationship was even stronger when restricting the comparison to studies initiating the exercise test within 1 h after phlebotomy, as conducted in studies I and II (R^2 = 0.90). Thus, collectively, these studies indicate that $\dot{V}O_{2max}$ decreases linearly with the BV removed, but with compensatory mechanisms blunting the effect of blood loss up to and including ~150 ml. This may enhance our understanding regarding the impact of small BV reductions on $\dot{V}O_{2max}$ and exercise capacity, induced by, for example, acute manipulations, dehydration or daily oscillations of PV.

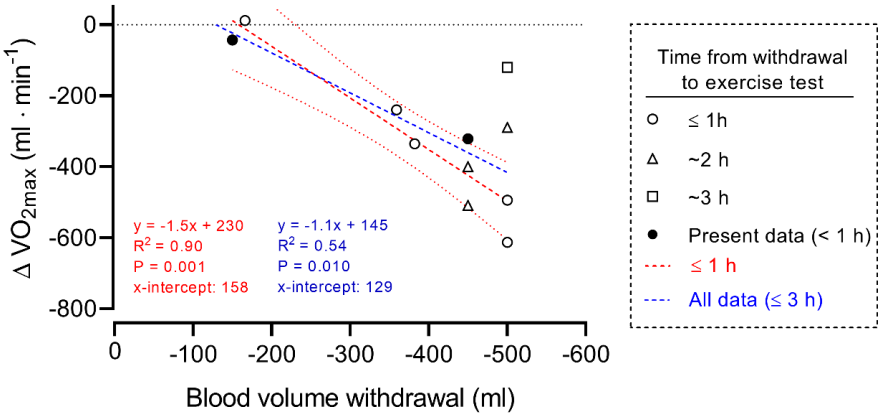

Fig. 4.3 The relationship between the magnitude of blood volume withdrawal and its impact on maximal O_2 uptake ($\dot{V}O_{2max}$) measured within the same day. The studies (Balke et al., 1954; Bonne et al., 2014; Hill et al., 2013; Krip et al., 1997; Montero et al., 2015a; Panebianco et al., 1995; Rowell et al., 1964; Skattebo et al., 2020; Woodson et al., 1978) are organised according to the time from blood withdrawal to the initiation of the exercise.

This threshold is probably dependent on the total BV of the subjects, which is supported by the fact that the two subjects with the largest individual reduction in $\dot{V}O_{2max}$

after removing 150 ml blood (see Fig. 4.2) were among the three subjects with the lowest BV and Hb_{mass}. Therefore, in women and untrained individuals with small body size and BV (< 5 l), this threshold might be slightly lower.

It must be recognised that most of the individual changes in $\dot{V}O_{2max}$ after withdrawing 150 ml blood were within the typical error of measurement, and the mean effect approached a statistically significant reduction. Therefore, it may be that the non-significant reduction of 1.1% was a real physiological response overturned by insufficient statistical power. However, the decrease in $\dot{V}O_{2max}$ was ~6 times larger despite only withdrawing 3 times more blood before BVR_{450ml} compared to BVR_{150ml}. Therefore, the main conclusion that acute compensatory mechanisms preserve $\dot{V}O_{2max}$ after small but not moderate BV withdrawals is unaffected by this notion.

4.3. Plasma volume shift explains the unchanged $\dot{V}O_{2max}$ after small blood loss

The preserved $\dot{V}O_{2max}$ after BVR_{150ml} was probably mediated by the movement of extracellular fluid from the interstitial to the intravascular space, which re-established PV (mean difference at $\dot{W}_{peak}$ from control: -12 ± 202 ml; P = 0.835; Fig. 4.4 b), almost secured euvolaemia (-50 ± 185 ml; P = 0.375; Fig. 4.4 c) and likely maintained the venous return to the heart as indicated by the unchanged O_2 pulse and HR_{peak} at $\dot{W}_{peak}$ compared with control (Fig. 4.5 a-b). Increased vasomotor activity acting on capacitance vessels and an efficient muscle-pump action may also have redistributed venous blood volumes and preserved SV despite the small hypovolaemia (Saltin, 1964; Saltin & Stenberg, 1964).

The unchanged $\dot{V}O_{2max}$, O_2 pulse and HR_{peak} point towards preserved $\dot{Q}_{max}$ after BVR_{150ml}. This is also supported by a small reduction in the estimated CaO_2 at maximal exercise (-4 ± 7 ml · l^{-1}; P = 0.040) caused by haemodilution (reduced [Hb]; see Table 4.3). To substantiate, to enable the subjects' mean $\dot{V}O_{2max}$ of 4.7 l · min^{-1}, it can be assumed that the subjects possessed a mean $\dot{Q}_{max}$ of around 28 l · min^{-1} (Mortensen et al., 2008; Munch et al., 2014). Therefore, theoretically, due to the small reduction in CaO_2, the systemic O_2 delivery may have been reduced by ~110 ml · min^{-1} (-4 ml · l^{-1} × 28 l · min^{-1}) even with an unchanged $\dot{Q}_{max}$. Therefore, the $\dot{Q}_{max}$ had to be preserved due to the unchanged $\dot{V}O_{2max}$, and the small reduction in CaO_2 may explain the minor non-significant $\dot{V}O_{2max}$ decay of 43 ml · min^{-1} after BVR_{150ml}.

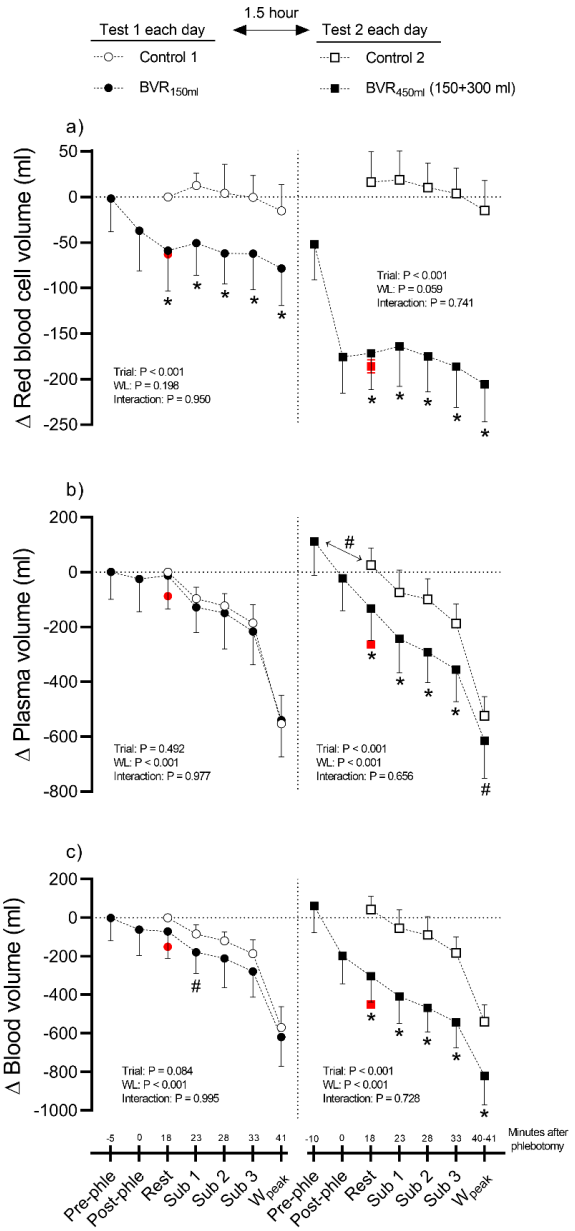

Fig. 4.4 The changes in red blood cell volume (a), plasma volume (b) and total blood volume (c) during the two control trials and the two blood volume reduction trials. All values are normalised to the resting measurement during control 1. The red symbols indicate the theoretical volumes at rest after phlebotomy if the haemoglobin concentration and the haematocrit had remained stable at the pre-phlebotomy level. Error bars indicate 95% confidence limits. The P-values for the ANOVA main effects of trial and workload (WL) as well as their interaction effect are inserted. * Significant change from control ($P \leq 0.05$). # Trend towards a change from control ($0.05 < P \leq 0.10$). n = 13

4.4. Compensatory mechanisms are insufficient after moderate blood loss

Several compensatory mechanisms were manifested after BVR_{450ml}, including PV expansion (Fig. 4.4 b) and trends towards increased HR_{peak} (3 ± 5 beats $\cdot$ min^{-1}; $P = 0.062$; Fig. 4.5 d) and decreased Vastus Lateralis TOI ($P = 0.080$; Fig. 4.5 f). The latter indicates increased leg O_2 extraction fraction (Boushel et al., 2001; Esaki et al., 2005), which was also detected after 450-ml blood donation in a previous study (McDonagh et al., 2016).

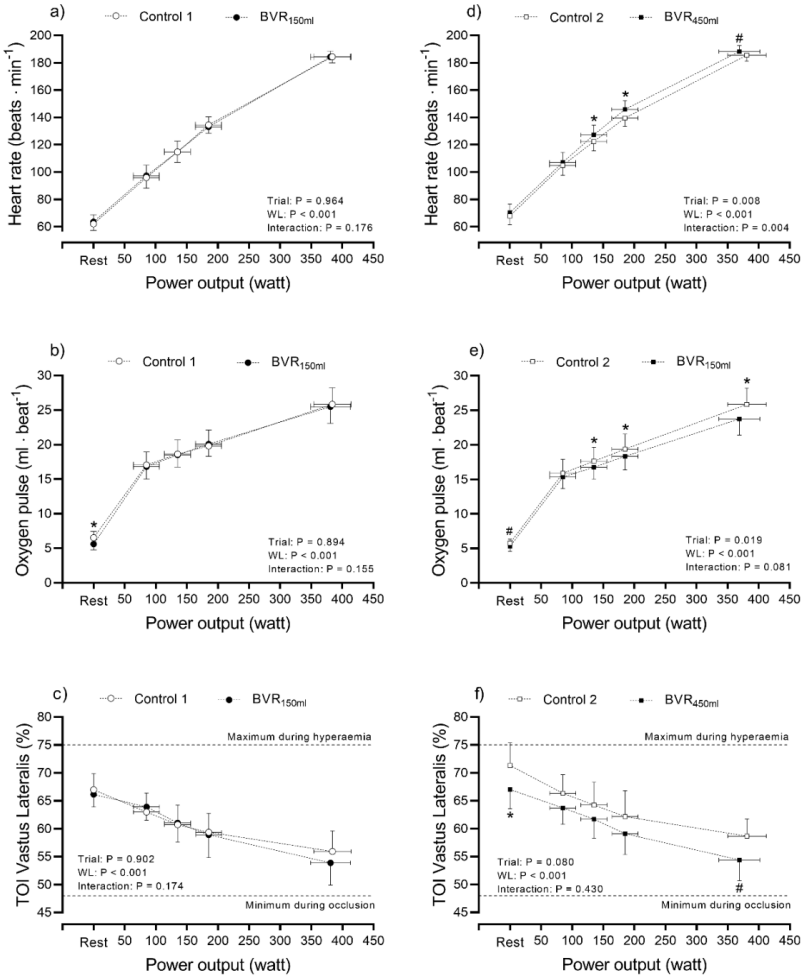

Fig. 4.5 The heart rate (a, c), O_2 pulse (b, e) and Vastus Lateralis tissue oxygenation index (TOI) (c, f) as a function of power output during the two control trials and the two blood volume reduction trials. Error bars indicate 95% confidence limits. The P-values for the ANOVA main effects of trial and workload (WL) as well as their interaction effect are inserted (submaximal workloads only). * Significant change from the control trial ($P \leq 0.05$). # Trend towards a change from the control trial ($0.05 < P \leq 0.10$). n = 12-13

At exhaustion, there was only a 281 ± 184 ml reduction in BV from control to after BVR_{450ml} ($P < 0.001$), indicating that PV-restoration attenuated 38% of the total blood loss. This is similar to the previous observation of only a ~300 ml reduction in BV 2 h after blood donation (Mora-Rodriguez et al., 2012), and suggests that transcapillary fluid shift may recover ~150-200 ml plasma rapidly after a regular blood donation of 450 ml.

Table 4.3. Measurements obtained at maximal exercise, and the body weight measured before each trial.

	First test each day		Second test each day	
	Control 1 (mean ± SD)	BVR_{150ml} (mean ± SD)	Control 2 (mean ± SD)	BVR_{450ml} (mean ± SD)
Peak power output (W)	384 ± 50	381 ± 53	381 ± 51	369 ± 54***
$\dot{V}O_{2max}$ (ml · min^{-1})	4700 ± 667	4656 ± 707	4749 ± 694	4429 ± 697***
$\dot{V}E_{peak}$ (l · min^{-1})	195 ± 29	193 ± 33	195 ± 32	190 ± 34#
BF (breaths · min^{-1})	63 ± 14	63 ± 17	62 ± 10	62 ± 20
RER_{peak}	1.19 ± 0.05	1.19 ± 0.06	1.15 ± 0.05	1.16 ± 0.06*
HR_{peak} (beats · min^{-1})	184 ± 8	184 ± 7	185 ± 7	188 ± 7#
O_2 pulse (ml · beat^{-1})	25.8 ± 3.9	25.5 ± 4.0	25.9 ± 4.0	23.8 ± 3.8***
[Hb] (gram · dl^{-1})	16.4 ± 1.0	16.0 ± 1.1#	16.3 ± 1.0	15.8 ± 1.1**
SpO_2 (%)	95 ± 2	95 ± 1	95 ± 1	95 ± 1
Estimated CaO_2 (ml · l^{-1})	212 ± 14	208 ± 14*	211 ± 14	205 ± 12**
Vastus lateralis TOI (%)	55.9 ± 6.1	53.9 ± 6.6	58.7 ± 5.1	54.4 ± 6.1#
$[La]_{peak}$ (mmol · l^{-1})	12.8 ± 1.4	12.7 ± 1.4	11.8 ± 1.5	11.5 ± 1.2
RPE	18.7 ± 1.2	19.0 ± 0.9	18.8 ± 0.8	19.3 ± 0.8#
Body weight (kg)	74.9 ± 6.6	75.1 ± 6.6	75.1 ± 6.4	75.2 ± 6.3

n = 13. [Hb], haemoglobin concentration; HR_{peak}, peak heart rate; CaO_2, arterial O_2 content; $[La]_{peak}$, peak blood lactate concentration; RER_{peak}, peak respiratory exchange ratio; RPE, rating of perceived exertion using the Borg scale (6-20); SpO_2, capillary O_2 saturation (fingertip); TOI, tissue oxygenation index (n = 12); $\dot{V}E_{peak}$, peak ventilation; $\dot{V}O_{2max}$, maximal O_2 uptake. *, ** and *** Significantly different from control ($P \leq 0.05$, $P < 0.01$ and $P < 0.001$, respectively). # Trend towards being different from control ($0.05 < P \leq 0.10$).

Despite these beneficial compensatory mechanisms, $\dot{V}O_{2max}$ reduced by 7% after BVR_{450ml}, which is similar to the average reduction of ~9% (range: 4-16%) observed in previous studies when $\dot{V}O_{2max}$ was measured within 3 h after 450-500 ml withdrawals (Balke et al., 1954; Hill et al., 2013; Krip et al., 1997; Panebianco et al., 1995; Rowell et al., 1964; Woodson et al., 1978). The combined effect of two mechanisms likely mediated this reduction: 1) a hypovolaemia-induced reduction of SV and thus $\dot{Q}_{max}$, as indicated by a significant reduction in the O_2 pulse ($P < 0.001$; Fig. 4.5 e); and 2) a reduction in the estimated CaO_2 caused by haemodilution (-6 ± 6 ml · l^{-1}; $P = 0.004$; Table 4.3), both of which reduce $\dot{V}O_{2max}$ by lowering the systemic O_2 delivery.

Again, assuming the subjects had a mean $\dot{Q}_{max}$ at around 28 l · min^{-1}, the reduction in CaO_2 may have caused a ~170 ml · min^{-1} reduction in systemic O_2 delivery alone (-6 ml · l^{-1} × 28 l · min^{-1}). Therefore, the $\dot{Q}_{max}$ was likely reduced by approximately 1-2 l · min^{-1} to

facilitate the observed reduction in $\dot{V}O_{2max}$ (-320 ml · min^{-1}). Of the very few investigations measuring $\dot{Q}_{max}$ after blood withdrawal, Bonne et al. (2014) and Montero et al. (2015a) observed a 1.7-2.3 l · min^{-1} (nitrous oxide rebreathing) reduction acutely after the withdrawal of 360-380 ml blood. In line with this, a 4.5 l · min^{-1} (acetylene rebreathing) reduction was detected alongside a ~600 ml · min^{-1} reduction in $\dot{V}O_{2max}$ 1 h after a 500 ml blood donation (Krip et al., 1997). Conversely, Ekblom et al. (1976) found an unchanged $\dot{Q}_{max}$ (dye-dilution) alongside a 240 ml · min^{-1} reduction in $\dot{V}O_{2max}$ 24 h after the withdrawal of 800 ml blood. The unchanged $\dot{Q}_{max}$ and the disproportionately small $\dot{V}O_{2max}$-reduction was caused by an "overnight" PV expansion that completely restored the BV, determined by Cr^{51} labelled RBCs and haematocrit, and the $\dot{V}O_{2max}$-reduction was entirely attributed to reduced CaO_2 (Ekblom et al., 1976). Therefore, in the first hours after blood withdrawal, $\dot{V}O_{2max}$ is lowered due to the combined effect of reduced $\dot{Q}_{max}$ and CaO_2, whereas in the following days when BV is restored by PV expansion, the reduced $\dot{V}O_{2max}$ and systemic O_2 delivery are attributed to euvolaemic haemodilution (Celsing et al., 1986; Ekblom et al., 1976; Krip et al., 1997).

4.5. Does endurance training increase the a-$\bar{v}O_2$diff?

In study I, no statistically significant change was detected in the a-$\bar{v}O_2$diff from pre- to post-ET (Fig. 4.1 d) despite robust increases in mitochondrial protein content and a trend towards an increased capillary-to-fibre ratio (Fig 4.6 a-b).

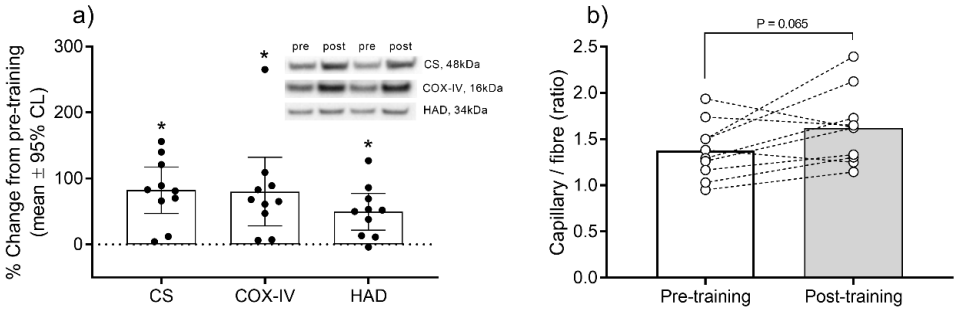

Fig. 4.6 The percentage change in protein content and representative Western blots for citrate synthase (CS), cytochrome c oxidase subunit 4 (COX-IV) and hydroxyacyl-CoA dehydrogenase (HAD) from pre- to post-training (a), as well as the capillary-to-fibre ratio pre- and post-training (b). The error bars indicate 95% confidence limits. Black and white circles indicate individual responses. * All proteins increased from pre- to post-training ($P < 0.001$). n = 10

This is in line with the majority of ET studies where the Fick principle has been used to calculate a-$\bar{v}O_2$diff from $\dot{V}O_{2max}$ and $\dot{Q}_{max}$. However, based on the average of all maximal exercise tests conducted pre- (n = 2; upright and supine cycling) and post-ET (n = 4; before and after phlebotomy), $\dot{V}O_{2max}$ increased by 318 ± 147 ml · min^{-1}. Of this increase, the increase in $\dot{Q}_{max}$ accounted for only 221 ± 202 ml · min^{-1}. Hence, on average, the a-$\bar{v}O_2$diff was elevated by 5 ± 12 ml · l^{-1} (154 ± 22 vs 159 ± 25 ml · l^{-1}) and accounted for 30% of the increase in $\dot{V}O_{2max}$. This points towards the weakness in the majority of studies aiming to assess whether a-$\bar{v}O_2$diff changes with ET, study I included; that a considerably large variability is introduced in the a-$\bar{v}O_2$diff parameter when calculated from $\dot{V}O_{2max}$ and $\dot{Q}_{max}$ compared to direct measurement. This is a challenge as ET studies often involve small sample sizes and are notably underpowered in detecting changes in parameters associated with large measurement errors. Therefore, Montero et al. (2015b) carried out a meta-analysis pooling the effects of nine studies and 114 subjects, but still did not observe a significant change in the a-$\bar{v}O_2$diff after ET (P = 0.23). However, several relevant studies were missing from their analysis (Douglas & Becklake, 1968; Ekblom et al., 1968; Kilbom & Åstrand, 1971; Murias et al., 2010a; Rowell, 1962; Saltin et al., 1968a); one was not considered since the ET lasted only two weeks (Jacobs et al., 2013) and some studies have been published subsequent to that literature search (Arbab-Zadeh et al., 2014; Astorino et al., 2017; Montero et al., 2015a; Skattebo et al., 2020). Therefore, when re-examining the a-$\bar{v}O_2$diff changes with ET, calculated by the Fick principle and using a large sample size (n = 249) and a meta-analytic approach, a significant change of 6.1 ml · l^{-1} was detected (95% confidence limits: 3.2-9.0; P < 0.001; Fig. 4.7), which equals a standardised mean difference of 0.26 (95% confidence limits: 0.08-0.44; P = 0.005). In only six out of these 26 subject groups, $\dot{Q}_{max}$ was measured using catheterisation techniques (dye dilution: 4; thermodilution: 1; radioactive indicator dilution: 1). Examining these catheterisation studies in isolation, including 47 subjects, the mean change in a-$\bar{v}O_2$diff was 8.9 ml · l^{-1} (95% confidence limits: 1.0-16.7; P = 0.03). Furthermore, in studies examining leg a-$v_fO_2$diff directly using catheters before and after ET (the lowermost section in Fig. 4.7), the mean change was 12.4 ml · l^{-1} (95% confidence limits: 6.1-18.6; P < 0.001) and displayed a trend towards being larger than the ET-effect on a-$\bar{v}O_2$diff (P = 0.08). Thus, collectively, study I and the previous studies indicate that a-$\bar{v}O_2$diff increases after ET, thus partly confirming hypothesis B, but the methods and the statistical power employed are often insufficient for its detection.

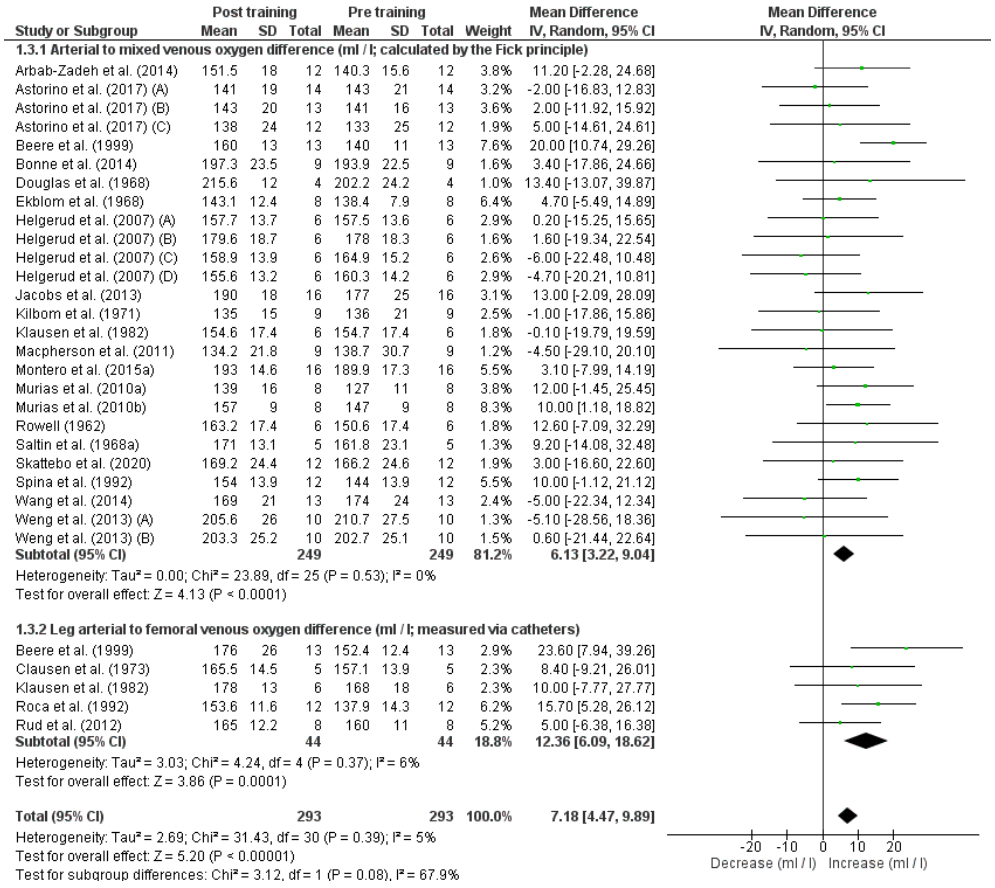

Fig. 4.7 A meta-analysis of studies measuring maximal O_2 uptake and maximal cardiac output and calculating arterial to mixed venous O_2 difference (a-$\bar{v}O_2$diff; Fick principle) before and after 10.3 ± 9.2 weeks (range: 2-52) of endurance training (ET) and/or high-intensity interval training in young (25 ± 3 yrs), healthy individuals (Arbab-Zadeh et al., 2014; Astorino et al., 2017; Beere et al., 1999; Bonne et al., 2014; Douglas & Becklake, 1968; Ekblom et al., 1968; Helgerud et al., 2007; Jacobs et al., 2013; Kilbom & Åstrand, 1971; Klausen et al., 1982; Macpherson et al., 2011; Montero et al., 2015a; Murias et al., 2010a, 2010b; Rowell, 1962; Saltin et al., 1968a; Skattebo et al., 2020; Spina et al., 1992; Wang et al., 2014; Weng et al., 2013). In comparison, studies assessing arterial to femoral venous O_2 difference (a-$v_fO_2$diff) during cycling before and after training are presented (Beere et al., 1999; Clausen et al., 1973; Klausen et al., 1982; Roca et al., 1992; Rud et al., 2012). It is disclosed that a previous meta-analysis has published a similar analysis investigating the changes in a-$\bar{v}O_2$diff with ET (Montero et al., 2015b). However, the previous meta-analysis included only 114 subjects compared to 249 subjects in the present analysis. The studies were weighted by their inverse variance and pooled using a random effects model using Review Manager software (RevMan v. 5.3; Cochrane Collaboration, Oxford, UK). Missing standard deviations (SD) were retrieved by contacting the corresponding authors or computed, according to Marinho et al. (2003).

Similar to the analysis of the data in study I, all the previous studies that calculated a-$\bar{v}O_2$diff before and after ET converge to indicate that 27% and 73% of the $\dot{V}O_{2max}$-improvement (mean change: 340 ml · min^{-1}; P < 0.001) are caused by a widening of the

a-$\bar{v}O_2$diff (mean change: 6.1 ml · l^{-1}) and elevation of $\dot{Q}_{max}$ (mean change: 1.6 l · min^{-1}; P < 0.001), respectively. Therefore, collectively, these data support the consensus that $\dot{V}O_{2max}$ is mainly limited by convective O_2 delivery (Montero et al., 2015b; Mortensen et al., 2005), but also support calculations indicating that 70-75% of the limitations lie within the central circulation and that 25-30% are determined by peripheral factors (di Prampero, 2003; di Prampero & Ferretti, 1990).

4.6. Endurance training increases O_2 extraction during exercise with small muscle mass

Because of the rationale that more of the muscle oxidative capacity is exploited during small vs large muscle mass exercise, we inferred that the improvement of leg O_2 extraction fraction is potentially greater after small muscle mass ET (such as during 1L-KE) and tested the hypotheses that (study III):

D. Leg O_2 extraction fraction will be higher in the trained leg (TL) compared with the untrained leg (CON) while exercising simultaneously at the same power output.
E. A higher leg O_2 extraction fraction will be facilitated by increased muscle oxidative capacity and larger recruitment of D_MO_2 in TL.
F. As the oxidative capacity will be gradually more exploited by increasing exercise intensity, a potential between-leg difference in O_2 extraction fraction will be more evident at high compared with low power outputs.

Post-training, $\dot{W}_{peak}$ and $\dot{V}O_{2peak}$ during 1L-KE was 15 ± 8% and 22 ± 17% higher during incremental exercise to exhaustion with TL compared to CON (P < 0.01; see Table 1 in paper III for more details), respectively. During the invasive studies, the O_2 extraction fraction was significantly higher in TL than in CON during 2L-KE exercise at high (O_2 extraction fraction: 3.2 ± 2.2%-points; a-$v_fO_2$diff: 7.1 ± 4.8 m · l^{-1}; both P < 0.001), but not at low power outputs (Fig. 4.8 a), thus confirming hypotheses D and F. Although not part of this PhD thesis, we repeated the protocol in hypobaric hypoxia after 1 h rest. Interestingly, the TL achieved a higher O_2 extraction fraction at high, but not at low-moderate power outputs also in hypoxia (Fig. 4.8 b; the hypoxic data will serve as material for future paper(s) and will not be discussed here). Importantly, the two legs were exercising with the same power outputs (fractional contribution is presented in the methods), indicating that the difference in training status exclusively caused the between-leg difference in O_2 extraction fraction.

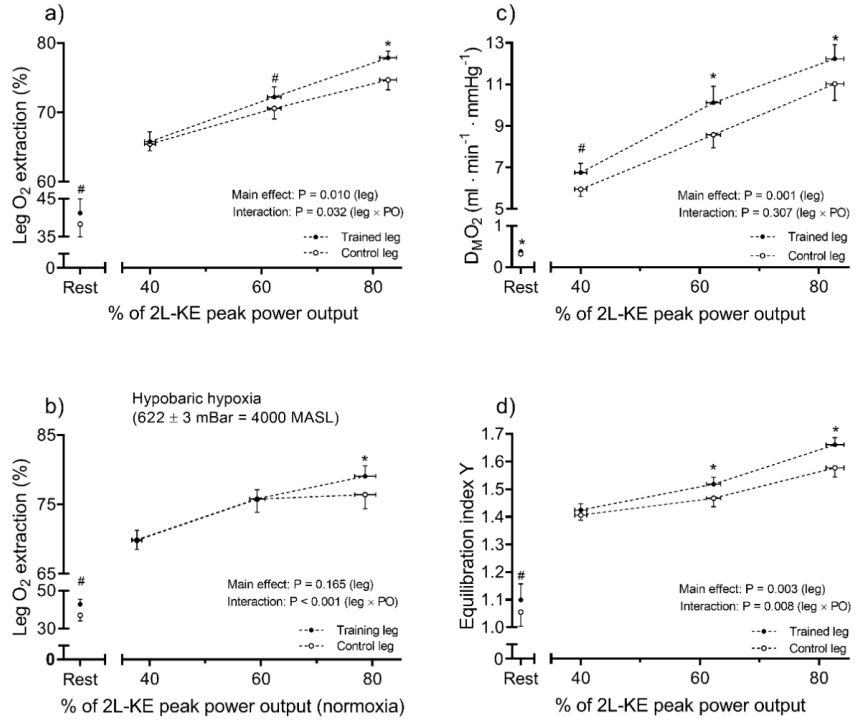

Fig. 4.8 Leg O_2 extraction fraction in normoxia (a; barometric pressure: 986 ± 6 mBar) and in hypobaric hypoxia manipulated using a hypobaric chamber (b; barometric pressure: 622 ± 3 mBar); recruitment of muscle diffusional O_2 conductance (D_MO_2) in normoxia (c); and the equilibration index Y in normoxia (d) 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), i.e., the diffusion limitation is lower in the trained leg. The data are means, and error bars denote standard error of the mean (n = 9). *Significant ($P \leq 0.05$) and #trend towards ($0.05 < P \leq 0.10$) difference between legs (post-hoc comparisons). MASL, metres above sea level.

Previous studies assessing trained and untrained legs separately during 1L-KE have reported increased (Blomstrand et al., 2011; Esposito et al., 2011; Kiens et al., 1993; Mourtzakis et al., 2004) or unchanged (Krustrup et al., 2004; Mortensen et al., 2012; Nyberg et al., 2017) O_2 extraction fraction after ET. Most of the studies reporting no improvement of O_2 extraction fraction used exercise protocols designed to assess vascular function at low to moderate intensities (Mortensen et al., 2012; Nyberg et al., 2017), which concurs with our findings since no significant enhancement of O_2 extraction was observed at these intensities. A difference in leg O_2 extraction fraction at high but not at low power outputs has been reported after one-legged cycling training (Rud et al., 2012). Similarly, Klausen et al. (1982) found an unchanged leg a-$v_fO_2$diff during submaximal exercise, but found a ~7 ml · l^{-1}

increase during maximal one-legged cycling after 8 weeks of one-legged ET. The latter concurs with the study by Boushel et al. (2014) in which maximal-exercise arm O_2 extraction fraction was raised from 62 to 68% after low-intensity training. During arm cycling, the active muscle mass is small (~6 kg) and the mass-specific blood flow is large, as during 1L-KE (Boushel & Saltin, 2013). Thus, collectively, study III and previous studies strongly suggest that the O_2 extraction fraction is improved after ET, particularly when the recruited muscle mass is low, and the exercise intensity is close to maximal.

4.7. Increased leg O_2 extraction during small muscle mass exercise is caused by elevated muscle oxidative capacity (and possibly capillarisation)

Peripheral O_2 extraction depends on the interaction between several factors including i) the kinetics of O_2 off-loading from Hb; ii) the erythrocyte capillary mean transit time (MTT), which is determined by the blood flow, the capillary density and the degree of matching of blood flow distribution to the metabolic demand; iii) the diffusional surface and D_MO_2 over the combined capillary wall, interstitium and sarcolemma barriers; and iv) the muscle oxidative capacity (Calbet et al., 2005).

A right-shifted ODC did not cause an increased O_2 extraction fraction since the $P_{50}O_2$ was slightly higher in CON (ANOVA main effect: $P = 0.005$) due to a lower femoral venous pH (ANOVA main effect: $P = 0.007$). However, after training, TL expressed a higher protein content of citrate synthase (45 ± 29%; $P < 0.001$), COX-IV (44 ± 46%; $P = 0.044$) and HAD (35 ± 28%; $P = 0.007$; Fig. 4.9) along with a higher capillary-to-fibre ratio (18 ± 23%; $P = 0.031$) and a trend towards higher capillary density (12 ± 17%; $P = 0.067$) than CON.

Therefore, the increased O_2 extraction fraction was likely mediated by the increased muscle oxidative capacity and/or capillarisation. 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 (Wagner, 2000). However, new evidence suggests that the muscle possesses a functional reserve in D_MO_2 that is utilised when breathing hypoxic gas during incremental exercise to exhaustion during cycling (Calbet et al., 2015b) or 1L-KE (Calbet et al., 2009). These studies indicate that greater D_MO_2 can be achieved with the same capillary-to-fibre ratio, meaning that the capillary-to-fibre ratio is not the primary regulator of D_MO_2 in healthy men. This interpretation is further supported by our findings of no association between the between-leg difference in the capillary-to-fibre ratio and the between-leg difference in O_2 extraction fraction during high-intensity exercise (Fig. 4.10 b).

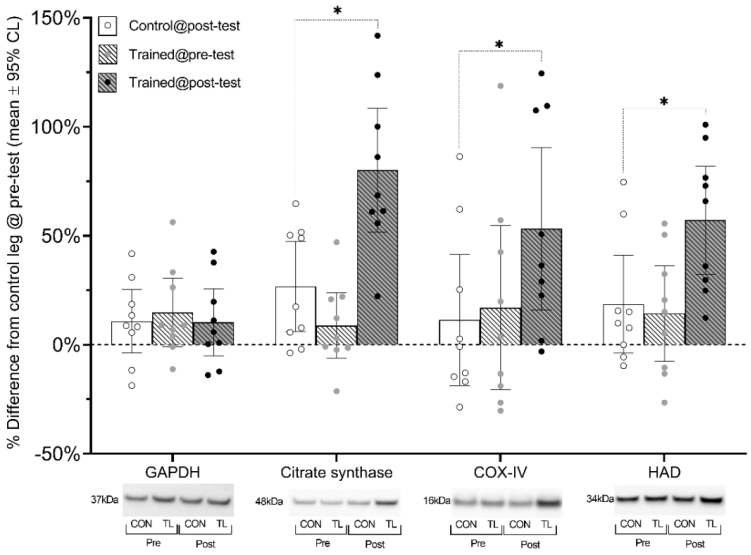

Fig. 4.9 The protein content of the mitochondrial enzymes citrate synthase, cytochrome-c-oxidase subunit 4 (COX-IV) and hydroxyacyl-CoA dehydrogenase (HAD), along with the loading control glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The individual and mean values are presented as percentage differences from the pre-test value of the control leg (n = 9). Error bars denote 95% confidence limits (CL). *Significant difference ($P \leq 0.05$) between legs at the post-test, i.e., at the time of the invasive experiment. Representative blots of the four proteins along with their observed molecular weights are presented.

Conversely, a significant correlation between the between-leg difference of mitochondrial enzymes (citrate synthase and COX-IV) and the difference in O_2 extraction fraction was found (Fig. 4.10 a). Therefore, it is likely that the enhanced muscle oxidative capacity in TL was the primary contributing factor to the improved O_2 extraction fraction. Increased leg O_2 extraction fraction after ET has coincided with increased muscle oxidative capacity before (increased citrate synthase activity) (Rud et al., 2012). Likewise, muscle oxidative capacity has been shown to be important for O_2 extraction in the isolated rat hind limb, especially when pump-perfused with high muscle blood flow and O_2 delivery (Hepple et al., 2002; McAllister & Terjung, 1990; Robinson et al., 1994) that mimics the situation observed during small muscle mass exercise in humans. 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 prolonged bed-rest, systemic $\overline{O}_2$ extraction is maintained during whole-body exercise even though the muscle oxidative capacity and $\dot{V}O_{2max}$ are substantially reduced (Ferretti et al., 1997; Saltin et al., 1968a). Therefore, it is argued that the muscles have a substantial reserve of oxidative capacity and that $\dot{V}O_{2max}$ is almost exclusively limited

by O_2 delivery (Boushel et al., 2011). This might be true during large muscle mass exercise but does not apply to the present experimental design using a small muscle mass exercise exploiting the oxidative capacity (Blomstrand et al., 1997).

The equilibration index Y increased with power output in both legs, indicating that perfusion and diffusion limitations were rising and decreasing, respectively. Interestingly, the equilibration index Y increased more with power output in TL (Fig. 4.8 d), indicating that the training-induced reduction of diffusion limitations was more marked during high-intensity exercise. Thus, the interactions between power output and O_2 extraction fraction and the equilibration index Y indicate that the benefit from increasing the muscle oxidative capacity is more apparent when the mitochondria operate closer to their maximal oxidative capacity; potentially allowing for the maintenance of efficient O_2 diffusion and O_2 extraction even in the most distal capillary regions with the lowest PO_2 gradients.

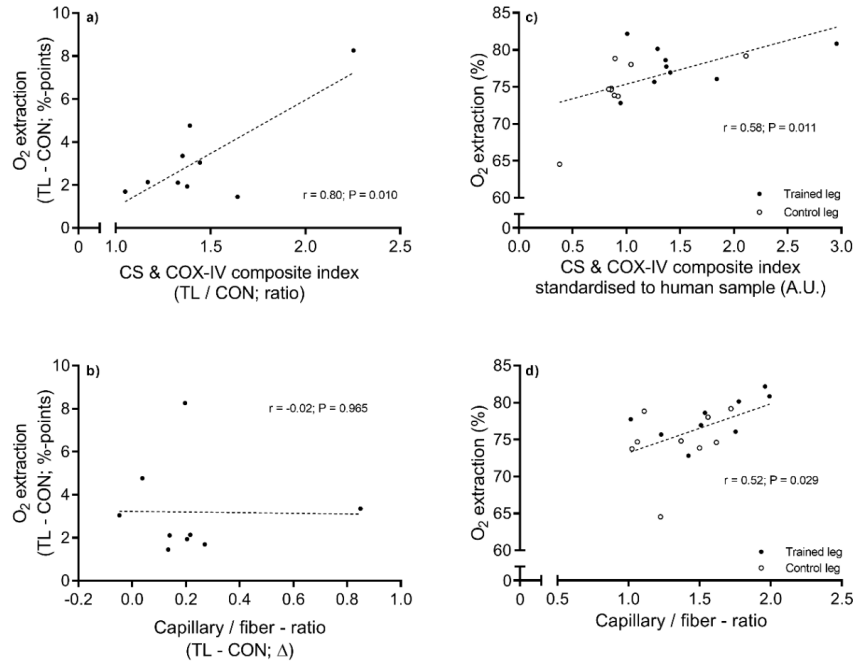

Fig. 4.10 Data are from the highest power output (normoxia). The relationship between the between-leg difference in O_2 extraction fraction and the between-leg ratio of mitochondrial protein content (a composite index of CS and COX-IV; a) and the between-leg difference in the capillary-to-fibre ratio (b). Note that the relationship in Fig. a was equally strong when assessed isolated for CS (r = 0.72; n = 9; P = 0.028) and COX-IV (r = 0.73; n = 9; P = 0.024). In Fig. c, the protein contents were standardised to a human control sample, which was loaded on each gel to allow for between-subject comparisons (presented as arbitrary units; A.U.). In Fig. d, the capillary-to-fibre ratio is plotted against O_2 extraction fraction. CS, citrate synthase; COX-IV, cytochrome-c-oxidase subunit 4; TL, trained leg; CON, control leg.

4.8. a-$\bar{v}O_2$diff and systemic $\bar{O}_2$ extraction cannot be used interchangeably

Since no previous study has attempted to systematically analyse the large pool of cross-sectional data existing on the limiting factors to $\dot{V}O_{2max}$, we carried out such an analysis (study IV) with particular emphasis on whether peripheral $\mathbf{O_2}$ extraction and systemic $\bar{\mathbf{O}}_2$ extraction fractions are modified with $\dot{V}O_{2max}$, by only including catheterisation studies.

From the analysis of the individual data on systemic responses, as expected, $\dot{Q}_{max}$ increased by 4.9 l · min^{-1} for each l · min^{-1} increase in $\dot{V}O_{2max}$ (Fig. 4.11 a; $P < 0.001$), explained by a linear increase in SV (Fig. 4.11 b; $P < 0.001$). These relationships were strong and complied with the "classic" view that O_2 delivery is the principal limitation to whole-body $\dot{V}O_{2max}$ (Mortensen et al., 2005; Saltin & Calbet, 2006).

The calculated systemic a-$\bar{v}O_2$diff showed a large variability for a given $\dot{V}O_{2max}$ and was, if anything, lower in those subjects displaying the highest $\dot{V}O_{2max}$ (> 5 l · min^{-1}) compared to those who were moderately- to well-trained ($\dot{V}O_{2max}$: 4-5 l · min^{-1}) (Fig. 4.11 c). This agrees with cross-sectional studies showing only a small difference between non-endurance-trained and active individuals (Ekblom & Hermansen, 1968; Ekblom et al., 1968) and no apparent difference between well-trained individuals and elite athletes (Ekblom & Hermansen, 1968). This has led previous investigators to argue that improved $\bar{O}_2$ extraction fraction does not contribute, or only minimally contributes, to the remarkably high $\dot{V}O_{2max}$ observed in elite athletes (Bassett & Howley, 1997; Lundby et al., 2017). However, in the present analysis, it was observed a reduction in CaO_2 with increasing $\dot{V}O_{2max}$ (Fig. 4.11 e), meaning that the potential to achieve a high a-$\bar{v}O_2$diff decreases with increasing $\dot{V}O_{2max}$. The decrease in CaO_2 was mainly explained by arterial hypoxemia (decreased arterial SO_2; Fig. 4.11 f; $P < 0.001$), which often accompanies a high $\dot{Q}_{max}$ (Nielsen, 2003; Powers et al., 1989), but also by a non-significant negative relationship between [Hb] and $\dot{V}O_{2max}$ (Fig. 4.11 g; $P = 0.232$), potentially mediated by ET-induced PV expansion causing haemodilution (Sawka et al., 2000) and a below-average haemoconcentration from rest to maximal exercise that may occur in well-trained individuals (Ekblom & Hermansen, 1968). After accounting for CaO_2, the calculated $\bar{O}_2$ extraction fraction (a-$\bar{v}O_2$diff / CaO_2) increased up to a $\dot{V}O_{2max}$ of ~4.5-5.0 l · min^{-1} and then approached a maximal value at ~90% (Fig. 4.11 d) when restricting the exponential decay model to plausible physiological limits ($\dot{V}O_{2max}$: 6-7 l · min^{-1}). This suggests that $\bar{O}_2$ extraction fraction serves a modifying role in determining $\dot{V}O_{2max}$.

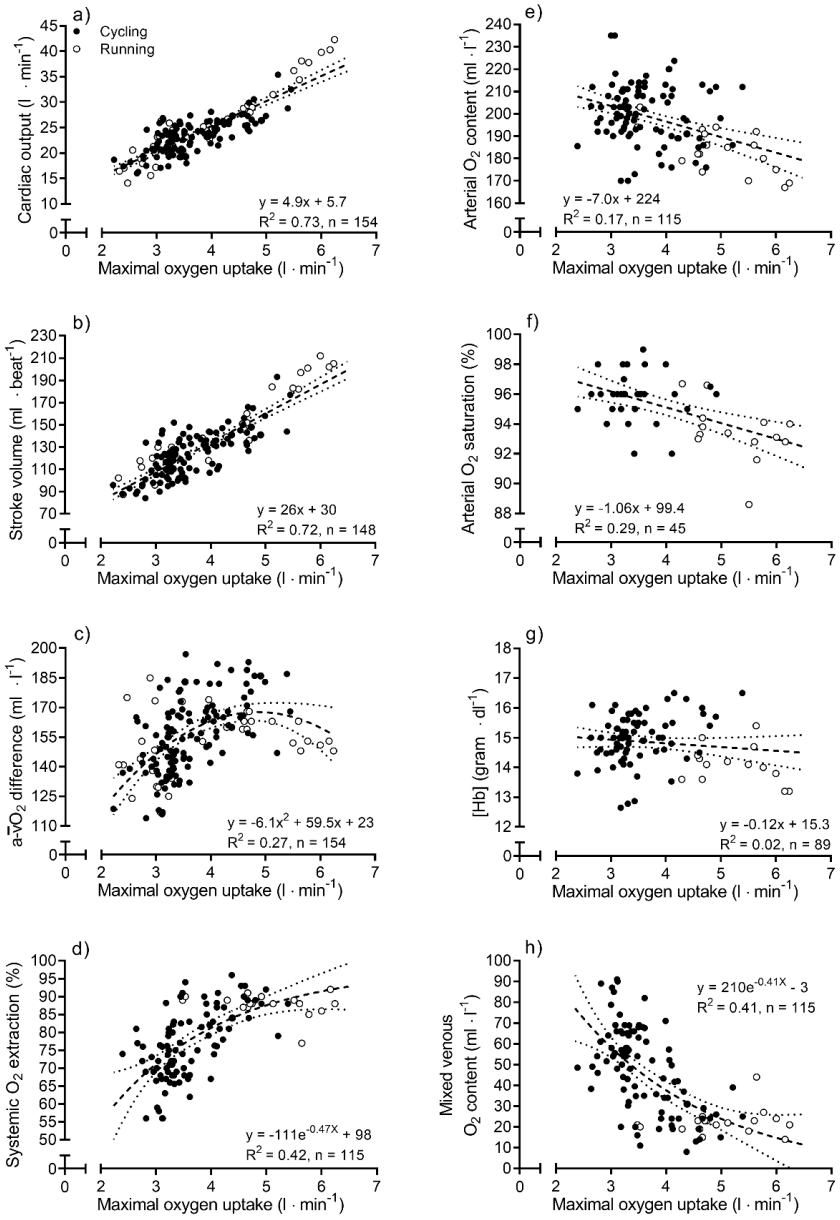

Fig. 4.11 The relationship between **individual values** (from studies reported in Table 3.2) of pulmonary maximal O_2 uptake and maximal cardiac output (a), stroke volume (b), calculated arterial to mixed venous O_2 difference (a-$\bar{v}O_2$ difference; c), **calculated systemic $\bar{O}_2$ extraction fraction (d)**, arterial O_2 content (e), arterial O_2 saturation (f), haemoglobin concentration ([Hb]; g) and the calculated mixed venous O_2 content (h). All data were obtained during maximal exercise. Inserted in each graph are the formulas for the regression equations along with the goodness of fit (R^2) and the number of data pairs (n).

4.9. Leg O_2 extraction fraction displays a close relationship with pulmonary $\dot{V}O_{2max}$

The pattern of increased systemic $\bar{O}_2$ extraction fraction (calculated using the Fick principle) with $\dot{V}O_{2max}$ was confirmed when measured directly using catheters (arterial and femoral venous blood sampling), with leg O_2 extraction fraction increasing progressively with pulmonary $\dot{V}O_{2max}$ until reaching ~90-95% (Fig. 4.12 a). Furthermore, studies reporting mean data of systemic $\bar{O}_2$ extraction fraction were superimposed on the regression equation from the individual data presented in Fig. 4.11d and these values also converged to a similar pattern (Fig. 4.13). Therefore, collectively, the original studies, this analysis and the meta-analysis presented in chapter 4.5 strongly suggest that $\bar{O}_2$ extraction fraction increases with $\dot{V}O_{2max}$ and ET, at least up to a certain level. It must be pointed out that these relationships were equally strong when $\dot{V}O_{2max}$ was standardised to body weight (Paper IV: Supplements).

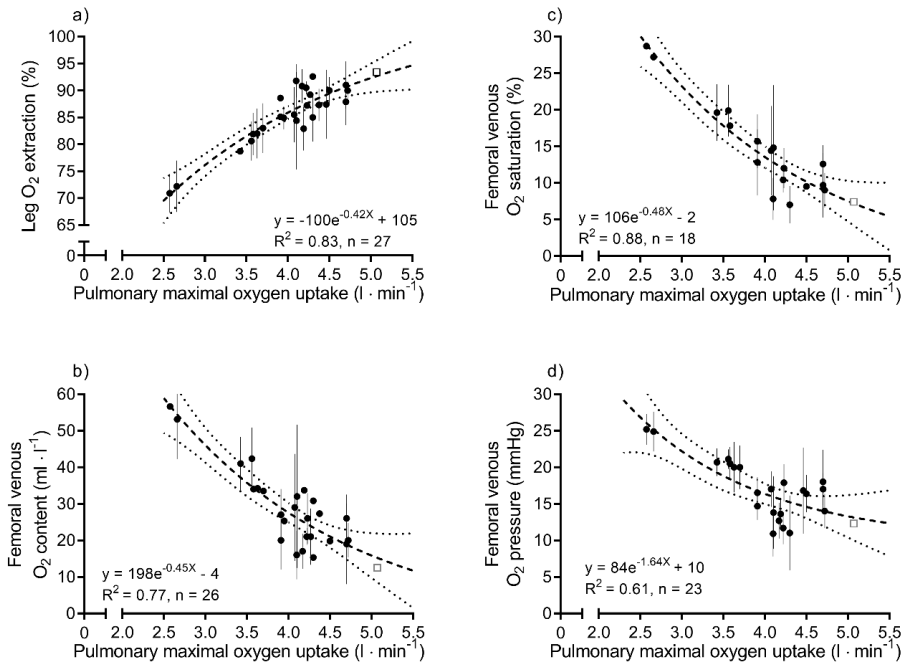

Fig. 4.12 The relationship between **the studies' mean values** of pulmonary maximal O_2 uptake and leg O_2 extraction fraction (a), femoral venous O_2 content (b), femoral venous O_2 saturation (c) and femoral venous O_2 pressure (d) measured by **arterial and femoral venous catheters**. Black and white symbols denote cycling and diagonal cross-country skiing, respectively. Data are mean values (± 95% confidence limits, where available) from studies reported in Table 3.3.

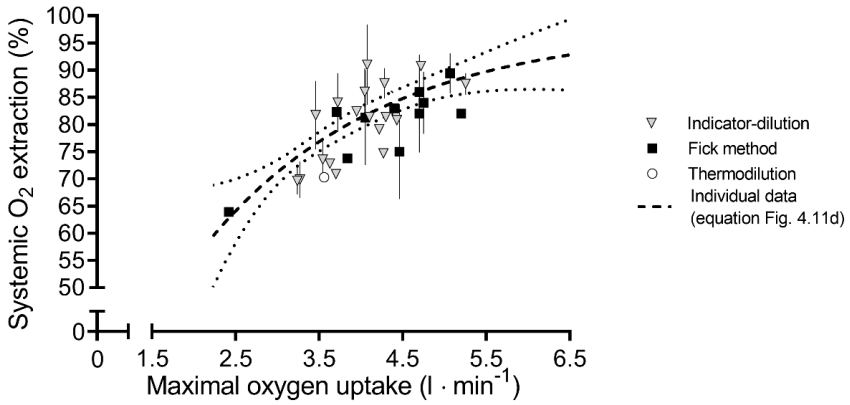

Fig. 4.13 Mean values (± 95% confidence limits, where available) of systemic O_2 extraction fraction vs maximal O_2 uptake from studies using the direct (pulmonary artery catheter) or the modified (right atrium catheter) Fick method (Calbet et al., 2004; Harms et al., 1998; Mortensen et al., 2008; Mortensen et al., 2005; Munch et al., 2014; Nielsen et al., 1999; Roca et al., 1989; Siebenmann et al., 2015; Sullivan et al., 1989; Sun et al., 2000; Sutton et al., 1988) the indicator dilution method (Calbet et al., 2003; Calbet et al., 2007; Calbet et al., 2015a; Calbet et al., 2006; Ekblom, 1970; Ekblom & Hermansen, 1968; Ekblom et al., 1975; Ekblom et al., 1976; Ekblom et al., 1968; Gleser, 1973; Gonzalez-Alonso & Calbet, 2003; Hermansen et al., 1970; Kanstrup & Ekblom, 1982; Lundby et al., 2008a; Lundby et al., 2008b; Saltin et al., 1968a; Stenberg et al., 1966; Åstrand et al., 1964) and the transpulmonary thermodilution method (Calbet et al., 2015b). The broken line is the regression equation obtained from Fig. 4.11d.

The highest recorded leg O_2 extraction fraction was 93% (group mean) (Calbet et al., 2005) and the regression model indicated a plateau at ~95% within physiological limits for pulmonary $\dot{V}O_{2max}$. Hence, a minimum of ~10 ml O_2 remains in each litre of femoral venous blood (Fig. 4.12 b) associated with a PO_2 of ~10 mmHg (Fig. 4.12 d), even for the best-trained individuals. In this situation, a PO_2 gradient persists between the blood and myoglobin (myoglobin/intracellular PO_2: ~1-2 mmHg) (Gayeski & Honig, 1988), where myoglobin-facilitated diffusion should proceed given the high myoglobin O_2 affinity (myoglobin $P_{50}O_2$: ~5 mmHg) and the low myoglobin SO_2 at maximal exercise (Gayeski & Honig, 1988). However, it is suggested that the primary site of resistance to O_2 diffusion is between the capillaries and the sarcoplasm and it has been estimated that the "critical capillary PO_2" needed to overcome this resistance may be as high as 10-20 mmHg (Gayeski & Honig, 1988; Poole & Jones, 2012; Roy & Popel, 1996; Wittenberg & Wittenberg, 1989). The remaining O_2 may, therefore, represent diffusional limitations across the combined capillary wall, interstitium and sarcolemma barriers, together with too short MTT for complete Hb-O_2 off-loading. This is supported by the need for an infinitesimal PO_2 gradient for O_2 to diffuse from the sarcoplasm to cytochrome c oxidase (Clark et al., 1987) and the

estimate that a mitochondrial PO_2 of ~1 mmHg may be sufficient to support maximal mitochondrial respiration (Gayeski et al., 1987; Nanadikar et al., 2019). The remaining O_2 may also represent muscle metabolism-perfusion mismatch (Cano et al., 2015; Heinonen et al., 2010) and, even more likely, an inevitable lower O_2 extraction from the blood perfusing the skin, connective tissue, fat and bone marrow of the leg causing venous admixture. In this context, the end-capillary PO_2, assessed using video microscopy, was found to be lower than the PO_2 both in the venule (O_2 microelectrode) and vein (blood gas) draining the muscle region of interest (Stein et al., 1993). Hence, the lowest femoral venous PO_2 values of ~10 mmHg indicates an even lower end-capillary PO_2 in the capillaries adjacent to the most metabolically active muscle regions during maximal exercise, possibly approaching ~5 mmHg. Therefore, no matter which limitation prevails, it is likely that the upper limit for leg O_2 extraction fraction is ~95%, caused by the above diffusional and distributional limitations and barriers.

4.10. Limitations to $\dot{V}O_{2max}$ by O_2 delivery and O_2 extraction varies with $\dot{V}O_{2max}$

Interestingly, the leg D_MO_2 and equilibration index Y, which quantitatively describes diffusion vs perfusion limitations to muscle $\dot{V}O_2$ (where $Y < 0.1$ indicates pure diffusion limitation, $0.1 < Y < 3$ indicates mixed perfusion-diffusion limitation and $Y > 3$ indicates pure perfusion limitation) (Piiper, 2000), was well above 1 in all subject groups and increased progressively with pulmonary $\dot{V}O_{2max}$ (Fig. 4.14 a-b; both $P < 0.01$). Therefore, all the healthy, young subjects included in the present analysis were characterised by more perfusion- than diffusion-limited leg muscles and became progressively more perfusion/O_2 delivery limited with a gradually higher $\dot{V}O_{2max}$. Thus, individuals with the highest $\dot{V}O_{2max}$ can only achieve a further substantial improvement in $\dot{V}O_{2max}$ by increasing O_2 delivery, a conclusion supported by the extremely low levels of Cv_fO_2 (Fig. 4.12 b) and $C\bar{v}O_2$ (Fig. 4.11 h) in these subjects. Therefore, the limiting factors to $\dot{V}O_{2max}$ change with training status and $\dot{V}O_{2max}$: i) untrained but healthy individuals display mixed perfusion-diffusion limitations; and ii) this diffusional limitation reduces as $\dot{V}O_{2max}$ is increased (Wagner, 2008). These conclusions are similar to those of Gifford et al. (2016), who found a clear relationship between OXPHOS measured in permeabilised muscle fibres *ex vivo* and $\dot{V}O_{2max}$ in untrained but not in trained individuals.

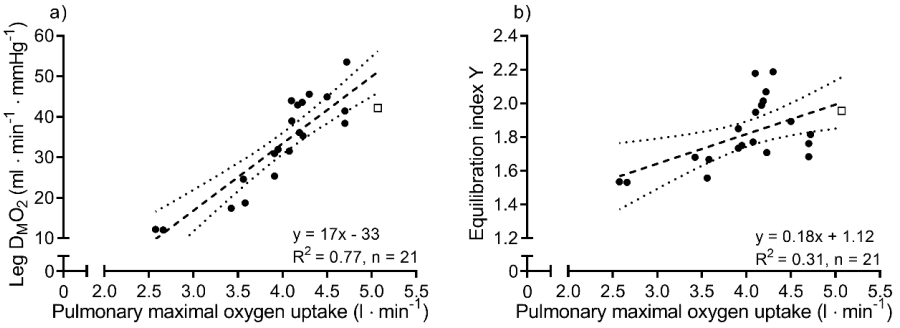

Fig. 4.14 The relationship between pulmonary maximal O_2 uptake and one-leg muscle O_2 conductance (D_MO_2; a) and the equilibration index Y (b) calculated using the Piiper and Scheid model (Piiper, 2000). Black and white symbols denote cycling and diagonal cross-country skiing, respectively. Data are mean values from studies reported in Table 3.3.

4.11. Methodological considerations

In study I, transcapillary fluid shifts may have occurred after phlebotomy, as indicated in study II, and it is uncertain whether BV normalisation was preserved during exercise. The findings were obtained from a small sample size, but the main finding was that the ET-induced increase in $\dot{V}O_{2max}$ was preserved despite removing the increase in Hb_{mass}, and this conclusion is based on methods with low typical error ($\dot{V}O_{2max}$ 2.9% and Hb_{mass} 1.1%). Impedance cardiography is associated with a larger measurement error than the invasive gold-standard methods (Del Torto et al., 2019; Richard et al., 2001). Besides, when calculating the a-$\bar{v}O_2$diff from $\dot{V}O_2$ and $\dot{Q}$ inherent of its measurements errors, a larger measurement error is expected as compared with deriving systemic a-$\bar{v}O_2$diff or leg a-$v_fO_2$diff using arterial and (mixed) venous blood sampling, as discussed in chapter 4.5. The echocardiography was conducted in the supine position, causing an abnormal venous return at rest. Therefore, the measured EDV and diastolic function parameters are not directly transferable to the upright position, especially during exercise.

In study II, the fluctuations of intravascular volumes were calculated assuming a stable Hb_{mass} during exercise, which may be corrupted by splenic contraction. However splenic contraction releases only a small amount of RBCs during exercise in humans, and the calculated PV reduction based on fluctuations in [Hb] and haematocrit has been previously found to be equivalent to that measured using radio-isotope-labelled human albumin (^{125}I-RISA) and RBCs (Cr^{51}) after heavy exercise (Stewart et al., 2003).

In study III, mitochondrial protein expressions analysed by Western blots were used as proxies to indicate muscle oxidative capacity. However, it may have been more appropriate to analyse the mitochondrial volume, citrate synthase activity or *ex vivo* OXPHOS using high-resolution respirometry (Larsen et al., 2012).

In study IV, the data were collected from several research groups and published over six decades (1958-2017), using a variety of gas analysers, flow sensors, methods to determine blood O_2 content and PO_2, and several procedures to analyse the indicator-dilution and blood temperature curves for $\dot{Q}_{max}$ and LBF measurements, respectively. Therefore, for a given $\dot{V}O_{2max}$, the between-subject variability presented here may be overestimated. Several different averaging strategies for $\dot{V}O_2$ and the associated variables were likely to have been applied (rarely stated in the manuscripts). Despite these potential sources of noise, the integrations of the obtained values fitted into the physiological range and agreed between studies, demonstrating the quality of these studies and the robustness of this analysis.

4.12. Ethical considerations

Studies I-III were carried out according to the Declaration of Helsinki, were approved by the Ethics Committee of the Norwegian School of Sport Sciences, and all subjects gave their written, informed consent before participation. In addition to the written information, the general outlines of the studies were explained orally to all subjects before inclusion. In studies including muscle biopsies, catheterisation and blood withdrawals, the understanding of what these procedures meant was emphasised. Also, it was especially emphasised that subjects could withdraw their consent at any time, which would not impact their further treatment.

In studies I and III, muscle biopsies from the Vastus Lateralis were obtained. This procedure may occasionally cause pain, despite the use of local anaesthetics, and as with all procedures making an incision in the skin, it carries some risk of infection (Tarnopolsky et al., 2011). These risks and discomforts were minimised by the muscle biopsies being sampled by experienced operators, under sterile conditions and using only a small incision (~1 cm) to allow for the introduction of the Bergström needle. No infection was reported. By using a 6 mm needle instead of the more common 5 mm needle, fewer “clips” for a given sample size are needed (Melendez et al., 2007; Tarnopolsky et al., 2011), thus potentially reducing discomfort. In most biopsies, only one “clip” was necessary.

In studies I and II, the blood volume was manipulated using blood withdrawals, with the largest withdrawal being ~500 ml. Blood pressure was measured regularly, and a medical doctor slowly withdrew the blood. No adverse physical reaction to the withdrawals occurred. A few vasovagal reactions occurred on the control day, caused by the insertion of the catheter in the antecubital vein. In these cases, the back of the blood sample chair was lowered, and the legs raised to prevent participants from fainting, and after stabilisation, ample time was given before initiating the exercise protocol. These subjects did not experience the same reaction on the experimental day involving blood withdrawals 1 week later.

In study III, the subjects were catheterised in both femoral veins and a femoral artery. This was conducted by a medical doctor with ~20 yrs' experience, who had conducted several hundred catheterisations in the past. An ultrasound device was used to check individual anatomy. A 20-gauge paediatric catheter was chosen to minimise bleeding during and after retraction. Pressure was employed on the puncture site during and for ~15 min after the removal of the catheters to counteract the development of a significant haematoma.

5. Conclusions

This thesis expands our current understanding regarding the impact of blood volume and O_2 extraction fraction on $\dot{V}O_{2max}$. The primary findings were:

Blood volume:

- $\dot{V}O_{2max}$ and $\dot{Q}_{max}$ may increase following short-term ET independent of Hb_{mass} and BV expansions, which emphasise that ET-induced changes in $\dot{V}O_{2max}$ are multifactorial (study I).
- $\dot{V}O_{2max}$ and maximal exercise capacity were preserved after an acute blood loss of 150 ml, caused by a rapid PV restoration (study II).
- The human body is not capable of preserving $\dot{V}O_{2max}$ after acute moderate blood loss (450 ml), even though compensatory mechanisms such as partial PV restoration, increased HR_{peak} and increased leg O_2 extraction (decreased TOI) were manifested (study II).
- Therefore, the reduction in $\dot{V}O_{2max}$ was more severe after a blood withdrawal of 450 ml compared to 150 ml, even after normalising the $\dot{V}O_{2max}$-decay to the blood volume removed (study II).

Oxygen extraction:

- No statistically significant change in the a-$\bar{v}O_2$diff occurred after 10 weeks of whole-body ET (cycling). However, our data indicated that ~30% and ~70% of the $\dot{V}O_{2max}$-improvement originated from changes in a-$\bar{v}O_2$diff and $\dot{Q}_{max}$, respectively (study I).
- This points towards a weakness in the majority of studies aiming to assess whether a-$\bar{v}O_2$diff changes with ET, study I included: that a considerably large variability is introduced in the a-$\bar{v}O_2$diff parameter when calculated from $\dot{V}O_{2max}$ and $\dot{Q}_{max}$ using the Fick principle (a-$\bar{v}O_2$diff = $\dot{V}O_{2max}$ / $\dot{Q}_{max}$). Thus, a very large sample size is likely needed to detect any statistically significant change.
- When meta-analysing study I together with previous studies comprising 249 subjects, a-$\bar{v}O_2$diff was significantly increased on average by 6 ml $\cdot$ l^{-1} and explained 27% of the increase in $\dot{V}O_{2max}$.

- In an exercise model that is not perfusion limited and not taxing $\dot{Q}_{max}$, O_2 extraction (catheters) increases after ET and is related to the elevation of muscle oxidative capacity (study III).
- At higher exercise intensities, which are associated with greater mitochondrial activation and lower time for Hb-O_2 off-loading, the benefit from an ET-induced increase in muscle oxidative capacity on O_2 extraction was most prominent (study III).

- Through the statistical analysis of previously published data on systemic and peripheral O_2 extraction fraction in relation to pulmonary $\dot{V}O_{2max}$ (study IV), the following observations were made:
    - a-$\bar{v}O_2$diff (calculated by the Fick principle) increased with $\dot{V}O_{2max}$ up to ~4.5 l · min^{-1} (moderately trained subjects), but did apparently not increase further with an elevation of $\dot{V}O_{2max}$.
    - However, after accounting for a decreasing CaO_2 with increasing $\dot{V}O_{2max}$, the calculated systemic $\bar{O}_2$ extraction fraction (a-$\bar{v}O_2$diff / CaO_2) increased up to a $\dot{V}O_{2max}$ of ~4.5-5.0 l · min^{-1} and approached a maximal value at ~90%.
    - This pattern was further strengthened by the direct measurements of leg O_2 extraction fraction using catheters, increasing progressively with pulmonary $\dot{V}O_{2max}$ until reaching ~90-95%.
    - The limiting factors to $\dot{V}O_{2max}$ change with $\dot{V}O_{2max}$: untrained, but healthy individuals display mixed perfusion-diffusion limitations, and this diffusion limitation reduces as $\dot{V}O_{2max}$ is increased.

6. Perspectives

A major aim of ET is to enhance exercise capacity by improving O_2 utilisation, and the findings of this thesis may impact our understanding of how O_2 delivery and O_2 extraction interact in limiting $\dot{V}O_{2max}$. For instance, black and white points are often made in the research literature to sell a strong message. Therefore, and since systemic O_2 delivery unquestionably dominates the limiting factors to whole-body $\dot{V}O_{2max}$ in healthy young subjects, in normoxia; this may have led to the widespread misconception that the peripheral adaptations to ET, such as angiogenesis and mitochondrial biogenesis, do not affect $\dot{V}O_{2max}$ but "only" improves factors such as substrate and metabolite exchange, ion-handling, fat oxidation and the lactate threshold, thus delaying muscle fatigue and improving endurance exercise performance. However, in untrained and moderately trained individuals, increased a-$\bar{v}O_2$diff may represent ~25-30% of the $\dot{V}O_{2max}$-improvements caused by ET. In the context of this discussion, it is emphasised that the parameters a-$\bar{v}O_2$diff and systemic $\bar{O}_2$ extraction fraction cannot be used interchangeably, as it occasionally is in the research literature. This is exemplified by using the regression equations presented in Fig. 4.11 to compare a typically sedentary subject to an elite endurance athlete with a large difference in $\dot{V}O_{2max}$ (3.0 vs 5.5 l · min^{-1}, or ~40 vs ~75-80 ml · kg^{-1} · min^{-1}): the elite athlete has a 1.83-fold higher $\dot{V}O_{2max}$, a 1.60-fold higher $\dot{Q}_{max}$ and a 1.26-fold higher $\bar{O}_2$ extraction fraction, but due to the lower CaO_2, the a-$\bar{v}O_2$diff is only 1.13-fold higher (Fig. 4.15).

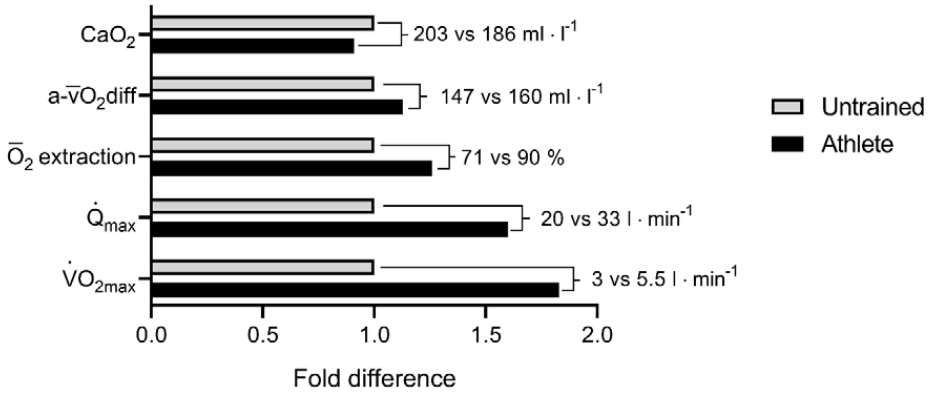

Fig. 4.15 A comparison of an untrained individual and an elite endurance athlete with maximal O_2 uptakes ($\dot{V}O_{2max}$) of 3.0 and 5.5 l · min^{-1}, respectively. The maximal cardiac output ($\dot{Q}_{max}$), systemic O_2 extraction fraction ($\bar{O}_2$ extraction), arterial to mixed venous O_2 content difference (a-$\bar{v}O_2$diff) and arterial O_2 content (CaO_2) were calculated using the regression equations presented in Fig. 4.11.

The limitations to whole-body $\dot{V}O_{2max}$ may change with training, with an accentuated O_2 delivery limitation and conversely a decreasing O_2 diffusional limitation with increasing $\dot{V}O_{2max}$. Therefore, the peripheral adaptations to ET will impact $\dot{V}O_{2max}$ less as training progress, but does not necessarily mean that they can be neglected. Although it may be observed leg O_2 extraction fractions as high as 90% in moderately trained individuals (55-60 $ml \cdot kg^{-1} \cdot min^{-1}$), it may take years of training before reaching 95% as observed in a few endurance athletes (Calbet et al., 2005; Gonzalez-Alonso & Calbet, 2003), especially considering these subjects have a substantially higher mass-specific muscle perfusion that probably would have disfavoured Hb-O_2 off-loading if no peripheral adaptations took place.

The impact of peripheral adaptations to ET on O_2 extraction fraction may be augmented during exercise involving a small muscle mass, as shown in study III. Such adaptations may be of particular importance for $\dot{V}O_{2max}$ in situations where the mitochondria do not possess a substantial excess oxidative capacity over O_2 delivery. This may have implications for untrained individuals and patients training while taking mitochondrion-toxic drugs like metformin or statins, which may blunt the expected mitochondrial biogenesis. It is also shown that training with small muscle mass (one-legged knee extension training of both legs, separately) may be particularly beneficial in chronic heart failure patients, even when assessed during large muscle mass exercise (two-legged cycling) after training (Esposito et al., 2011; Tyni-Lenne et al., 1999); likely attributed to their limited muscle oxidative capacity and limited potential for central adaptations, especially with symptom-limited training.

Few studies have investigated peripheral O_2 extraction fraction using catheters in women during whole-body maximal exercise (Proctor et al., 2004; Proctor et al., 2001), with no subject group exceeding a mean $\dot{V}O_{2max}$ of 40 $ml \cdot kg^{-1} \cdot min^{-1}$, to my knowledge. Since a higher intrinsic mitochondrial respiration is measured in women than men (higher mitochondrial quality) (Cardinale et al., 2018), this may impact their O_2 extraction capacity and compensate for the lower CaO_2 but is yet to be explored.

The acute BV manipulations indicate that $\dot{V}O_{2max}$ is unchanged after small Hb_{mass} reductions of 22-25 g, due to compensatory mechanisms. Whether $\dot{V}O_{2max}$ is insensitive to an acute upregulation of this amount of Hb_{mass} is yet to be explored, although, reinfusion of 135 ml packed RBCs equivalent to 26 g Hb_{mass} increases time-trial performance by 4.7% (Bejder et al., 2019). The compensatory mechanisms observed after acute blood loss may represent important preventive mechanisms also preserving $\dot{V}O_{2max}$ after, e.g., PV reduction following dehydration induced by prolonged exercise or hyperthermia, or daily oscillations of PV.

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Paper I

Blood volume expansion does not explain the increase in peak oxygen uptake induced by 10 weeks of endurance training

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Abstract

Purpose: The endurance training (ET)-induced increase in peak oxygen uptake ($\dot{V}O_{2peak}$) and cardiac output ($\dot{Q}_{peak}$) during upright cycling are reversed to pre-ET levels after removing the training-induced increase in blood volume (BV). We hypothesised that ET-induced improvements in $\dot{V}O_{2peak}$ and $\dot{Q}_{peak}$ are preserved following phlebotomy of the BV gained with ET during supine but not during upright cycling. Arteriovenous O_2 difference (a-vO_2diff; $\dot{V}O_2/\dot{Q}$), cardiac dimensions and muscle morphology were studied to assess their role for the $\dot{V}O_{2peak}$-improvement.

Methods: Twelve untrained subjects ($\dot{V}O_{2peak}$: 44±6 ml·kg^{-1}·min^{-1}) completed 10 weeks of supervised ET (three sessions/week). Echocardiography, muscle biopsies, haemoglobin mass (Hb_{mass}) and BV were assessed pre- and post-ET. $\dot{V}O_{2peak}$ and $\dot{Q}_{peak}$ during upright and supine cycling were measured pre-ET, post-ET and immediately after Hb_{mass} was reversed to the individual pre-ET level by phlebotomy.

Results: ET increased the Hb_{mass} (3.3±2.9%; P=0.005), BV (3.7±5.6%; P=0.044) and $\dot{V}O_{2peak}$ during upright and supine cycling (11±6% and 10±8%, respectively; P≤0.003). After phlebotomy, improvements in $\dot{V}O_{2peak}$ compared with pre-ET were preserved in both postures (11±4% and 11±9%; P≤0.005), as was $\dot{Q}_{peak}$ (9±14% and 9±10%; P≤0.081). The increased $\dot{Q}_{peak}$ and a-vO_2diff accounted for 70% and 30% of the $\dot{V}O_{2peak}$-improvements, respectively. Markers of mitochondrial density (CS and COX-IV; P≤0.007) and left ventricular mass (P=0.027) increased.

Conclusion: The ET-induced increase in $\dot{V}O_{2peak}$ was preserved despite removing the increases in Hb_{mass} and BV by phlebotomy, independent of posture. $\dot{V}O_{2peak}$ increased primarily through elevated $\dot{Q}_{peak}$ but also through a widened a-vO_2diff, potentially mediated by cardiac remodelling and mitochondrial biogenesis.

Keywords: blood volume; cardiac output; echocardiography; haemoglobin mass; maximal oxygen uptake; peripheral adaptations; supine cycling

Abbreviations

a-vO_2diff: arteriovenous oxygen difference

BV: blood volume

CO: carbon monoxide

COX-IV: cytochrome c oxidase subunit 4

CS: citrate synthase

EDV: end-diastolic volume

ESV: end-systolic volume

ET: endurance training

HAD: hydroxyacyl-CoA dehydrogenase

[Hb]: haemoglobin concentration

Hb_{mass}: haemoglobin mass

HR: heart rate

HR_{peak}: peak heart rate

[La]: blood lactate concentration

LV: left ventricle

MCHC: mean corpuscular haemoglobin concentration

MTT: mean transit time

PV: plasma volume

$\dot{Q}$: cardiac output

$\dot{Q}_{peak}$: peak cardiac output

RBCV: red blood cell volume

RER_{peak}: peak respiratory exchange ratio

RPE: rating of perceived exertion

RV: right ventricle

SD: standard deviation

SV: stroke volume

VE_{peak}: peak ventilation

$\dot{V}O_2$: oxygen uptake

$\dot{V}O_{2peak}$: peak oxygen uptake

$\dot{W}_{peak}$: peak power output

Introduction

During whole-body exercise, the oxidative capacity of skeletal muscle exceeds the oxygen (O_2) delivery; as illustrated by the two-fold higher mass-specific O_2 delivery and peak O_2 uptake ($\dot{V}O_{2peak}$) during dynamic one-legged knee-extension compared to cycling exercise (approximately 2.5 vs 20 kg active muscle mass, respectively) (Boushel and Saltin 2013; Cardinale et al. 2019). Yet endurance training (ET) induces remarkable increases in mitochondrial enzymes and capillary density, commonly improving these by ~40% and ~10-20% after a few months of ET, respectively (Granata et al. 2018; Klausen et al. 1981). Such adaptations are likely more important for endurance performance than $\dot{V}O_{2peak}$, but despite a long-standing debate (Bassett and Howley 2000), it remains uncertain whether $\dot{V}O_{2peak}$ is limited by central or by combined central-peripheral factors. According to the Fick equation, every change in $\dot{V}O_{2peak}$ is matched by a concomitant change in peak cardiac output ($\dot{Q}_{peak}$) and/or arteriovenous O_2 difference (a-vO_2diff). Most studies find $\dot{Q}_{peak}$ increased following short-term ET, whereas more heterogeneous findings exist for the a-vO_2diff (Montero and Diaz-Canestro 2016).

At the commencement of ET, improvement in stroke volume (SV) and $\dot{Q}_{peak}$ are thought to be mainly facilitated by the blood volume (BV) expansion that increases venous return and preload to the heart and its filling rates (Bonne et al. 2014; Krip et al. 1997). However, cardiac remodelling has been reported already after three months of ET, which, together with the reduced pericardial constraints and enhanced cardiac compliance after long periods of ET (Arbab-Zadeh et al. 2014), suggests multifactorial mechanisms for the enhancement of $\dot{Q}_{peak}$ with ET (Saltin et al. 1968).

The ET-induced increase in BV can be removed by phlebotomy to assess its importance for the ET-induced changes in $\dot{Q}_{peak}$ and $\dot{V}O_{2peak}$. When using this experimental design, the 7-10% improvement in $\dot{V}O_{2peak}$ and $\dot{Q}_{peak}$ after 6 weeks of ET were reversed to pre-ET levels after phlebotomy (Bonne et al. 2014; Montero et al. 2015a). Concomitantly, no change in cardiac morphology (Bonne et al. 2014) nor in the a-vO_2diff were found despite robust peripheral adaptations (Montero et al. 2015a). Therefore, the authors suggested that the improvement in $\dot{V}O_{2peak}$ was explained by increased $\dot{Q}_{peak}$ attributed to the BV expansion alone. However, acute hypovolemia due

to phlebotomy may increase the sympathetic tone leading to arteriolar and venous constriction (Fortrat et al. 1998; Zollei et al. 2004) and offset an ET-induced drop in peripheral vascular resistance in these studies. In this context, reduced BV and impaired haemodynamic control after bed rest can lead to a reduction in $\dot{Q}_{peak}$ and $\dot{V}O_{2peak}$ during upright cycling (Saltin et al. 1968), which is reversed when measured during supine cycling (Bringard et al. 2010) due to gravitational effects on central BV, preload and thus SV (Warburton et al. 2002). The supine position increases the central venous pressure and the baroreflex loading compared to the upright position (Ray et al. 1993), thus potentially avoiding increases in sympathetic tone and total peripheral resistance in the face of reduced BV.

We investigated the importance of the ET-induced increases in haemoglobin mass (Hb_{mass}) and BV for the changes in $\dot{V}O_{2peak}$ and $\dot{Q}_{peak}$. Maximal exercise was conducted before and after removing an amount of blood corresponding to the measured individual increase in Hb_{mass} induced by ET. We hypothesised that the increases in $\dot{V}O_{2peak}$ and $\dot{Q}_{peak}$ would return to pre-ET levels after phlebotomy during upright cycling but remain elevated during supine cycling owing to improved venous return. Furthermore, we hypothesised that the change in $\dot{V}O_{2peak}$ is mostly facilitated by elevated $\dot{Q}_{peak}$, but also by a widened a-vO_2diff. Potential mechanisms for the changes in a-vO_2diff and $\dot{Q}_{peak}$ were studied in muscle biopsies (capillarisation and mitochondrial enzymes) and by echocardiography, respectively.

Materials and methods

Ethical approval

The study was approved by the Ethics Committee of the Norwegian School of Sport Sciences (ref. 13-220817) and The Norwegian Centre for Research Data (ref. 55151). Oral and written informed consents were obtained from all subjects before the start of this investigation, which was carried out in accordance with the Declaration of Helsinki.

Subjects

Twelve untrained subjects, defined as conducting ≤ 1 ET session per week during the previous year, were recruited and completed the ET period (7♂; age: 29.2 ± 5.9 years; weight: 72.4 ± 13.1 kg; height: 1.75 ± 0.11 m; body fat: 27 ± 5%; $\dot{V}O_{2peak}$: 44.2 ± 5.9 ml · kg^{-1} · min^{-1}). One subject performed all tests except the phlebotomy procedure and the post-phlebotomy testing. Therefore, data from this individual were used only when presenting individual responses and the muscle biopsy analyses. All subjects were non-smokers and reported no contraindications to ET or maximal exercise testing.

Experimental design

The experimental design is summarised in Fig. 1. Before and after 10 weeks of ET, Hb_{mass}, BV, body composition and cardiac dimensions were assessed, and a biopsy from the *m. vastus lateralis* was obtained under resting conditions. Maximal exercise testing was performed during upright and supine cycling before and after ET as well as directly after removing the BV necessary to counteract the individual increase in Hb_{mass} elicited by the ET. Before pre-ET measurements, all subjects were familiarised with supine cycling (two sessions) and maximal exercise during upright cycling.

<<Fig. 1 here>>

Exercise training

During the 10-week ET period, the subjects underwent three supervised training sessions per week. Session 1 consisted of 60 min of continuous exercise between 70-80% of peak heart rate (HR_{peak}) (Fig. 2a). Session 2 included 4 × 8 min intervals with a target intensity between 85-90% of HR_{peak} (Fig. 2b). Lastly, session 3 consisted of 4-6 × 4 min intervals with a target intensity ≥ 90% of HR_{peak} (4 repetitions in weeks 1-3, 5 repetitions in weeks 4-6, and 6 repetitions in weeks 7-9; Fig. 2c). The intervals were interspersed by 3 or 2 min active recovery at ~70% of HR_{peak}, respectively. A short tapering was performed in week 10 to maximise performance during the post-ET testing. The continuous session was shortened to 40 min, and the number of repetitions during the 8 and 4 min intervals were reduced to 3 and 4 repetitions, respectively. All subjects conducted 27-30 ET sessions (compliance: 94.4 ± 3.6%).

<<Fig. 2 here>>

Measurements and procedures

Body composition

Body composition was assessed by dual-energy X-ray absorptiometry (Lunar iDXA, enCORE software version 17; GE Healthcare, Chicago, IL, USA) after overnight fasting.

Haematology and blood withdrawal

Blood from an antecubital vein was collected in the morning under standardised, seated conditions. Haematological variables were analysed for EDTA blood (3 ml; BD, Franklin Lakes, NJ, USA) using a Sysmex XN-9000 (Sysmex, Kobe, Japan). Serum samples (SST II Advance 5 ml; BD) were placed at room temperature for coagulation (30 min), centrifuged at 1500 G for 10 min and stored at 4°C until analysed for ferritin concentration (Advia Chemistry XPT; Siemens Healthineers, Erlangen, Germany).

Hb_{mass} was measured in duplicate on separate days using a carbon monoxide (CO) rebreathing method (Prommer and Schmidt 2007; Schmidt and Prommer 2005). First, the subjects rested seated for 10 min, followed by capillary blood sampling in two 125-µl pre-heparinised tubes (Clinitubes; Radiometer, Copenhagen, Denmark) from a pre-heated fingertip. The subjects then inhaled a bolus of 1.0 (♀) or 1.2 (♂) ml per kg body weight of 99.97% chemically pure CO (AGA Norge, Oslo, Norway) administered via a 100-ml plastic syringe (Omnifix; Braun, Kronberg im Taunus, Germany) to a spirometer (Blood tec GmbH, Germany). In this closed circuit, the CO was rebreathed for 2 min together with 3 l of pure O_2 (AGA Norge) while checking for leakages using a CO analyser (Draeger, Lübeck, Germany). Two capillary blood samples were collected, 6 and 8 min after the administration of CO. All blood samples were immediately analysed in duplicate for percent carboxyhaemoglobin using an ABL80 CO-OX FLEX (Radiometer). After rebreathing, the CO not absorbed by the body was calculated by multiplying the CO concentration of the rebreathing bag by the bag volume and the subject's estimated residual lung volume (Miller et al. 1998). The CO exhaled between the time-point of disconnecting from the spirometer to the blood sampling was estimated by multiplying the difference in end-tidal CO concentration before and after rebreathing by the estimated alveolar ventilation (West 2008). The Hb_{mass} was calculated by dilution of CO in blood (Schmidt and Prommer 2005) with correction for

loss of CO to myoglobin (0.3% of the administered CO per minute) (Prommer and Schmidt 2007). The coefficient of variation of the duplicate Hb_{mass} determinations, expressed as the percent typical error (standard deviation of the difference scores/$\sqrt{2}$), was 1.10%. To derive intravascular volumes (BV; red blood cell volume, RBCV; plasma volume, PV), the formulae given by Siebenmann et al. (2017) were used with no correction for venous to whole-body haematocrit.

The phlebotomy trial was conducted 3.9 ± 2.2 days after the post-ET trial. A BV equal to each individual's ET-induced increase in Hb_{mass} (ΔHb_{mass} / [Hb]) was removed by phlebotomy via an 18 G catheter (BD) indwelling in an antecubital vein. Immediately after the phlebotomy, echocardiography was conducted, followed by exercise testing starting 45 min after the phlebotomy.

Exercise testing

Upright and supine cycling were conducted in random counterbalanced order on the same day, interspersed by 45 min of passive rest. During upright cycling (Excalibur Sport; Lode B.V., Groningen, The Netherlands), the test started with a 3-min resting measurement while seated on the bike. Thereafter, three 5-min submaximal workloads (♂ 50-150 W, ♀ 50-100 W) were conducted directly followed by a maximal test with step-increments of 25 W every minute until exhaustion. The mean workload during the last 60 s was defined as the peak power output ($\dot{W}_{peak}$). During supine cycling (Angio 2000; Lode B.V.), the subjects were fastened with a 4-point harness lying on a bench. The axis of rotation and thus the legs were raised ~20 cm above the heart. The structure of the protocol was similar to upright cycling, with the submaximal workloads (♂ 50-100 W, ♀ 50-75 W) followed by step-increments of 20 W every minute. After reaching exhaustion, the subjects cycled at 40-50 W for 5 min to speed up their recovery. $\dot{V}O_2$ was measured over the last 2.5 min at each submaximal stage and continuously during the resting measurements and incremental tests, using open-circuit indirect calorimetry with a mixing chamber (Oxycon Pro; Jaeger Instrument, Friedberg, Germany) (Foss and Hallén 2005). Before each test, the gas analysers and flow transducer were calibrated according to the instruction manual.

SV, HR and $\dot{Q}$ were continuously monitored by impedance cardiography and an integrated electrocardiogram (ECG) using a PhysioFlow Q-link device (Manatec

Biomedical, Paris, France). This method is evaluated against the direct Fick method (Charloux et al. 2000; Richard et al. 2001; Siebenmann et al. 2015) and uses the cyclic variations in transthoracic impedance during the cardiac cycle to estimate SV, as these pulsatile variations represent the changes in volume and velocity of the aortic BV (Charloux et al. 2000; Richard et al. 2001). Six electrodes (PF-50; Manatec Biomedical) were placed on each subject's neck, chest and back after the skin was cleaned with alcohol and rubbed with an abrasive ECG preparation gel (Custo prep; Custo med, Ottobrunn, Germany). Equal placement of electrodes in all tests was ensured by tracking their positions on transparent plastic sheets according to skin and anatomical landmarks. The subjects wore a tight mesh t-shirt to avoid displacement of the electrodes and their attached leads. A fan was placed in front of the subjects for heat dissipation to counteract the accumulation of sweat during exercise, to ensure that the electrodes maintained adhesiveness throughout the test. After instrumentation, the subjects rested on the ergometer for 5 min before autocalibration of the software (version 2.7.4). Immediately after the autocalibration, blood pressure was measured in duplicate (ProBP 3400 series; Welch Allyn, Skaneateles, NY, USA) and the mean values were fed to the software.

$\dot{V}O_2$, SV, HR and $\dot{Q}$ were recorded using 10-second averages. On the submaximal workloads, the average of the last 2 min served as the steady-state values. During the incremental tests to exhaustion, the highest 30-s average was taken as the peak value. The a-vO_2diff was calculated as the ratio between $\dot{V}O_2$ and $\dot{Q}$ according to the Fick equation. The peak capillary blood lactate concentration ($[La]_{peak}$) was measured 1 min after exhaustion (Biosen C-line; EKF Diagnostic, Cardiff, UK). The typical error for $\dot{W}_{peak}$ and $\dot{V}O_{2peak}$ measured during upright cycling (familiarisation vs pre-ET) was 3.0% and 2.9%, respectively.

Transthoracic echocardiography

All subjects underwent three echocardiographic studies (pre-ET, post-ET and directly after phlebotomy) (Vivid E95; GE Vingmed Ultrasound AS, Horten, Norway) using a 2.5 MHz (M5Sc) and an active matrix 4D volume phased array transducer. Echocardiographic views were obtained using greyscale harmonic imaging according to

the recommendations of the European Association of Cardiovascular Imaging (Lang et al. 2015). Recordings were digitally stored for offline post-hoc analysis (EchoPac; GE Vingmed Ultrasound AS), carried out by a blinded observer. From 2D echocardiography, left ventricular (LV) dimensions and LV diastolic function parameters were assessed. Right ventricular (RV) areas and fractional area change were assessed in the 4-chamber view. Tissue Doppler was used to assess wall motion velocities at the mitral annulus level and mean values from the septal and lateral walls are reported. LV mass was calculated using Devereux' formula (Devereux et al. 1986). 3D data sets, including LV volumes and ejection fraction, were obtained using a dedicated semi-automated algorithm.

Strain analysis was performed using 2D speckle tracking echocardiography by automatic tracking of acoustic markers on a frame-by-frame basis throughout the cardiac cycle. The endocardial borders were traced in the end-systolic frame of the 2D images from the apical 4-, 2-chamber, and apical long-axis views for the assessment of longitudinal strain. The operator manually adjusted segments where the automatic tracking failed. Peak systolic LV global longitudinal strain was averaged from 16 LV segments. The frame rate was 61 ± 5 Hz.

Skeletal muscle biopsy

Biopsies (~100-200 mg) were collected from the mid-portion of *m. vastus lateralis* after local anaesthesia, using the Bergström technique with manual suction (n = 10). The tissue was immediately dissected free from visible fat and connective tissue. An appropriate sample for immunohistochemistry was embedded in OCT (CellPath, Newtown, UK) and quickly frozen in isopentane cooled on liquid nitrogen to freezing point (approx. -120^{o}C). Tissue allocated for Western Blotting was immediately snap-frozen in liquid nitrogen. All tissue samples were stored at -80^{o}C until further analyses.

Immunohistochemistry

Serial 8 μm transverse cross sections were cut at -20^{o}C (Leica CM1860 UV; Leica Biosystems, Danvers, MA, USA), mounted on microscope slides (Superfrost Plus; Thermo Fischer Scientific, Waltham, MA, USA), air-dried and stored at -80^{o}C until analyses. The sections were blocked for 60 min with 1% bovine serum albumin (Sigma

Life Science, St Louis, MO, USA) in a phosphate-buffered saline (Sigma Life Science) and 0.05% Tween-20 (VWR, West Chester, PA, USA) solution (PBS-t). Primary antibodies against 1) myosin heavy chain type 1 (1:500 dilution; BA-D5, obtained from DSHB, Iowa City, IA, USA) (Schiaffino et al. 1989) and dystrophin (1:500; ab15277; Abcam, Cambridge, UK) or 2) the endothelial marker CD31 (1:100; M0823; Dako A/S, Glostrup, Denmark) and dystrophin were diluted in the blocking solution and incubated overnight at 4^{o}C. The sections were then washed 3×10 min in PBS-t, incubated with secondary antibodies (1:200; Alexa Fluor 488, A11001 and A11012; Invitrogen Molecular Probes, Carlsbad, CA, USA) for 60 min and again washed 3×10 min in PBS-t before mounted with Prolong Gold antifade reagent with DAPI (Life Technologies Corp., Carlsbad, CA, USA) and covered with glass. The sections were visualised under a 10× / 0.30 NA air objective (UplanFL N; Olympus corp.; Tokyo, Japan) 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., ON, Mississauga, Canada). Fibre cross-sectional areas, fibre types and manual identification of capillaries were conducted and analysed using TEMA software (CheckVision, Denmark). The investigator was blinded for subject identity and time point. Capillarisation was expressed as capillary-to-fibre ratio, capillaries in contact with each fibre and capillary density (capillaries per mm^2). A mean of 198 ± 56 (range: 96-345) fibres were analysed for each cross section.

Protein immunoblot

For Western blotting analyses, ~60 mg of muscle tissue was homogenised in 1 ml T-PER (Tissue Protein Extraction Reagent, 78510; Thermo Fischer Scientific) and 20 µL Halt Protease & Phosphatase Inhibitor Cocktail (78440; Thermo Fischer Scientific). The tissue lysate was extracted, aliquoted and stored at -80^{o}C until further analyses. The protein concentration was measured using a commercial kit (BioRad DC Protein Assay, 5000116; Bio-Rad Laboratories, Hercules, CA, USA) and a FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany). Standard Western blotting procedures were applied for quantification of citrate synthase (CS), cytochrome c oxidase subunit 4 (COX-IV) and hydroxyacyl-CoA dehydrogenase (HAD): 20µg of protein was separated by 4-12% gradient Bis-Tris gels (Invitrogen, Life Technologies)

for ~45 min at 200 V in cold buffer (NuPage MES SDS Running Buffer; Invitrogen, Life Technologies). Proteins were subsequently transferred onto a PVDF membrane (Bio-Rad Laboratories) at 30 V for 90 min in cold buffer (NuPage Transfer Buffer; Invitrogen, Life Technologies). Membranes were blocked at room temperature for 2 h in a 5% fat-free skimmed milk (Merck, Darmstadt, Germany) and 0.1% TBS-t solution (TBS: Bio-Rad Laboratories; Tween-20: VWR). Thereafter, the membranes were divided into three pieces based on molecular weight (Protein Ladder 310005; GeneON, Ludwigshafen am Rhein, Germany) and then incubated overnight (4^{o}C) with primary antibodies against CS (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, Danvers, MA, USA) secondary antibody was applied for 1 h at room temperature followed by visualisation using an HRP detection system (Super Signal West Dura Extended Duration Substrate; Thermo Fischer 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 and post samples were loaded on the same gel in duplicate, and mean values were used for statistical analysis.

Statistical analyses

Data in text and tables are presented as mean ± standard deviation (SD) and in graphs as mean ± standard error of the mean. The data were initially assessed for normal distribution using the D'Agostino-Pearson test. For variables only measured pre-ET and post-ET, group changes were analysed with a paired Student's t-test. Peak and submaximal responses measured on three time points were analysed using repeated measures ANOVA and two-way repeated measures ANOVA (workload x time point), respectively. ANOVAs was followed by the Dunnett's multiple comparisons test, comparing the control situation (pre-ET) with the post-ET and the phlebotomy trial. The alpha-level was set to ≤ 0.05 and values between > 0.05 and ≤ 0.10 were considered to indicate trends. GraphPad Prism 8 (GraphPad Software, CA, USA) was used for statistical analysis.

Results

Body Composition

Subjects weight was reduced by 1.4 ± 1.8 kg (P = 0.028) during the ET period, which was entirely accounted for by a reduction in fat mass (-1.8 ± 1.4 kg; P = 0.002; Table 1) as lean body mass remained unchanged (0.1 ± 1.3 kg; P = 0.895).

Haematological adaptations

The Hb_{mass} increased by 24 ± 22 g (P = 0.005; Fig. 3) and BV increased by 181 ± 288 ml (P = 0.044; Table 1) from pre- to post-ET. To re-establish pre-ET levels of Hb_{mass} during the phlebotomy exercise trial, 166 ± 139 ml of whole blood was phlebotomised, which caused the BV to be unchanged compared with pre-ET levels (difference: 15 ± 195 ml; P = 0.953). Due to a small increase in MCHC (P = 0.022) after ET (i.e. higher Hb concentration in the RBCs), the RBCV was decreased (difference: -89 ± 95 ml; P = 0.020) and the PV was slightly increased (difference: 104 ± 192; P = 0.174) after phlebotomy compared with pre-ET levels.

<<Table 1 and Fig. 3 here>>

Maximal exercise tests

After ET, $\dot{W}_{peak}$ during upright and supine cycling was increased (12 ± 7%; P < 0.001 and 8 ± 8%; P = 0.014, respectively) and remained elevated after phlebotomy (9 ± 7%; P = 0.001 and 7 ± 8%; P = 0.048, respectively; Fig. 4a). Similarly, $\dot{V}O_{2peak}$ increased by 11 ± 6% (0.36 ± 0.20 l · min^{-1}; P < 0.001) and 10 ± 8% (0.25 ± 0.20 l · min^{-1}; P = 0.003) during upright and supine cycling, respectively, and both remained elevated after phlebotomy (11 ± 4% and 11 ± 9%, respectively; both P < 0.005; Fig. 4b). During upright cycling, $\dot{Q}_{peak}$ increased by 10 ± 10% (1.8 ± 1.5 l · min^{-1}; P = 0.005) after ET and showed a trend towards remaining elevated after phlebotomy (9 ± 14%; P = 0.081; Fig. 4c). During supine cycling, $\dot{Q}_{peak}$ was not changed after ET (4 ± 9%; P = 0.376) but was increased after phlebotomy compared to pre-ET (9 ± 10%; P = 0.023; Fig. 4c). This effect was driven by two outliers lowering the mean value during the post-ET assessment. The peak a-vO_2diff during upright and supine cycling was unchanged after

ET, both before (P = 0.788 and P = 0.228, respectively) and after (P = 0.528 and P = 0.850, respectively) phlebotomy (Fig. 4d).

<<Fig. 4 here>>

At the three measurement time-points, a similar level of exertion was evident during the incremental exercise tests, as indicated by similar peak ventilation (VE_{peak}), peak respiratory exchange ratio (RER_{peak}), HR_{peak}, rating of perceived exertion (RPE) and $[La]_{peak}$ (Table 2).

<<Table 2 here>>

Submaximal exercise

During submaximal exercise, SV was increased after ET both during upright ($F_{1.6, 16.2}$ = 3.7; P = 0.054; see Fig. 5 for post-hoc tests) and supine cycling ($F_{1.4, 14.1}$ = 8.2; P = 0.008), which was accompanied by a reduction in HR (upright: $F_{1.4, 14.4}$ = 4.9; P = 0.032; supine: $F_{1.4, 14.0}$ = 15.9, P < 0.001). The a-vO_2diff was unchanged during submaximal exercise (upright: $F_{1.9, 19.4}$ = 1.0; P = 0.369; supine: $F_{1.6, 16.4}$ = 0.6; P = 0.517).

<<Fig. 5 here>>

Cardiac morphology and function

The LV mass increased by 13 ± 17 g after ET (P = 0.027) and no change was observed for LV and RV chamber volumes (Table 3). Hence, an increase in LV mass-to-volume ratio (LV mass / EDV) was observed, especially after phlebotomy (P = 0.013), indicating concentric cardiac remodelling. All LV and RV diastolic- and systolic function parameters were unchanged.

<<Table 3 here>>

Muscular adaptations

ET increased the protein content of the mitochondrial enzymes CS (P < 0.001), COX-IV (P = 0.007) and HAD (P = 0.003; Fig. 6). Fibre cross-sectional area increased (Table 4), both in type I (P = 0.039) and type II (P = 0.023) muscle fibres. Despite increased cross-sectional area, the capillary density (capillaries · mm^{-2}) was maintained (P = 0.772), due to a trend towards increased capillary-to-fibre ratio (P = 0.065).

<<Table 4 and Fig. 6 here>>

Discussion

The primary findings of the present study are: (1) The ET-induced increase in $\dot{V}O_{2peak}$ was preserved despite removing the ET-induced increases in Hb_{mass} and BV by phlebotomy. Therefore, improvements in $\dot{V}O_{2peak}$ and $\dot{Q}_{peak}$ cannot be exclusively attributed to BV expansion. (2) The improvements in $\dot{V}O_{2peak}$ were mainly accounted for by the increase in $\dot{Q}_{peak}$ and SV_{peak}, independent of posture. (3) ET induced concentric cardiac remodelling including an increase in LV mass, with preserved chamber dimensions measured at rest, which probably contributed to the increase in SV_{peak}.

Why was $\dot{V}O_{2peak}$ maintained after reducing Hb_{mass} to pre-training values?

Despite normalising Hb_{mass} and BV, the ET-induced improvements in $\dot{V}O_{2peak}$ and $\dot{Q}_{peak}$ were maintained during upright cycling. This contradicts studies using a similar experimental design, which suggest that $\dot{V}O_{2peak}$ and $\dot{Q}_{peak}$ return to pre-ET levels after removing the ET-induced elevations in BV (Bonne et al. 2014) and RBCV (Montero et al. 2015a). We speculate that the discrepancies between studies originate from the magnitude of the ET-induced BV expansion. For Bonne et al. (2014) and Montero et al. (2015a), the ET-induced increases in BV were 382 ml (7%) and 310 ml (6%), respectively, compared to 181 ml (4%) in the present study. Yet improvements in $\dot{V}O_{2peak}$ (9-11%) and $\dot{Q}_{peak}$ (7-10%) during upright cycling were similar in the three studies. Thus, ET-induced increases in $\dot{V}O_{2peak}$ and $\dot{Q}_{peak}$ of this magnitude does not depend on BV expansion alone, and $\dot{Q}_{peak}$ improved partly due to different mechanisms in the three studies. This is supported by increased LV mass in the present study, as opposed to no change in the study by Bonne et al. (2014), and suggests that multifactorial mechanisms explain the ET-induced increases in $\dot{Q}_{peak}$ and $\dot{V}O_{2peak}$ as suggested in classical studies (Saltin et al. 1968).

Because of the small BV withdrawal, little hypovolemia-induced impairment on venous return was likely elicited in our subjects. Supported by no reductions in $\dot{Q}_{peak}$,

$\dot{V}O_{2peak}$ and submaximal SV during upright cycling after phlebotomy. Consequently, we were unable to test one of our main hypotheses: that, when $\dot{V}O_{2peak}$ and $\dot{Q}_{peak}$ during upright cycling were reversed to pre-ET levels after phlebotomy; the improvements would be preserved during supine cycling owing to the beneficial gravitational effects on venous return.

Apparently, it may be that the cardiovascular system can maintain venous return to the heart despite small BV-reductions by redistributing venous volumes through increased vasomotor activity acting on capacitance vessels. This is supported by the unchanged LV EDV, SV and peak mitral inflow velocity during early diastole from before to just after the phlebotomy procedure. There is, however, some uncertainty in extrapolating these responses measured during supine rest to upright peak exercise, where LV filling times are shorter and exert a major challenge on cardiac preload (Gledhill et al. 1994). Similarly, indications of maintained venous return despite small reductions in BV have been observed in studies examining fluid loss after heat stress and prolonged exercise (Saltin 1964; Saltin and Stenberg 1964). In those studies, a small to moderate reduction in PV was not sufficient to decrease $\dot{Q}_{peak}$, assessed in normothermic conditions. Thus, it was argued that an effective contribution from the muscle pump and increased vasomotor activity enabled a normal SV despite reduced BV. In contrast to small BV losses, phlebotomy of one unit of blood (450 ml) or more reduces $\dot{V}O_{2peak}$ by lowering venous return (Kanstrup and Ekblom 1984; Krip et al. 1997). Therefore, a certain threshold might exist, at which small reductions in BV are not detrimental to venous return, acting as a mechanism for the circulation to cope with small BV losses and PV reductions induced by, e.g. dehydration.

Although Hb_{mass} and BV were restored to pre-ET levels, transcapillary fluid shifts may occur during and after the phlebotomy procedure. The first exercise trial started 45 min after phlebotomy as compared with ~15 min in the studies by Bonne et al. (2014) and Montero et al. (2015a). To obtain an indication of fluid shifts, we measured [Hb] repeatedly during the phlebotomy day in 6 of the subjects. From before (15.6 ± 1.0 g $\cdot$ dl^{-1}) to just after the phlebotomy procedure, the venous [Hb] was slightly decreased (15.2 ± 1.0 g $\cdot$ dl^{-1}; $P = 0.19$), with no further change until initiation of the first (15.2 ± 1.2 g $\cdot$ dl^{-1}) and second (15.2 ± 1.1 g $\cdot$ dl^{-1}) cycling exercise trials. Therefore, at the end of, or gradually during the phlebotomy procedure, a gross

movement of fluid from the interstitium to the plasma appears to have counteracted the blood withdrawal. Hence, the PV (and BV) may have been slightly higher during the phlebotomy exercise trials than reported in the present study, which may have contributed to the maintained $\dot{Q}_{peak}$. However, since the reduction in [Hb] exclusively occurred from before to just after the phlebotomy, this mechanism has likely also affected the subjects in the Bonne et al. (2014) and Montero et al. (2015a) studies. Also, the Hb_{mass} was re-established to pre-ET levels independent of potential PV shifts.

We have focused on the role of BV for $\dot{Q}_{peak}$, i.e. one of the main determinants of venous return and cardiac preload. However, an ET-induced reduction in afterload through a lowering of total peripheral resistance may also increase $\dot{Q}_{peak}$. For instance, after ET of both legs separately, Klausen et al. (1982) found a reduction in mean arterial pressure (MAP) and total peripheral resistance that likely contributed to the increased $\dot{Q}_{peak}$ after ET. This mechanism can also have facilitated the increased $\dot{Q}_{peak}$ in the present study.

The magnitude of haematological adaptations

The changes in BV reported after 6-8 weeks ET (3-4 sessions · $week^{-1}$) typically range from 140-550 ml (Bonne et al. 2014; Helgerud et al. 2007; Montero et al. 2017; Montero et al. 2015a; Montero and Lundby 2017b), whereas a meta-analysis reported a mean increase of 267 ml after ~15 weeks of ET (range: 1-51 weeks) (Montero and Lundby 2017a). A complex interplay of mechanisms causes the BV expansion in ET that may be affected by the training program, the training status of the subjects and their nutritional status (Montero and Lundby 2018; Sawka et al. 2000). Although our BV expansion was within the expected range, the smaller increase compared to that by Bonne et al. (2014) and Montero et al. (2015a) is unlikely caused by iron deficiency, as indicated by the normal and maintained ferritin levels. Lean body mass was maintained, indicating sufficient protein and caloric intake during the ET period. Over 10 weeks, the subjects performed 27-30 ET sessions as compared to only 18-20 sessions over 6 weeks in the studies by Bonne et al. (2014) and Montero et al. (2015a), using a similar training intensity. Hence, the training intensity and the total volume and length of the training intervention cannot explain the different findings.

Central vs peripheral limitations to $\dot{V}O_{2peak}$.

There were no statistically significant changes in estimated a-vO_2diff from pre- to post-ET, potentially indicating no substantial contribution of peripheral adaptations to the improvements in $\dot{V}O_{2peak}$. Based on the average of all maximal exercise tests conducted pre- (n = 2; upright and supine cycling) and post-ET (n = 4; before and after phlebotomy), $\dot{V}O_{2peak}$ increased by 318 ± 147 ml · min^{-1}. Of this increase, the increase in $\dot{Q}_{peak}$ account for 221 ± 202 ml · min^{-1}. Hence, on average, a-vO_2diff was elevated by 5 ± 12 ml · l^{-1} (154 ± 22 vs 159 ± 25 ml · l^{-1}) and account for 30% of the increase in $\dot{V}O_{2peak}$. Therefore, our data support that $\dot{V}O_{2peak}$ is mainly limited by convective O_2 delivery (Montero et al. 2015b; Mortensen et al. 2005), but also supports calculations indicating that 70-75% of the limitations lie within the central circulation and that 25-30% are determined by peripheral factors (di Prampero 2003; di Prampero and Ferretti 1990).

O_2 extraction and blood flow are interdependent. For example, by decreasing $\dot{Q}_{peak}$ and leg blood flow using β-adrenergic blockade, systemic and leg a-vO_2diff increases during sub-maximal and maximal exercise, facilitated by increased erythrocyte capillary mean transit time (MTT) (Ekblom et al. 1972; Pawelczyk et al. 1992). In the present study, muscle fibre hypertrophy was accompanied by only a minor increase in the capillary-to-fibre ratio, causing no change in capillary density. If we calculate the capillary volume within the leg muscle mass engaged during cycling (Boushel et al. 2014) and subtract a non-leg blood flow of 6.5 l · min^{-1} from the total $\dot{Q}_{peak}$ (Calbet et al. 2007; Calbet et al. 2006; Lundby et al. 2008; Mortensen et al. 2005), there would be a trend towards shorter erythrocyte MTT after ET during upright peak exercise (508 ± 138 vs 452 ± 132 ms before and after ET, respectively). Therefore, due to reduced time for O_2 unloading, peripheral adaptations such as increased muscle oxidative capacity (CS and COX-IV) may have been crucial in maintaining the pre-ET level of a-vO_2diff. This is substantiated by a correlation between the percent change in a-vO_2diff during upright cycling and the percent change in CS content from before to after ET (r = 0.73; n = 10; P = 0.017). Further improvements in a-vO_2diff, at least large enough to evoke statistical significance, may likely only be detected if peripheral adaptations largely surpass the changes in $\dot{Q}_{peak}$ and peripheral blood flow.

After years of training, elite endurance athletes have a higher leg O_2 extraction than untrained individuals (> 90% vs ~70%, respectively) (Calbet et al. 2005; Roca et al. 1992). A similar situation can be evoked by relative short periods of one-legged ET inducing robust peripheral adaptations without stimulating the central circulation, and improve leg a-vO_2diff by 5-10 ml · l^{-1} (Klausen et al. 1982; Rud et al. 2012). Thus ET improves the muscles' ability to extract O_2 but may be masked by improvements in $\dot{Q}_{peak}$ and peripheral blood flow after short periods of whole-body ET (Montero et al. 2015b). Furthermore, the improvements seen after 7-8 weeks of one-legged ET by Rud et al. (2012) and Klausen et al. (1982) were in the range of 5-10 ml · l^{-1} as assessed by arterial and femoral venous blood sampling. Accordingly, small potential improvements in systemic a-vO_2diff, as indicated in the present study (5 ml · l^{-1}), may be difficult to detect when calculated from $\dot{V}O_{2peak}$ and non-invasively determined $\dot{Q}_{peak}$.

Cardiac remodelling

LV mass increased without any change in EDV. This contradicts the classic model of athletic cardiac remodelling that predicts increased EDV following ET due to the haemodynamic stimulus of volume loading on the ventricles (Morganroth et al. 1975). However, longitudinal studies demonstrate concentric remodelling at the commencement of ET, with the adaptations gradually switching into eccentric remodelling. For instance, after 3 months of ET, Arbab-Zadeh et al. (2014) found a 10% increase in LV mass-to-volume ratio before it returned to pre-ET levels after 9-12 months of ET. Therefore, with an increase in LV mass-to-volume ratio of 14% after 10 weeks of ET, our data support that the initial ET-induced cardiac remodelling is concentric (Arbab-Zadeh et al. 2014; Bjerring et al. 2019; Weiner et al. 2015).

Despite unchanged LV EDV and diastolic and systolic functional parameters at rest, the submaximal exercise SVs and SV_{peak} were increased after ET. This indicates an increased capacity of the heart to utilise the Frank-Starling mechanism to increase SV from rest to peak exercise after ET (Crawford et al. 1985; Rerych et al. 1980). This could be due to increased chamber compliance (Arbab-Zadeh et al. 2014; Levine et al. 1991), increased filling rates (Ferguson et al. 2001; Gledhill et al. 1994), a lower rise in MAP during exercise due to reduced total peripheral resistance (Klausen et al. 1982) or a combination. Since the SV_{peak} was elevated even after phlebotomy, increased filling

rates through expanded BV are unlikely to have made any major contribution. However, with the present experimental design, we cannot determine whether increased LV mass, enhanced venous return through other mechanisms than elevated BV, reduced total peripheral resistance, or improved qualitative properties of the heart (e.g. contractility, compliance, faster ventricular relaxation) were facilitating the elevated SV_{peak} and $\dot{Q}_{peak}$.

Study considerations

Although Hb_{mass} and BV were restored to pre-ET levels by phlebotomy, transcapillary fluid shifts may have occurred, and it is uncertain whether BV normalisation was preserved during exercise. Some of the subjects were unfamiliar with cycling exercise before the study, and no one had tried supine cycling. Therefore, we cannot exclude the possibility that some subjects became better able to maintain venous return and cardiac filling at peak exercise after ET due to familiarisation. The findings were obtained from a small sample size. But the main finding was that the ET-induced increase in $\dot{V}O_{2peak}$ was preserved despite removing the increase in Hb_{mass}, and this conclusion is based on methods with low typical error ($\dot{V}O_{2peak}$ 2.9% and Hb_{mass} 1.1%). Impedance cardiography is associated with a larger measurement error than the invasive gold-standard methods (Del Torto et al. 2019; Richard et al. 2001). Besides, when calculating the a-vO_2diff from $\dot{V}O_2$ and $\dot{Q}$ inherent of its measurements errors, a larger measurement error is expected as compared with deriving a-vO_2diff using arterial and venous blood sampling.

Conclusion

Improvements in $\dot{V}O_{2peak}$ and $\dot{Q}_{peak}$ following short-term ET do not depend on Hb_{mass} and BV expansions in untrained individuals. $\dot{V}O_{2peak}$ increased primarily through increased $\dot{Q}_{peak}$ but also through a widened a-vO_2diff, explaining ~70% and ~30% of the improvements, respectively, and were potentially mediated by cardiac remodelling and mitochondrial biogenesis.

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Competing interests

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

Author Contributions

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Tables

Table 1. Haematological variables and body composition measured before and after 10 weeks of endurance training.

	Pre-training (mean ± SD)	Post-training (mean ± SD)	% Change (mean ± SD)
Body composition			
Body weight (kg)	74.2 ± 12.1	72.7 ± 11.3	-1.8 ± 2.6*
Lean mass (kg)	51.2 ± 9.0	51.3 ± 9.5	0.0 ± 2.6
2-leg lean mass (kg)	18.0 ± 3.9	18.0 ± 4.0	-0.2 ± 1.8
Fat mass (kg)	20.5 ± 4.3	18.8 ± 4.4	-9.0 ± 8.2*
Haematology			
Hb_{mass} (g)	795 ± 196	820 ± 196	3.3 ± 2.9*
BV (ml)	5098 ± 929	5279 ± 947	3.7 ± 5.6*
RBCV (ml)	2388 ± 548	2370 ± 528	-0.3 ± 5.0
PV (ml)	2712 ± 432	2909 ± 464	7.5 ± 9.2*
[Hb] ($g \cdot dl^{-1}$)	15.5 ± 1.5	15.4 ± 1.3	-0.2 ± 3.6
Haematocrit (%)	46.5 ± 3.7	44.6 ± 3.3	-3.8 ± 4.5*
MCHC ($g \cdot dl^{-1}$)	33.3 ± 1.8	34.5 ± 0.9	4.0 ± 4.8*
S-Ferritin ($\mu g \cdot l^{-1}$)	87.7 ± 58.8	86.9 ± 52.9	11.9 ± 41.7

N = 11. BV, blood volume; [Hb], haemoglobin concentration; Hb_{mass}, haemoglobin mass; MCHC, mean corpuscular haemoglobin concentration; PV, plasma volume; RBCV, red blood cell volume. * Significant change from pre- to post-training ($P \leq 0.05$).

Table 2. Variables indicating the level of exertion at exhaustion.

Variable	Pre-training (mean ± SD)	Post-training (mean ± SD)	Phlebotomy (mean ± SD)
Upright cycling			
HR_{peak}	196 ± 7	193 ± 9	193 ± 10
VE_{peak} ($l \cdot min^{-1}$)	146 ± 40	150 ± 39#	148 ± 33
RER_{peak}	1.22 ± 0.06	1.21 ± 0.05	1.19 ± 0.06
$[La]_{peak}$ ($mmol \cdot l^{-1}$)	12.3 ± 1.9	12.7 ± 2.1	12.8 ± 2.1
RPE	19.5 ± 0.7	19.5 ± 0.7	19.6 ± 0.5
Supine cycling			
HR_{peak}	184 ± 9	181 ± 12	181 ± 13
VE_{peak} ($l \cdot min^{-1}$)	118 ± 31	123 ± 29	122 ± 27
RER_{peak}	1.20 ± 0.06	1.18 ± 0.05	1.15 ± 0.05*
$[La]_{peak}$ ($mmol \cdot l^{-1}$)	10.9 ± 2.1	11.5 ± 2.2	11.1 ± 2.8
RPE	19.5 ± 0.8	19.6 ± 0.7	19.5 ± 0.8

N=11. HR_{peak}, peak heart rate (10-s average); $[La]_{peak}$, peak blood lactate concentration; RPE, rating of perceived exertion using the Borg scale (6-20); RER_{peak}, peak respiratory exchange ratio (30-s average); VE_{peak}, peak ventilation (30-s average). * Significantly different from pre-training ($P \leq 0.05$). # Trend towards being different from pre-training ($0.05 < P \leq 0.10$).

Table 3. Cardiac morphology and function measured at rest before and after 10 weeks of endurance training, as well as directly after phlebotomy.

Variable	Pre-training (mean ± SD)	Post-training (mean ± SD)	Phlebotomy (mean ± SD)
Left ventricular morphology			
LV mass (g)	123 ± 37	137 ± 37*	
IVSd (mm)	7.4 ± 0.8	8.1 ± 0.8	
LV PWd (mm)	7.3 ± 0.9	7.7 ± 0.9	
LV EDD (mm)	49.1 ± 6.5	50.1 ± 6.2	49.9 ± 6.6
3D EDV (ml)	124 ± 35	124 ± 40	117 ± 39
3D ESV (ml)	49 ± 15	50 ± 19	46 ± 17
LV mass-to-volume ratio ($g \cdot ml^{-1}$)	1.02 ± 0.26	1.12 ± 0.20	1.19 ± 0.21*
Left ventricular systolic function			
3D ejection fraction (%)	61 ± 3	60 ± 3	61 ± 4
3D stroke volume (ml)	75 ± 21	74 ± 21	71 ± 23
Global longitudinal strain (%)	-22.5 ± 1.4	-22.1 ± 1.3	-21.1 ± 1.8#
Left ventricular diastolic function			
E ($cm \cdot s^{-1}$)	72.9 ± 14.5	73.2 ± 17.9	69.2 ± 13.3
A ($cm \cdot s^{-1}$)	47.2 ± 5.4	47.3 ± 5.0	46.2 ± 10.4
E / A ratio	1.6 ± 0.4	1.6 ± 0.4	1.6 ± 0.4
E' ($cm \cdot s^{-1}$)	12.5 ± 2.1	12.0 ± 2.6	11.6 ± 1.9
A' ($cm \cdot s^{-1}$)	7.8 ± 1.1	7.5 ± 1.4	7.2 ± 1.5
E / E' ratio	5.8 ± 0.6	6.1 ± 1.0	6.1 ± 1.2
Right ventricular morphology and function	±	±	±
RV end-diastolic area (cm^2)	19.3 ± 4.3	20.5 ± 3.0	20.3 ± 4.1
RV end-systolic area (cm^2)	11.2 ± 2.3	12.0 ± 1.8	11.5 ± 2.4
RV fractional area change (%)	42 ± 4	42 ± 3	43 ± 2
TAPSE (cm)	2.35 ± 0.18	2.31 ± 0.22	2.35 ± 0.26
Atrial morphology			
Left atrial volume (ml)	46 ± 9	50 ± 8	45 ± 8
Right atrial area (cm^2)	15.7 ± 2.9	15.1 ± 2.4	15.5 ± 2.8

N = 11, except for global longitudinal strain and RV parameters (N = 10). E and A, peak mitral inflow velocity during early diastole and during atrial systole, respectively; E' and A', myocardial peak velocity during early diastole and atrial systole, respectively; EDV, end-diastolic volume; ESV, end-systolic volume; EDD, end-diastolic diameter; IVSd, interventricular septum thickness in end-diastole; LV, left ventricular; PWd, posterior wall thickness in end-diastole; RV, right ventricular; TAPSE, tricuspid annular plane systolic excursion. * Significantly different from pre-training ($P \leq 0.05$). # Trend towards being different from pre-training ($0.05 < P \leq 0.10$).

Table 4. Muscle morphology and capillarisation before and after 10 weeks of endurance training.

Variable	Pre-training (mean ± SD)	Post-training (mean ± SD)
Fibre size (μm^2)		
All fibres	4176 ± 818	4799 ± 1032*
Type I	3870 ± 468	4479 ± 720*
Type II	4406 ± 1218	5027 ± 1562*
Capillaries around a fibre		
All fibres	3.4 ± 0.8	3.8 ± 0.8
Type I	3.4 ± 0.7	3.8 ± 0.8
Type II	3.3 ± 0.7	3.7 ± 1.0
Capillaries · mm^{-2}	337 ± 86	345 ± 67
Capillary-to-fibre ratio	1.38 ± 0.31	1.62 ± 0.40#

N = 10; * Significantly ($P \leq 0.05$) or # trend towards ($0.05 < P \leq 0.10$) change from pre- to post-training.

Figure legends

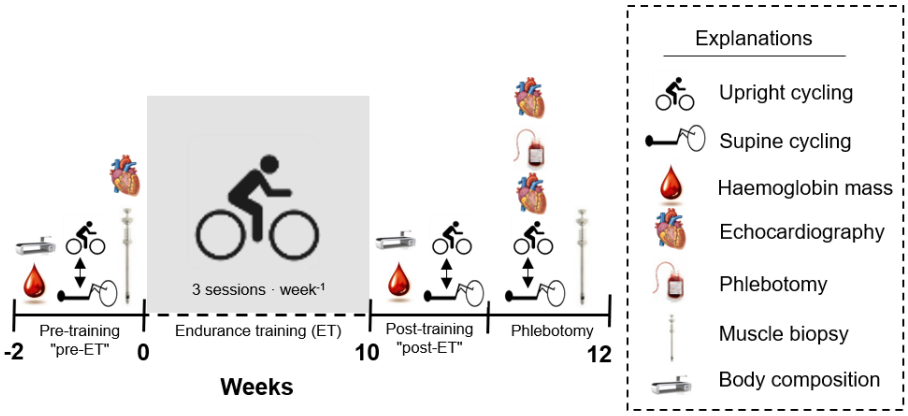

Fig. 1 The experimental design of the study. During the phlebotomy trial, echocardiography was conducted first (the post-ET echocardiography), following which the subjects were phlebotomised. This was followed by a second echocardiography. The first of two cycling exercises was initiated precisely 45 min after phlebotomy

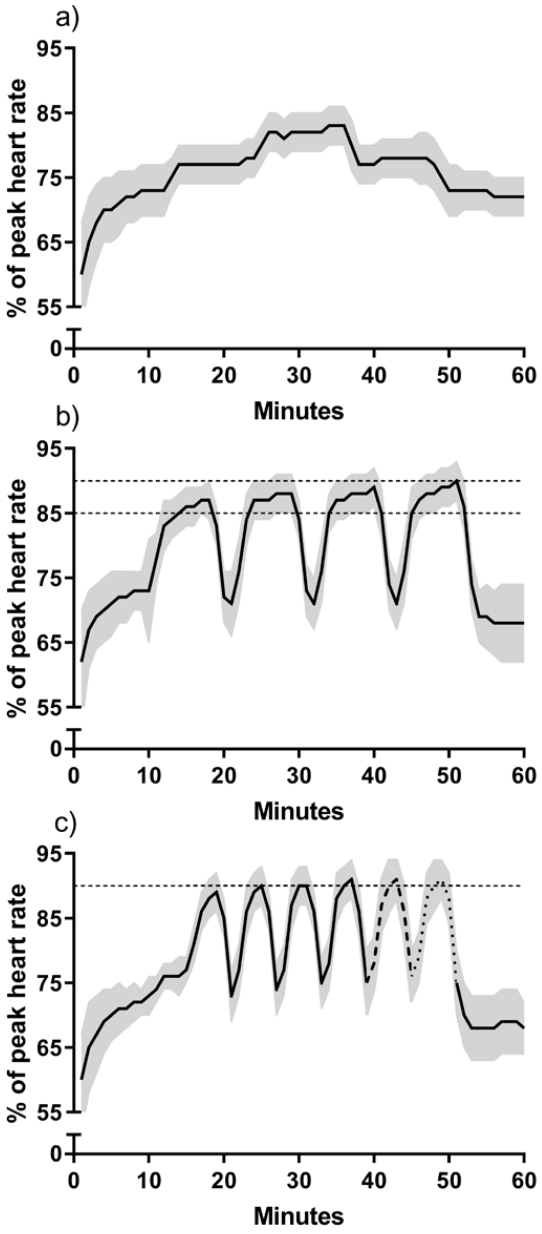

Fig. 2 Percentage of peak heart rate during continuous moderate-intensity (a); 8-min interval (b); and 4-min interval (c) sessions conducted during the training period. The black lines and the grey-shaded areas denote their mean values and standard deviations, respectively

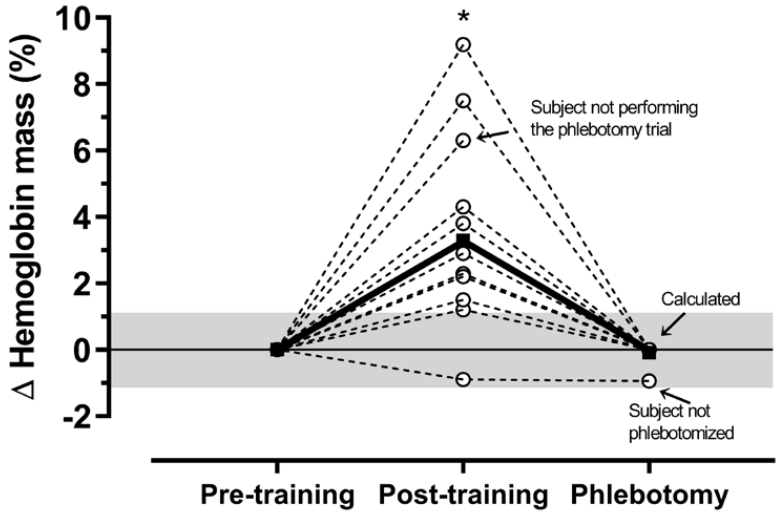

Fig. 3 Individual (white circles and dashed lines) and mean changes (black squares and solid line) in haemoglobin mass from before to after 10 weeks of endurance training. The grey area represents the percent typical error for this variable, as calculated from the duplicate measurements (1.10%). *Significantly different from pre-training (P = 0.005). N = 11-12

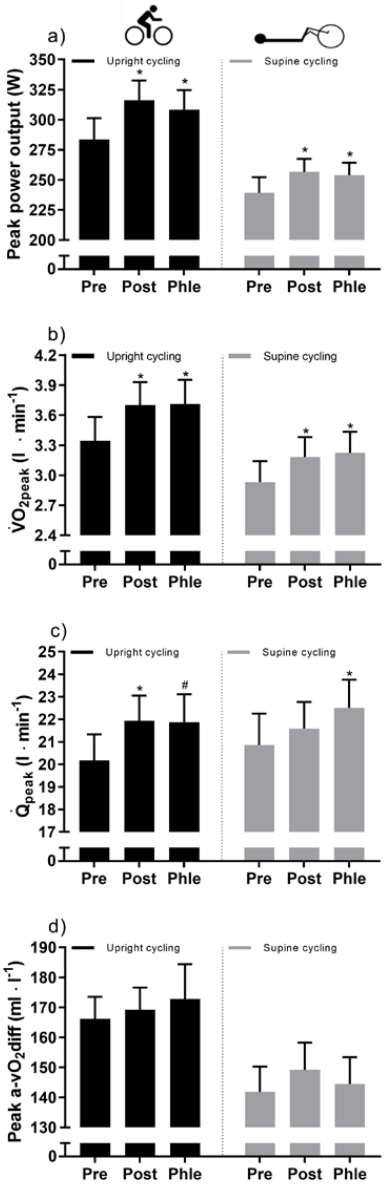

Fig. 4 Peak values of power output (a); oxygen uptake ($\dot{V}O_{2peak}$) (b); cardiac output ($\dot{Q}_{peak}$) (c); and arteriovenous O_2 difference (a-$\dot{v}O_2$diff) (d) during incremental exercise tests to exhaustion before training (Pre), after training (Post) and after training and phlebotomy (Phle). Error bars indicate the standard error of the mean. * Significant change from pre-training ($P \leq 0.05$). # Trend towards change from pre-training ($0.05 < P \leq 0.10$). N = 11

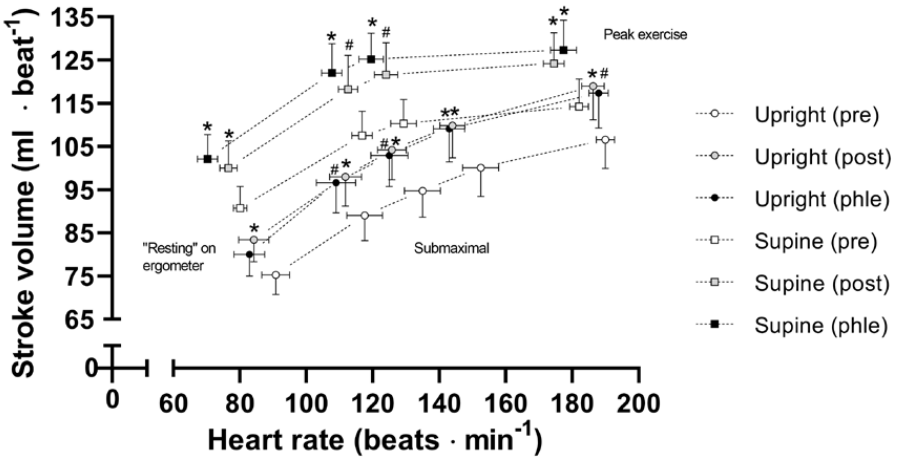

Fig. 5 The stroke volume during upright and supine cycling as a function of heart rate during pre-training, post-training and phlebotomy exercise trials. Error bars indicate standard error of the mean. * Significant change in stroke volume from pre-training ($P \leq 0.05$). # Trend towards change in stroke volume from pre-training ($0.05 < P \leq 0.10$). N = 11

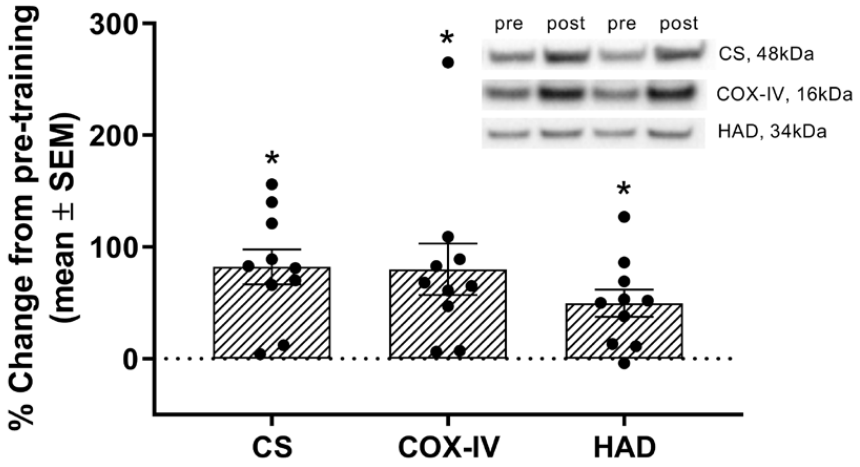

Fig. 6 The percentage change in protein content and representative Western blots for citrate synthase (CS), cytochrome c oxidase subunit 4 (COX-IV) and hydroxyacyl-CoA dehydrogenase (HAD) from pre- to post-training. The error bars indicate standard error of the mean. Black circles indicate individual responses. * All proteins increased from pre- to post-training ($P < 0.001$). N = 10

Paper II

Effects of 150 and 450 ml acute blood losses on plasma volume shift and oxygen uptake during maximal exercise

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Abstract

Purpose: This study aimed to determine whether maximal oxygen uptake ($\dot{V}O_{2max}$) and exercise capacity are maintained after small acute blood loss (~150 ml) and elucidate potential compensatory mechanisms.

Methods: Thirteen young, healthy subjects (weight: 75±7 kg; height: 1.79±0.07 m; $\dot{V}O_{2max}$: 63±9 ml·kg^{-1}·min^{-1}; mean±SD) performed submaximal and maximal exercise on a cycle ergometer in three experimental conditions: in euvolaemia (CON; blood volume, BV: 6.0±0.7 l) and after acute BV reductions of 150 ml (BVR_{150ml}) and 450 ml (BVR_{450ml}). Plasma volume (PV) shifts were monitored by measuring haematocrit, haemoglobin concentration and haemoglobin mass.

Results: $\dot{V}O_{2max}$ was unchanged after BVR_{150ml} (-1±2%; P=0.124) but reduced by 7±3% after BVR_{450ml} (P<0.001) compared to CON. This equalled a 2.5-fold larger $\dot{V}O_{2max}$-reduction after BVR_{450ml} than BVR_{150ml} after normalising to the BV removed (P=0.029). Peak power output did only decrease after BVR_{450ml} (P<0.001). At maximal exercise, BV was restored after BVR_{150ml} compared to CON (-50±185 ml; P=0.375), attributed to transcapillary fluid-shifts expanding PV and counteracting blood withdrawal. This mechanism was not fully capable of restoring BV after BVR_{450ml} (-281±184 ml; P<0.001). A small increase in peak heart rate (3±5 beats·min^{-1}; P=0.062) and reduction in Vastus Lateralis tissue oxygenation index (-4±8 %-points; P=0.080) were evident after BVR_{450ml}, the latter indicating increased leg O_2 extraction.

Conclusion: $\dot{V}O_{2max}$ and maximal exercise capacity were preserved after removing 150 ml blood, which was caused by a rapid PV restoration. Compensatory mechanisms were also evident after removing 450 ml blood, including increased PV, heart rate and O_2 extraction, but were inadequate in maintaining $\dot{V}O_{2max}$.

Keywords: blood volume; cardiac output; haemoglobin mass; maximal oxygen uptake; plasma volume, VO2max

Abbreviations

a-$\bar{v}O_2$diff: arterial to mixed venous oxygen difference

BV: blood volume

CO: carbon monoxide

[Hb]: haemoglobin concentration

Hb_{mass}: haemoglobin mass

hct: haematocrit

HR: heart rate

HR_{peak}: peak heart rate

[La]: blood lactate concentration

MCHC: mean corpuscular haemoglobin concentration

PV: plasma volume

$\dot{Q}$: cardiac output

$\dot{Q}_{max}$: maximal cardiac output

RBC: red blood cell

RBCV: red blood cell volume

RER_{peak}: peak respiratory exchange ratio

RPE: rating of perceived exertion

SD: standard deviation

SV: stroke volume

VE_{peak}: peak ventilation

$\dot{V}O_2$: oxygen uptake

$\dot{V}O_{2max}$: maximal oxygen uptake

$\dot{W}_{peak}$: peak power output

Introduction

It is well established that the circulating blood volume (BV) and the total haemoglobin mass (Hb_{mass}) are of principal importance for maximal oxygen uptake ($\dot{V}O_{2max}$) and maximal exercise capacity in humans (Ekblom et al. 1972; Schmidt and Prommer 2010). A standard blood donation of 400-500 ml reduces $\dot{V}O_{2max}$ by ~5-10% (Balke et al. 1954; Ekblom et al. 1972; Gordon et al. 2014; Panebianco et al. 1995) and the reinfusion of ~360-450 ml of freeze-preserved packed red blood cells (RBC) increases $\dot{V}O_{2max}$ by ~2-6% (Buick et al. 1980; Ekblom et al. 1976; Spriet et al. 1986). The underlying mechanisms responsible are the accompanying changes in stroke volume (SV), maximal cardiac output ($\dot{Q}_{max}$) and blood O_2 carrying capacity, thus changing the systemic O_2 delivery (Ekblom et al. 1976; Spriet et al. 1986).

BV expansion can be observed after a few weeks of endurance training (ET) and is proposed to play a significant role in the enhancement of $\dot{V}O_{2max}$ and maximal exercise capacity (Montero et al. 2017). However, not all investigations detect a change in BV after ET despite robust increases in $\dot{V}O_{2max}$ and exercise capacity (Helgerud et al. 2007; Jacobs et al. 2013; Shoemaker et al. 1996), suggesting that the enhancement of $\dot{V}O_{2max}$ is multifactorial (Saltin et al. 1968). The role of the ET-induced BV expansion can be investigated experimentally by manipulating the BV to the pre-ET level immediately followed by an assessment of $\dot{V}O_{2max}$ and maximal exercise capacity. Conflicting findings have emerged when using this experimental design, including the ET-induced $\dot{V}O_{2max}$ gains being completely abolished (Bonne et al. 2014; Montero et al. 2015) to remain ~10% elevated above the pre-ET level and unaffected by the phlebotomy (Skattebo et al. 2020). One explanation for the discrepancies may originate from the magnitude of the BV expansions and the resulting BV withdrawal before the re-assessment of $\dot{V}O_{2max}$ in these studies. In the previous study conducted in our lab, removal of ~170 ml blood was necessary to counteract the ET-induced BV expansion (Skattebo et al. 2020) compared to ~360-380 ml in the two other studies (Bonne et al. 2014; Montero et al. 2015). Therefore, compensatory mechanisms may exist that fully preserves $\dot{V}O_{2max}$ when the blood loss is sufficiently low, but exceeding this threshold will obviously result in decreased $\dot{V}O_{2max}$ caused by reduced systemic O_2 delivery. Indications of maintained venous return despite small BV reductions have been

observed in studies examining fluid loss after heat stress and prolonged exercise (Saltin 1964; Saltin and Stenberg 1964). In those studies, a small to moderate reduction in plasma volume (PV) was not sufficient to decrease $\dot{Q}_{max}$, assessed in normothermic conditions. Thus, it was argued that an effective contribution from the muscle pump and increased vasomotor activity redistributed venous blood volumes and enabled a normal SV. Therefore, a certain threshold might exist, at which small BV reductions are not detrimental to $\dot{Q}_{max}$, $\dot{V}O_{2max}$ and maximal exercise capacity, acting as a mechanism for the circulation to cope with small BV losses and PV reductions.

To address this question experimentally, we removed 150 ml blood and immediately tested its impact on $\dot{V}O_{2max}$ and maximal exercise capacity compared to an euvolaemic control trial and a more severe blood loss (450 ml) in the same subjects. Potential transcapillary fluid shifts after blood withdrawal and during exercise were monitored by measuring haemoglobin concentration ([Hb]) and haematocrit (hct) repeatedly, by knowing the Hb_{mass} (CO rebreathing) and by calculating the intravascular volumes (BV, PV and RBCV) (Dill and Costill 1974). We hypothesised that $\dot{V}O_{2max}$ and peak power output ($\dot{W}_{peak}$) during incremental exercise to exhaustion are maintained after removing 150 ml blood, but decreased after removing 450 ml.

Materials and methods

Ethical approval

The study was approved by the Ethics Committee of the Norwegian School of Sport Sciences (ref. 100-290819) and The Norwegian Centre for Research Data (ref. 418763). Oral and written informed consents were obtained from all subjects before the start of this investigation, which was carried out in accordance with the Declaration of Helsinki.

Subjects

Thirteen moderately to well-trained men were recruited and completed all tests (age: 27.3 ± 4.0 years; weight: 74.7 ± 6.5 kg; height: 1.79 ± 0.07 m; $\dot{V}O_{2max}$: 63.4 ± 8.7 ml · kg^{-1} · min^{-1}; Hb_{mass}: 891 ± 127 g; BV: 5991 ± 736 ml). An additional two subjects were recruited but dropped out due to factors unrelated to the study. All subjects were non-smokers and reported no contraindications to maximal exercise testing.

Experimental design

The subjects visited the laboratory on three occasions at the same time of day (08:00 or 14:00): on a familiarisation day, a control day and an experimental day, each separated by 1 week (Fig. 1). Exercise testing was repeated twice on each day, separated by 90 min rest. Each trial included three submaximal workloads followed by a maximal test with step-increments every minute until exhaustion for determination of $\dot{W}_{peak}$ and $\dot{V}O_{2max}$. The two control trials were conducted in euvolaemia (normal BV), whereas the 1st and the 2nd experimental trial were preceded by the removal of 150 ml blood (referred to as BVR_{150ml}) and 450 ml blood (150 ml + 300 ml; BVR_{450ml}), respectively. In this way, the 1st and the 2nd control trial served as controls for BVR_{150ml} and $BVR_{450ml,}$ respectively. Between trials, the recovery and the food intake were standardised. *Ad libitum* water intake was recorded on the control day and was repeated on the experimental day. The subjects were instructed to abstain from strenuous exercise and caffeine consumption the last 24 h and 12 h before lab visits, respectively. Hb_{mass} was measured on the familiarisation day for calculations of intravascular volumes.

<<Fig. 1 here>>

Measurements and procedures

Exercise trials

The trials started with a 3-min resting measurement while seated on the cycle ergometer (Excalibur Sport; Lode B.V., Groningen, The Netherlands). Afterwards, the three 5-min submaximal workloads were directly followed by a maximal test with step-increments of 25 W every minute until exhaustion, with the starting level being the third submaximal workload +25 W. The submaximal workloads were individually set to induce a capillary blood lactate concentration ([La]; Biosen C-line; EKF Diagnostic, Cardiff, UK) $\leq$ 2.5 mmol $\cdot$ l^{-1} on the third workload and a time to exhaustion of ~8 min on the maximal test (based on the familiarisation day). The mean workload during the last 60 s was defined as $\dot{W}_{peak}$. After reaching exhaustion, the subjects cycled at 50-100 W for 10 min to speed up their recovery.

$\dot{V}O_2$ was measured over the last 2.5 min at each submaximal stage and continuously during the resting measurements and incremental tests, using open-circuit indirect calorimetry with a mixing chamber (Oxycon Pro; Jaeger Instrument, Friedberg, Germany) (Foss and Hallén 2005). On the submaximal workloads, the average of the last 2 min served as the steady-state values. During the incremental tests to exhaustion, the highest 30-s average was taken as $\dot{V}O_{2max}$. Before each trial, the gas analysers and flow transducer were calibrated according to the instruction manual. Heart rate (HR) was measured continuously (electrocardiogram), and the highest 10-s average was defined as peak HR (HR_{peak}). The average 30-s HR during $\dot{V}O_{2max}$ was used to calculate the O_2 pulse ($\dot{V}O_2$ / HR) as an indicator of SV (O_2 pulse = SV · a-$\bar{v}O_2$diff). Blood samples were drawn at rest, during the last 20 s on each submaximal stage and at exhaustion. $[La]_{peak}$ was measured 1 min after exhaustion.

As a substitute measure of arterial O_2 saturation, peripheral capillary O_2 saturation (SpO_2) was measured using a pulse oximeter placed on the pre-heated right index finger (901-M; Masimo, Irvine, CA, USA). The arterial O_2 content (CaO_2) at maximal exercise was estimated as: (1.34 × [Hb] × SpO_2) + 3 ml dissolved in plasma. The tissue oxygenation index (TOI) was obtained from the Vastus Lateralis muscle as the ratio of oxygenated to total tissue Hb and myoglobin via spatially resolved near-infrared spectroscopy (NIRS; PortaMon; Artinis Medical Systems, Elst, The Netherlands). NIRS reflects primarily capillary oxygenation but is also affected by the arteriolar and venular blood and muscle myoglobin. The Vastus Lateralis TOI correlates well with the femoral venous O_2 saturation and may serve as a substitute measure of leg O_2 extraction (Boushel et al. 2001; Esaki et al. 2005). The oxygenation parameters were obtained using three light-emitting diodes (wavelengths: 760 and 850 nm) spaced 30, 35 and 40 mm from the detector. The NIRS optode was covered with Saran wrap, placed mid-way between the trochanter major and the lateral epicondyle and secured with elastic bandages around the thigh. Equivalent positioning across days was ensured by using skin and anatomical landmarks. The data was recorded at 10 Hz and analysed using the Oxysoft software (v. 3.0.95; Aritnis). One minute after the termination of exercise, an 8.5 cm-wide cuff (Zimmer Biomet, Warsaw, IN, USA) was manually inflated (VBM Medizintechnik, Sulz am Neckar, Germany) to 300 mmHg to occlude the arterial blood flow for 6 min (prolonged up to 8 min if necessary) until the minimum

TOI was reached. After cuff-release, the maximum TOI was recorded during hyperaemia. The subjects had a skin- and subcutaneous tissue thickness of 5.3 ± 1.8 mm (range: 3.1–9.7) below the optode, assessed using an ultrasound device (Vivid E95; GE Vingmed Ultrasound AS, Horten, Norway).

The typical error for $\dot{W}_{peak}$ and $\dot{V}O_{2max}$, calculated across the four exercise trials in euvolaemia (familiarisation and control trials) (Hopkins 2015), was 1.7 ×/÷ 1.3% (95% confidence limits presented as ×/÷ factor) and 1.9 ×/÷ 1.3%, respectively.

Blood sampling and phlebotomy

During the control- and the experimental day, blood samples (3 ml, EDTA; BD, Franklin Lakes, NJ, USA) were obtained via an 18 G catheter (BD) indwelling in an antecubital vein that was regularly flushed with normal saline (0.9% NaCl) to maintain patency. [Hb] was measured in duplicate (triplicate if large deviations occurred) on a hemoximeter (ABL80 CO-OX FLEX; Radiometer, Copenhagen, Denmark) and hct was measured in triplicate by the micro-centrifugation method (6 min at 12,800 rpm; Hettich, Tuttlingen, Germany) and adjusted for trapped plasma (3%). The intra-assay typical error was 1.6 ×/÷ 1.1% and 0.5 ×/÷ 1.1% for [Hb] and hct, respectively (n = 296-309). The same catheter was used for phlebotomy of 150 ml blood before BVR_{150ml}, and an additional 300 ml blood before BVR_{450ml} (450 ml in total). The blood was drawn slowly over 5 min (BVR_{150ml}) or 10 min (BVR_{450ml}) during which the blood pressure was monitored (ProBP 3400 series; Welch Allyn, Skaneateles, NY, USA). The subjects rested seated for 10 min after phlebotomy before moving to the cycle ergometer. The 150 ml blood removed before BVR_{150ml} consisted of 63 ± 4 ml RBCs and 87 ± 4 ml plasma. Before BVR_{450ml}, 186 ± 12 ml RBCs and 264 ± 12 ml plasma were removed in total. This represented 2.5 ± 0.3% (range: 2.1-3.2) and 7.4 ± 0.9% (range: 6.3-9.5) of the Hb_{mass} and 2.5 ± 0.3% (2.1-3.2) and 7.6 ± 1.0% (range: 6.4-9.6) of the subjects' total BV.

The circulating RBCV, PV and BV during exercise trials were calculated assuming a constant Hb_{mass} over the experimental period (Eastwood et al. 2008; Prommer et al. 2008). The reductions in intravascular volumes induced by phlebotomy and blood sampling were included in these calculations, and it was assumed that all changes in [Hb] and hct could be accounted for by fluctuations of PV occurring due to,

e.g., sweating, transcapillary fluid shifts, swelling/shrinking of RBCs, intestinal fluid uptake and urine production (Dill and Costill 1974; Stewart et al. 2003). Calculated PV reduction based on fluctuations in [Hb] and hct is found equivalent to that measured using radio-isotope-labelled human albumin (^{125}I-RISA) and RBCs (Cr^{51}) after heavy exercise (Stewart et al. 2003).

Haemoglobin mass and blood volume

Hb_{mass} was measured using a carbon monoxide (CO) rebreathing method (Prommer and Schmidt 2007; Schmidt and Prommer 2005). The subjects rested seated for 10 min, followed by capillary blood sampling in two 125-µl pre-heparinised tubes (Clinitubes; Radiometer) from a pre-heated fingertip. The subjects inhaled a bolus of 1.2 ml per kg body weight of 99.97% chemically pure CO (AGA Norge, Oslo, Norway) administered via a 100-ml plastic syringe (Omnifix; Braun, Kronberg im Taunus, Germany) to a spirometer (Blood tec GmbH, Germany). In this closed circuit, the CO was rebreathed for 2 min together with 3 l of pure O_2 (AGA Norge) while checking for leakages using a CO analyser (Draeger, Lübeck, Germany). Two capillary blood samples were collected, 6 and 8 min after the administration of CO. All blood samples were immediately analysed in duplicate for percent carboxyhaemoglobin using an ABL80 CO-OX FLEX (Radiometer). After rebreathing, the CO not absorbed by the body was calculated by multiplying the CO concentration of the rebreathing bag by the bag volume and the subject's estimated residual lung volume (Miller et al. 1998). The CO exhaled between the time-point of disconnecting from the spirometer to the blood sampling was estimated by multiplying the difference in end-tidal CO concentration before and after rebreathing by the estimated alveolar ventilation (West 2008). The Hb_{mass} was calculated by dilution of CO in blood (Schmidt and Prommer 2005) with correction for loss of CO to myoglobin (0.3% of the administered CO per min) (Prommer and Schmidt 2007). The typical error of duplicate Hb_{mass} determinations conducted by Ø.S. in our lab is typically 1.1-1.6 % (Koivisto et al. 2018; Skattebo et al. 2020).

Statistical analyses

Data in text and tables are presented as mean ± standard deviation (SD) and in graphs as mean ± 95% confidence limits. The data were initially assessed for normal distribution

using the D'Agostino-Pearson test. Maximal responses and resting measurements were analysed using a paired sample t-test and only planned comparisons were conducted: control 1 vs BVR_{150ml} and control 2 vs BVR_{450ml}. A paired sample t-test was used to analyse whether the change in $\dot{V}O_{2max}$ from control 1 to BVR_{150ml} was different from the change from control 2 to BVR_{450ml} (Δ value vs Δ value). The submaximal $\dot{V}O_2$, HR, TOI, blood volumes (BV, PV and RBCV) and haematological parameters (Hb, hct and MCHC) were analysed using a two-way repeated measures ANOVA (trial x workload) followed by the Bonferroni's multiple comparisons test. Control 1 vs BVR_{150ml} and control 2 vs BVR_{450ml} were analysed separately. The alpha-level was set to ≤ 0.05 and values between > 0.05 and ≤ 0.10 were considered to indicate trends. GraphPad Prism 8 (GraphPad Software, CA, USA) and Microsoft Office Excel 2016 (Microsoft Corporation, WA, USA) were used for statistical analysis.

Results

Maximal exercise tests

$\dot{V}O_{2max}$ was unchanged after BVR_{150ml} (-1.1 ± 2.3%; P = 0.124) but reduced by 6.9 ± 3.1% after BVR_{450ml} (P < 0.001; Fig. 2) compared to control, respectively. $\dot{W}_{peak}$ only decreased after BVR_{450ml} (P < 0.001; Table 1). The reduction in $\dot{V}O_{2max}$ per millilitre blood removed was larger after BVR_{450ml} than after BVR_{150ml} (-0.7 ± 0.3 vs -0.3 ± 0.6 ml · min^{-1} · ml^{-1}, respectively; P = 0.029) and per gram Hb_{mass} removed (-4.9 ± 1.9 vs -2.0 ± 4.3 ml · min^{-1} · g^{-1}, respectively; P = 0.024). The estimated CaO_2 at maximal exercise (based on [Hb] and SpO_2) was reduced after BVR_{150ml} (-4 ± 7 ml · l^{-1}; P = 0.040) and BVR_{450ml} (-6 ± 6 ml · l^{-1}; P = 0.004), with no significant differences between trials (P = 0.294). After BVR_{450ml}, the HR_{peak} and Vastus Lateralis TOI displayed trends towards being increased (3 ± 5 beats · min^{-1}; P = 0.062; Fig. 3) and decreased (P = 0.080; Fig. 4), respectively, the latter indicating increased leg O_2 extraction. After BVR_{450ml}, the O_2 pulse was decreased (P < 0.001), indicating decreased SV. The peak ventilation, breathing frequency, respiratory exchange ratio, rating of perceived exertion and $[La]_{peak}$ were similar across trials (Table 1).

The body weight-indexed $\dot{V}O_{2max}$ (ml · kg^{-1} · min^{-1}) at control was not related to the reduction in $\dot{V}O_{2max}$ (in ml · min^{-1}) after BVR_{150ml} (R^2 = 0.01; P = 0.740) nor

BVR_{450ml} (R^2 = 0.01; P = 0.795), indicating that the reduction in $\dot{V}O_{2max}$ induced by a fixed amount of blood withdrawal is independent of $\dot{V}O_{2max}$ and training status.

<<Table 1 and Fig. 2 here>>

Submaximal exercise

Blood withdrawal did not affect submaximal $\dot{V}O_2$ (main effect of trial: P = 0.478-0.621). After BVR_{450ml}, the HR was increased at the 2nd and 3rd submaximal workload (Fig. 3), whereas the O_2 pulse was decreased (2nd: P = 0.037; 3rd: P = 0.004). The Vastus Lateralis TOI was reduced at rest (P = 0.022) and displayed a trend towards being reduced during submaximal exercise after BVR_{450ml} (main effect of trial: P = 0.080; Fig. 4).

<<Fig. 3-4 here>>

Haematological fluctuations during exercise

The changes in [Hb], hct and mean corpuscular haemoglobin concentration (MCHC) during the trials are presented in Fig 5. Despite removing 150 ml blood, the calculated BV at rest was unchanged after BVR_{150ml} compared to control 1 (mean difference: -71 ± 234 ml; P=0.296; Fig. 6c). This was caused by transcapillary fluid shift occurring during and right after the phlebotomy inducing haemodilution (Fig. 5a-b) and re-established the PV to the pre-phlebotomy level (mean difference: -12 ± 202 ml; P = 0.835; Fig. 6b). From rest to exhaustion, the PV reduced similarly in control 1 and after BVR_{150ml} (-553 ± 173 vs -528 ± 110 ml, respectively) due to a similar haemoconcentration (Δ[Hb]; 10.3 ± 3.2% vs 10.0 ± 2.1%, respectively; P = 0.730). The BV at $\dot{W}_{peak}$ was similar (mean difference: -50 ± 185 ml; P = 0.375) despite a significantly lower RBCV after BVR_{150ml} (mean difference: -63 ± 59 ml; P = 0.002; Fig. 6a).

Despite similar body weight during all trials (Table 1; P ≥ 0.30) and identical fluid intake between the two control tests and between BVR_{150ml} and BVR_{450ml} (6.9 ± 4.0 dl water), the PV showed a trend towards being elevated before the 2nd phlebotomy compared to before control 2 (86 ± 164 ml; P = 0.083; comparison indicated in Fig. 6b)

causing a mild euvolaemic anaemia. After the 2nd phlebotomy, the PV was lower than in control 2 at rest (-158 ± 173 ml; P = 0.006) and during submaximal exercise (mean differences: -169 to -193 ml; all P < 0.01; Fig. 6b), but showed only a trend towards being different at $\dot{W}_{peak}$ (-90 ± 165 ml; P = 0.073). This was caused by a slightly larger PV reduction from rest to exhaustion in control 2 than after BVR_{450ml} (-550 ± 119 vs -482 ± 128 ml, respectively; P = 0.200). The haemoconcentration was 10.5 ± 2.5% and 9.7 ± 2.5% in these trials, respectively (P = 0.400). The RBCV and BV were lower at $\dot{W}_{peak}$ after BVR_{450ml} compared to control 2 (-191 ± 46; P < 0.001 and -281 ± 184 ml; P < 0.001, respectively), indicating that 38 ± 41 % of the total blood withdrawal (450 ml) was attenuated due to PV-restoration.

<<Fig. 5-6 here>>

Discussion

To the best of our knowledge, this is the first study that has investigated the effect of a small (150 ml) vs a moderate (450 ml) blood withdrawal on $\dot{V}O_{2max}$ during maximal exercise. The novel finding is that 150 ml blood loss was almost entirely compensated for by transcapillary fluid shift that re-established PV and likely contributed to the unchanged $\dot{V}O_{2max}$ and $\dot{W}_{peak}$. PV was not restored after the removal of 450 ml blood. Therefore, hypovolaemia and a lower O_2 transport capacity of blood caused $\dot{V}O_{2max}$ to be reduced by ~7% despite compensatory mechanisms being evident, including increased HR_{peak} and leg O_2 extraction (decreased TOI). The reduction in $\dot{V}O_{2max}$ per millilitre of blood removed was larger after removing 450 compared to 150 ml blood. Together, these findings demonstrate that compensatory mechanisms enable the circulation to cope with small BV reductions to preserve $\dot{V}O_{2max}$ and maximal exercise capacity, but with these being inadequate when the blood loss is too large

The sensitivity of $\dot{V}O_{2max}$ to BV reductions

The findings of the present study comply with previous work, showing a negligible effect of small BV reductions on $\dot{V}O_{2max}$ and $\dot{W}_{peak}$ (Skattebo et al. 2020). This also agrees with findings showing that small to moderate PV reductions induced by heat

stress or prolonged exercise does not decrease $\dot{Q}_{max}$ and $\dot{V}O_{2max}$ when assessed in normothermic conditions (Saltin 1964; Saltin and Stenberg 1964). Our results also agree with previous studies investigating the acute effect of blood withdrawal on $\dot{V}O_{2max}$ when the exercise test is initiated within 3 h after phlebotomy (Fig. 7). A statistical analysis of these studies shows a linear decrease in $\dot{V}O_{2max}$ as a function of the BV removed with the x-intercept at ~150 ml (Balke et al. 1954; Bonne et al. 2014; Hill et al. 2013; Krip et al. 1997; Montero et al. 2015; Panebianco et al. 1995; Rowell et al. 1964; Skattebo et al. 2020; Woodson et al. 1978). This relationship was even stronger when restricting the comparison to studies initiating the exercise test within 1 h after phlebotomy (R^2 = 0.90). Thus, collectively, these studies indicate that $\dot{V}O_{2max}$ decreases linearly with the BV removed, but with compensatory mechanisms being able to blunt the effect of blood loss up to and including ~150 ml. Of course, this threshold is probably dependent on the total BV of the subjects. This is supported by the fact that the two subjects with the largest individual reduction in $\dot{V}O_{2max}$ after removing 150 ml blood (see Fig. 2) were among the three subjects with the lowest BV and Hb_{mass}.

<<Fig. 7 here>>

Compensatory mechanisms preserving $\dot{V}O_{2max}$

The preserved $\dot{V}O_{2max}$ after BVR_{150ml} was probably mediated by the movement of extracellular fluid from the interstitial to the intravascular space that re-established PV, almost secured euvolaemia and likely maintained the venous return. An effective contribution from the muscle pump and increased vasomotor activity may also have redistributed venous blood volumes and enabled a normal SV despite the small hypovolaemia (Saltin 1964; Saltin and Stenberg 1964). Simultaneously, no change in the Vastus Lateralis TOI was observed, indicating an unchanged leg O_2 extraction. Although not measured, an unchanged $\dot{Q}_{max}$ is also supported by the unchanged O_2 pulse and the small haemodilution-induced reduction in estimated CaO_2 at maximal exercise (-4 ml · l^{-1}). To substantiate, with a mean $\dot{V}O_{2max}$ of 4.7 l · min^{-1} it can be assumed the subjects had a mean $\dot{Q}_{max}$ at around 28 l · min^{-1} (Mortensen et al. 2008; Munch et al. 2014), which implicates that the systemic O_2 delivery could have been reduced by ~110 ml · min^{-1} (-4 ml · l^{-1} × 28 l · min^{-1}) even with an unchanged $\dot{Q}_{max}$.

Therefore, the small reduction in CaO_2 and the unchanged O_2 pulse and O_2 extraction postulates that $\dot{Q}_{max}$ had to be maintained due to the unchanged $\dot{V}O_{2max}$.

Physiological responses after a moderate blood loss (450 ml)

$\dot{V}O_{2max}$ was reduced by ~7% after withdrawing 450 ml blood, which is similar to previous findings showing an average reduction of ~9% (range: 4-16%) when $\dot{V}O_{2max}$ was measured within 3 h after 450-500 ml blood donation (Balke et al. 1954; Hill et al. 2013; Krip et al. 1997; Panebianco et al. 1995; Rowell et al. 1964; Woodson et al. 1978). The reduction equalled 4.9 ml · min^{-1} per g Hb_{mass} removed from the circulation, which is almost identical to the slope between Hb_{mass} and $\dot{V}O_{2max}$ in subjects with a wide range in physical fitness and body size (4.4 - 4.8 ml · min^{-1} · g^{-1}) (Gore et al. 1997; Lundby and Robach 2015; Schmidt and Prommer 2010), confirming a dependency of $\dot{V}O_{2max}$ on Hb_{mass} by approximately 4-5 ml · min^{-1} · g^{-1}.

At rest, after removing 450 ml blood, [Hb] was reduced by 0.4 g · dl^{-1} compared to before the 1st phlebotomy. The transcapillary fluid shifts induced by hypovolaemia was likely the cause of this decay, but a contribution from post-exercise albumin retention, which increased the oncotic pressure and therefore fluid retention within the intravascular space after BVR_{150ml}, is also plausible (Gillen et al. 1991), and may explain why the PV was slightly increased before starting the 2nd phlebotomy compared to rest in control 2 (see arrow in Fig. 6b). Blood donations of 450 ml have previously been shown to decrease [Hb] with 0.3-0.4 g · dl^{-1} within 2 h (Mora-Rodriguez et al. 2012; Panebianco et al. 1995), although, some studies observed a delayed response with decreased [Hb] being evident first the following day(s) (Eliassen et al. 2018; Hill et al. 2013). Mora-Rodriguez et al. (2012) observed a reduction in BV of only 287 ml 2 h after the withdrawal of 450 ml blood, which is identical to the decrease in BV observed at rest after BVR_{450ml} in the present investigation (~300 ml).

The reduction in $\dot{V}O_{2max}$ after BVR_{450ml} was likely mediated by the combined effect of a reduced SV, as indicated by the reduced O_2 pulse and increased HR, and a haemodilution-induced reduction of CaO_2. The small reduction in the estimated CaO_2 (6 ml · L^{-1}) may, by itself, explain a ~170 ml · min^{-1} reduction in systemic O_2 delivery (-6 ml · l^{-1} × 28 l · min^{-1}), meaning that $\dot{Q}_{max}$ was likely decreased by ~1-2 l · min^{-1} to facilitate the observed reduction in $\dot{V}O_{2max}$ (-320 ml · min^{-1}). Very few investigations

have measured $\dot{Q}_{max}$ after blood withdrawal. Ekblom et al. (1976) observed a 240 ml · min^{-1} reduction in $\dot{V}O_{2max}$ 24 h after the withdrawal of 800 ml blood. This was accompanied by an unchanged $\dot{Q}_{max}$ (determined by dye-dilution) attributed to the "over-night" PV expansion indicated by the 1.7 g · dl^{-1} reduction in [Hb] and an unchanged BV determined by Cr^{51} labelled RBCs. Therefore, the reduction in $\dot{V}O_{2max}$ was entirely attributed to reduced CaO_2 that lowered the systemic O_2 delivery. Conversely, when measured 1 h after the removal of 500 ml blood, $\dot{Q}_{max}$ was reduced by 4.5 l · min^{-1} (acetylene rebreathing) alongside with the ~600 ml · min^{-1} reduction in $\dot{V}O_{2max}$ (Krip et al. 1997). The likely explanation for the different findings was that hypovolaemia was not yet offset by PV expansion in the study by Krip et al. (1997), confirmed using Evans blue dye. Therefore, in the early phases after blood withdrawal ($\geq$ 450 ml), $\dot{V}O_{2max}$ is lowered due to the combined effect of reduced $\dot{Q}_{max}$ and CaO_2, whereas, in the following days when BV is restored, $\dot{V}O_{2max}$ and systemic O_2 delivery are reduced entirely attributed to euvolaemic anaemia (Celsing et al. 1986; Ekblom et al. 1976; Krip et al. 1997).

Some of the reduction in systemic O_2 delivery was likely counteracted by elevated leg O_2 extraction since the Vastus Lateralis TOI was reduced at rest and tended to be reduced during submaximal and maximal exercise, in line with previous findings (McDonagh et al. 2016).

Conclusion

In conclusion, $\dot{V}O_{2max}$ and maximal exercise capacity were preserved after removing 150 ml blood, which was caused by a rapid PV restoration. Several compensatory mechanisms were also evident after removing 450 ml blood, including an almost complete PV restoration and a slightly increased HR_{peak} and leg O_2 extraction (decreased TOI), but these mechanisms were insufficient to maintain $\dot{V}O_{2max}$. Therefore, the reduction in $\dot{V}O_{2max}$ was more severe after a blood withdrawal of 450 ml compared to 150 ml, even after normalising to the volume of blood removed.

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Additional information

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Competing interests

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

Author Contributions

Conception and design of the experiment: Ø.S., J.H., C.C.

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Analysis of data: Ø.S., E.S.J.

Interpretation of data: Ø.S., J.H., C.C., E.S.J.

Writing the first draft: Ø.S.

Revising the manuscript: All authors.

All authors have read and approved the final version of the manuscript.

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Tables

Table 1. Measurements obtained at maximal exercise, and the body weight measured before each trial.

	First test each day		Second test each day	
	Control 1 (mean ± SD)	BVR_{150ml} (mean ± SD)	Control 2 (mean ± SD)	BVR_{450ml} (mean ± SD)
Peak power output (watt)	384 ± 50	381 ± 53	381 ± 51	369 ± 54***
$\dot{V}O_{2max}$ ($ml \cdot min^{-1}$)	4700 ± 667	4656 ± 707	4749 ± 694	4429 ± 697***
VE_{peak} ($l \cdot min^{-1}$)	195 ± 29	193 ± 33	195 ± 32	190 ± 34#
BF ($breaths \cdot min^{-1}$)	63 ± 14	63 ± 17	62 ± 10	62 ± 20
RER_{peak}	1.19 ± 0.05	1.19 ± 0.06	1.15 ± 0.05	1.16 ± 0.06*
HR_{peak} ($beats \cdot min^{-1}$)	184 ± 8	184 ± 7	185 ± 7	188 ± 7#
O_2 pulse ($ml \cdot beat^{-1}$)	25.8 ± 3.9	25.5 ± 4.0	25.9 ± 4.0	23.8 ± 3.8***
[Hb] ($gram \cdot dl^{-1}$)	16.4 ± 1.0	16.0 ± 1.1#	16.3 ± 1.0	15.8 ± 1.1**
SpO_2 (%)	95 ± 2	95 ± 1	95 ± 1	95 ± 1
Estimated CaO_2 ($ml \cdot l^{-1}$)	212 ± 14	208 ± 14*	211 ± 14	205 ± 12**
Vastus lateralis TOI (%)	55.9 ± 6.1	53.9 ± 6.6	58.7 ± 5.1	54.4 ± 6.1#
$[La]_{peak}$ ($mmol \cdot l^{-1}$)	12.8 ± 1.4	12.7 ± 1.4	11.8 ± 1.5	11.5 ± 1.2
RPE	18.7 ± 1.2	19.0 ± 0.9	18.8 ± 0.8	19.3 ± 0.8#
Body weight (kg)	74.9 ± 6.6	75.1 ± 6.6	75.1 ± 6.4	75.2 ± 6.3

N = 13. [Hb], haemoglobin concentration; HR_{peak}, peak heart rate; CaO_2, arterial O_2 content; $[La]_{peak}$, peak blood lactate concentration; RER_{peak}, peak respiratory exchange ratio; RPE, rating of perceived exertion using the Borg scale (6-20); SpO_2, capillary O_2 saturation (fingertip); TOI, tissue oxygenation index (n = 12); VE_{peak}, peak ventilation; $\dot{V}O_{2max}$, maximal oxygen uptake. *, ** and *** Significantly different from control ($P \leq 0.05$, $P < 0.01$ and $P < 0.001$, respectively). # Trend towards being different from control ($0.05 < P \leq 0.10$). The body weight was measured before phlebotomy.

Figures

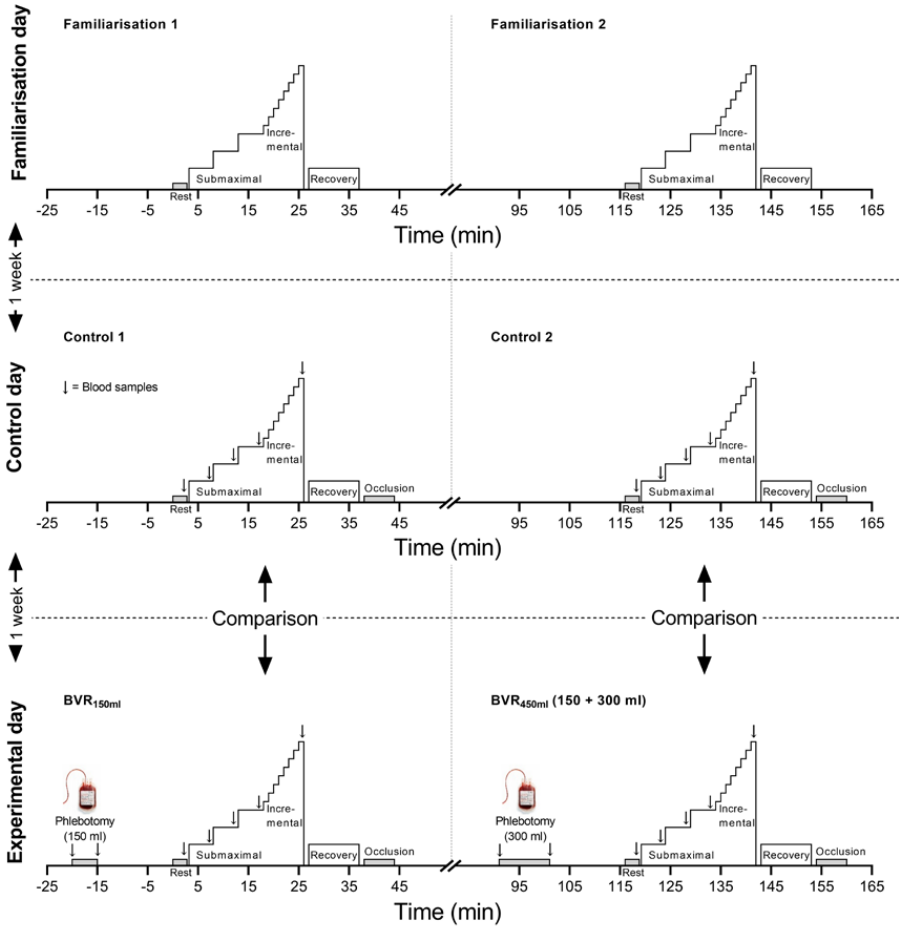

Fig. 1 The experimental design of the study. The three submaximal workloads (90 ± 32, 138 ± 36 and 185 ± 42 W) were followed by step-increments of 25 W every min until exhaustion. After exhaustion, the subjects cycled at 50-100 W for 10 min to speed up recovery.

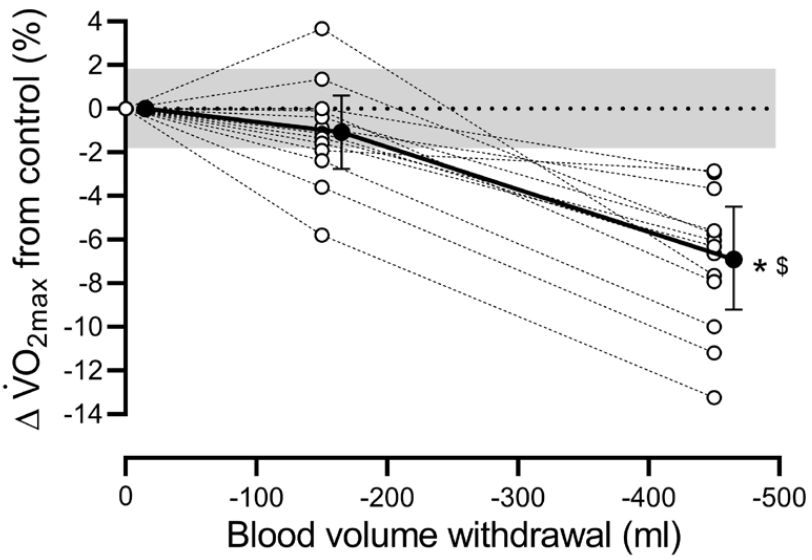

Fig. 2 The individual (white circles and dotted lines) and mean (black circles and solid line) differences in maximal oxygen uptake ($\dot{V}O_{2max}$) compared to the control trials as a function of the blood volume removed. Error bars indicate 95% confidence limits. The grey-shaded area indicates the typical error of measurement for $\dot{V}O_{2max}$ (± 1.9%). * Significant change from the control trial ($P < 0.001$). $ Significantly larger decrease after removing 450 ml compared to 150 ml blood ($P < 0.001$). N = 13

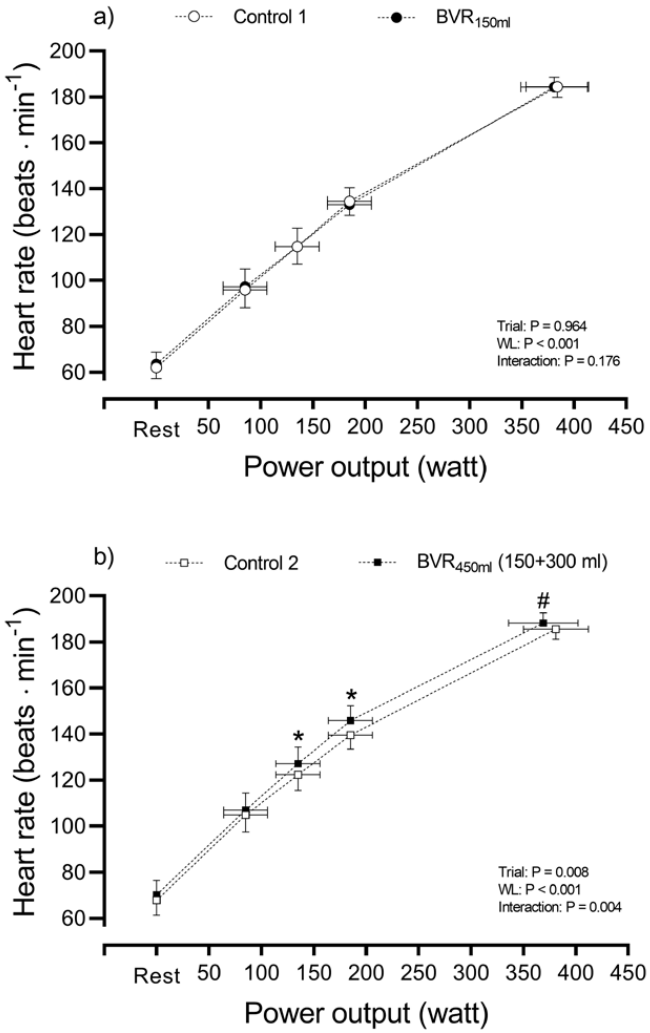

Fig. 3 The heart rate as a function of power output during the two control trials and the two blood volume reduction trials (a: BVR_{150ml} and b: BVR_{450ml}). Error bars indicate 95% confidence limits. The P-values for the ANOVA main effects of trial and workload (WL) as well as their interaction effect are inserted. * Significant change from the control trial ($P \leq 0.05$). # Trend towards a change from the control trial ($0.05 < P \leq 0.10$). N = 13

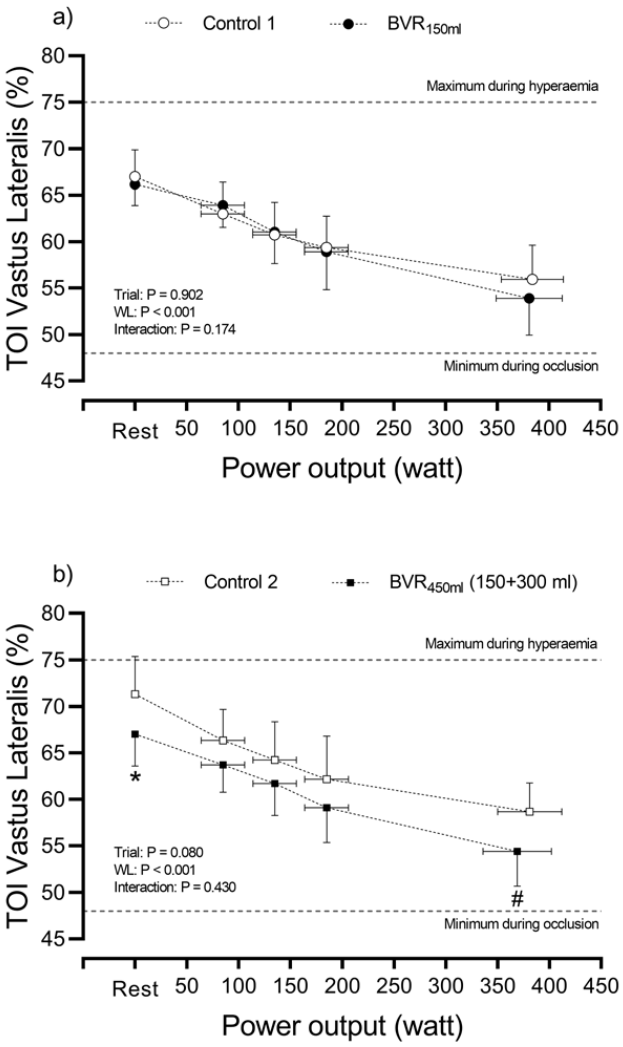

Fig. 4 The Vastus Lateralis tissue oxygenation index (TOI) as a function of power output during the two control trials and the two blood volume reduction trials (a: BVR_{150ml} and b: BVR_{450ml}). Error bars indicate 95% confidence limits. The P-values for the ANOVA main effects of trial and workload (WL) as well as their interaction effect are inserted. * Significant change from the control trial ($P \leq 0.05$). # Trend towards a change from the control trial ($0.05 < P \leq 0.10$). N = 12

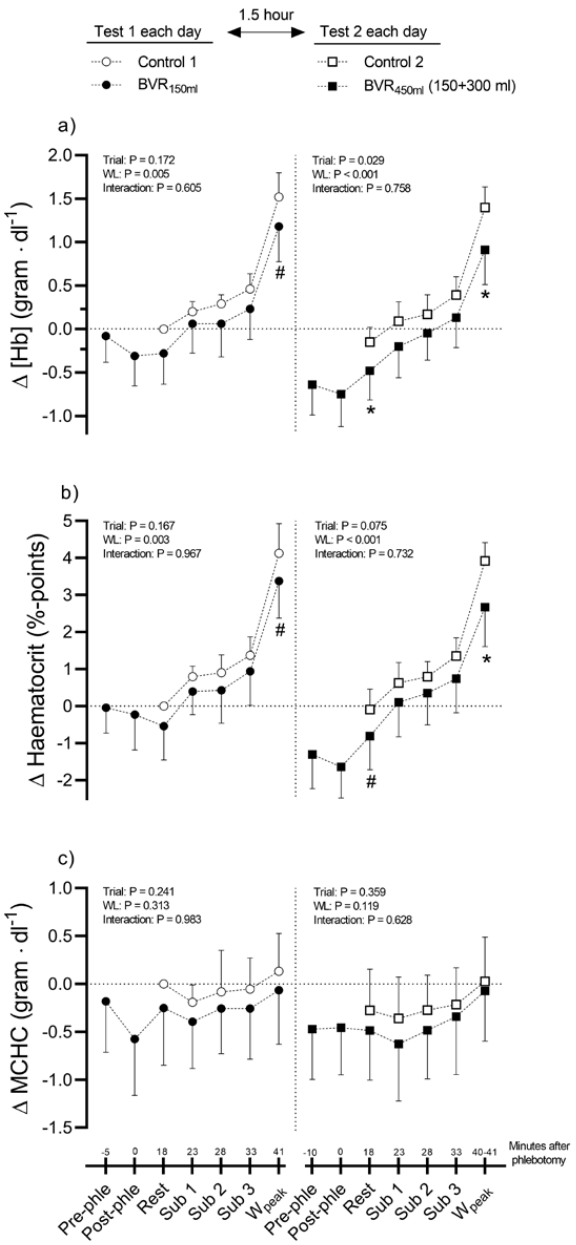

Fig. 5 The changes in the haemoglobin concentration ([Hb]; a), haematocrit (b) and mean corpuscular haemoglobin concentration (MCHC; c) during the two control trials and the two blood volume reduction trials. All values are normalised to the resting measurement during control 1. Error bars indicate 95% confidence limits. The P-values for the ANOVA main effects of trial and workload (WL) as well as their interaction effect are inserted. * Significant change from the control trial ($P \leq 0.05$). # Trend towards a change from the control trial ($0.05 < P \leq 0.10$). N = 13

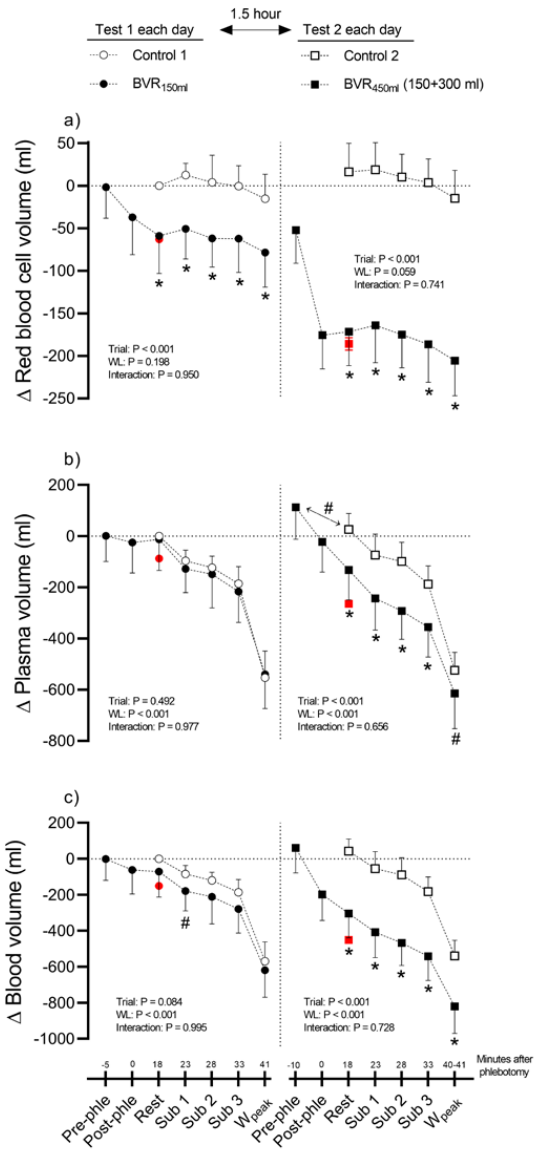

Fig. 6 The changes in red blood cell volume (a), plasma volume (b) and total blood volume (c) during the two control trials and the two blood volume reduction trials. All values are normalised to the resting measurement during control 1. All volumes were calculated using the changes in haematocrit and haemoglobin concentration, and by assuming a stable haemoglobin mass (carbon monoxide rebreathing) apart from the reductions induced by phlebotomy and blood sampling during the experiments. The red symbols indicate the theoretical volumes after phlebotomy if the haemoglobin concentration and the haematocrit had remained stable at the pre-phlebotomy level. Error bars indicate 95% confidence limits. The P-values for the ANOVA main effects of trial and workload (WL) as well as their interaction effect are inserted. * Significant change from the control trial ($P \leq 0.05$). # Trend towards a change from the control trial ($0.05 < P \leq 0.10$). N = 13

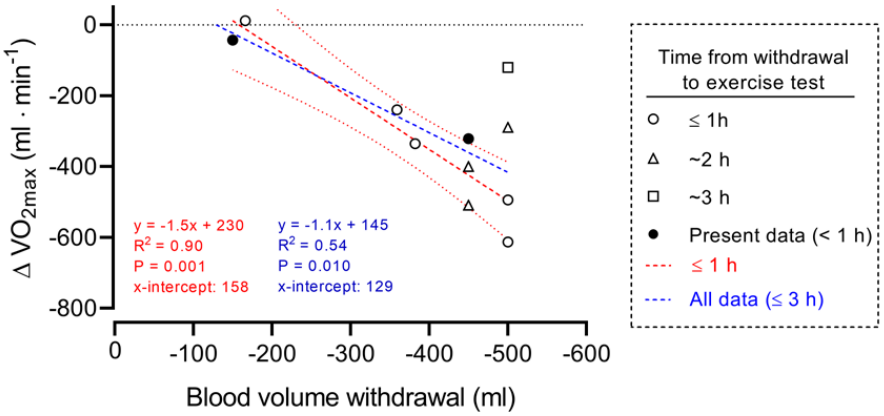

Fig. 7 The relationship between the magnitude of blood volume withdrawal and its impact on maximal oxygen uptake ($\dot{V}O_{2max}$) measured within the same day. The studies (Balke et al. 1954; Bonne et al. 2014; Hill et al. 2013; Krip et al. 1997; Montero et al. 2015; Panebianco et al. 1995; Rowell et al. 1964; Skattebo et al. 2020; Woodson et al. 1978) are organised according to the time from blood withdrawal to the initiation of the exercise.

Paper III

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

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Abstract

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 to 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 $P<0.05$). 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±1.6%-points; $P=0.010$; 40-83% of $\dot{W}_{peak}$) with the largest between-leg difference at 83% of $\dot{W}_{peak}$ (O_2 extraction: 3.2±2.2%-points; arteriovenous O_2 difference: 7.1±4.8 $mL \cdot L^{-1}$; $P<0.001$). 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 ($P<0.01$), 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=72-0.73; $P=0.03$), but not with the difference in the capillary-to-fibre ratio ($P=0.965$). 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

Introduction

During dynamic exercise involving a large muscle mass (>15 kg; i.e., 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 two-fold larger O_2 delivery per unit muscle mass is observed during 1L-KE compared to cycling (500 vs 240 mL $\cdot$ min^{-1} $\cdot$ 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- 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 immobilisation 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) characterised by a substantially lower maximal mitochondrial respiratory capacity (OXPHOS; measured in permeabilised muscle fibres *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,[16-18] these data, in conjunction with animal data,[19]

suggest that the potential for improvement is greater during exercise with a small muscle mass.

When conducting whole-body endurance training (e.g., cycling and running), both central and peripheral adaptations occur. 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 one-legged 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: 1) the O_2 delivery should be similar and; 2) every improvement in O_2 extraction should originate from the changes in muscle oxidative capacity and/or capillarisation. Similar experiments involving simultaneous exercise with one trained and one untrained leg have been conducted in the past.[17,20,21] However, in two of these studies, care was not taken to balance the power output between the trained and the untrained leg (the trained leg performed more work),[20,21] and all three studies used two-legged cycling where the muscles possess a large oxidative reserve capacity, even before training.[14]

Based on the above premises, we aimed to increase 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 minimising the perfusion limitations as it does not tax $\dot{Q}_{max}$.[3] During three low- to high-intensity workloads, equalling 40 ± 3%, 62 ± 4% and 83 ± 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 analysed for mitochondrial enzymes and capillarisation. We hypothesised 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 hypothesised that a between-leg difference in O_2 extraction would be more evident at high compared with low power outputs.

Materials and methods

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; $\dot{V}O_{2max}$: 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 (53552). Before study initiation, oral and written informed consents were obtained from all participants, and the study was conducted according to the Declaration of Helsinki.

Experimental design

After six weeks of 1L-KE endurance training, the participants performed 2L-KE exercise at three different power outputs: 40 ± 3%, 62 ± 4% and 83 ± 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 $\dot{V}O_{2max}$ 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, WI, USA), a muscle biopsy from each thigh was sampled, and the quadriceps femoris muscle mass was estimated anthropometrically[22] and adjusted as proposed by Radegran.[23]

Before testing, several short familiarisation 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.

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 35-65 min during the training period. Another session consisted of 10 min warm-up, 20 min "all-out" at the highest possible power output and a cool-down of 5 min. The third session consisted of a 10 min warm-up, and 4-6 repetitions of 5 min at 85% of $\dot{W}_{peak}$, which were interspersed with recovery periods of 2 min and followed by a 5-min cooldown. From weeks 1 to 6, the total work performed increased from 197 ± 23 kJ 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.

Non-invasive physical tests

Pulmonary $\dot{V}O_{2max}$ was measured during cycling (Excalibur Sport; Lode B.V., Groningen, The Netherlands) with step increments of 25 W · min^{-1} after 15 min 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 min and 45 min, respectively. The incremental tests began at 30 W or 60 W, after a 15 min 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.

Catheterisation 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 anaesthesia (2% lidocaine) using the Seldinger technique. Briefly, a 20-gauge catheter (Arrow ref. # ES-04150; Teleflex Medical, Wayne, PA, USA) 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, Irvine, CA, USA) positioned at the height of the parasternal fourth intercostal space. Via an amplifier (Gould instrument systems, Cleveland, OH, USA), blood pressures were sampled at 100 Hz using a PC with LabVIEW software (National Instruments, Austin, TX, USA).

Blood sampling and analytic procedures

Blood was sampled anaerobically using heparinised syringes (safePICO; Radiometer, Copenhagen, Denmark) and immediately analysed for haemoglobin concentration ([Hb]), haemoglobin 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, Denmark). Blood O_2 content (ctO_2) was calculated as: $(1.34 \times [Hb] \times SO_2) + (0.003 \times PO_2)$. 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.[24] Blood samples were taken simultaneously from both veins and the artery.

Femoral arterial blood flow

LBF was measured in the femoral artery of both legs using ultrasound Doppler (Vivid E95; GE Vingmed Ultrasound AS, Horten, Norway) 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 maximised according to the vessel width but kept clear of the vessel walls. The insonation angle was minimised 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 to 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 anonymised before LBF was analysed using software incorporated in the Vivid E95.

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,[25,26] using the measured arterial and femoral venous PO_2. D_MO_2 [$\dot{V}O_2$ / (mean capillary PO_2 – mitochondrial PO_2)] is recognised 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 utilisation by the cytochromes. The calculated D_MO_2 depends on the recruited exercise muscle mass,[5] F_IO_2 and exercise intensity.[2,27] Since the observed D_MO_2 is higher during cycling than 1L-KE (reversed if standardising to the active muscle mass), and also higher in hypoxia than normoxia; 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.[27] 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.[28] 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)].[28]

Pulmonary gas exchange

Pulmonary $\dot{V}O_2$ was measured using a metabolic cart with a mixing chamber (Oxycon Pro; Jaeger Instrument, Friedberg, Germany). Before each test, the gas analysers and

flow transducer (Triple V; Erich Jaeger GmbH) were calibrated according to the instruction manual.

Knee extension ergometer

Knee extension exercises were performed on an electromagnetically braked ergometer modified after Hallén.[29] Briefly, the ergometer isolates leg muscle contractions to quadriceps femoris during knee extensions.[30] The hip-angle was ~128°, and the upper body was strapped using a four-point seat belt to minimise the engagement of muscle mass to stabilise 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.

Exercise protocol and timing of measurements (main experiment)

First, a baseline measurement was conducted after 10 min rest seated in the KE ergometer. Thereafter, three 8-min exercise bouts at low (47 ± 7 W; i.e., 23.5 W by each leg), moderate (73 ± 10 W; i.e., 36.5 W by each leg) and high (97 ± 10 W; i.e., 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 Fig. 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 min, B-mode imaging of the left femoral artery began, followed by Doppler measurements from precisely 4 min on the left leg and approximately from 5 min on the right leg. Two blood samples from

each vessel were drawn during the Doppler measurements (~4.5 min and ~5.5 min), and the duplicates were averaged. Blood pressures were calculated as ~30-sec 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.[31] 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 = 0.008) and high (8.6 ± 1.7 vs 6.9 ± 2.3; P < 0.001) intensity exercise elicited significantly higher perceived intensity in CON than in TL.

<<***Fig. 1 near here***>>

Skeletal muscle biopsy

Muscle biopsies (~100-200 mg) were collected from the mid-portion of *vastus lateralis* after local anaesthesia, 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, Newtown, UK) and quickly frozen in isopentane cooled on liquid nitrogen to freezing point (approx. -120^{o}C). Tissue allocated for Western Blotting was immediately snap-frozen in liquid nitrogen. All tissue samples were stored at -80^{o}C until further analysis.

Immunohistochemistry

Serial 8 μm transverse cross sections were cut at -20^{o}C (Leica CM1860 UV; Leica Biosystems, Danvers, MA, USA), mounted on microscope slides (Superfrost Plus; Thermo Fischer Scientific, Waltham, MA, USA), air-dried and stored at -80^{o}C until further analysis. The sections were blocked for 60 min with 10% goat serum (ab7481; Abcam, Cambridge, UK) and 1% bovine serum albumin (Sigma Life Science, St Louis, MO, USA) in a phosphate-buffered saline (Sigma Life Science) and 0.05% Tween-20 (VWR, West Chester, PA, USA) solution (PBS-t). Primary antibodies against 1) myosin heavy chain type 1 (1:500 dilution; BA-D5, developed by Schiaffino, S., obtained from DSHB, Iowa City, IA, USA) and dystrophin (1:500; ab15277; Abcam, Cambridge, UK), or 2) the endothelial marker CD31 (1:100; M0823; Dako A/S, Glostrup, Denmark) and dystrophin were diluted in the blocking solution and incubated overnight at 4^{o}C.

The sections were then washed 3×10 min in PBS-t, incubated with secondary antibodies (1:200; Alexa Fluor 488, A11001 and A11012; Invitrogen Molecular Probes, Carlsbad, CA, USA) for 60 min and again washed 3×10 min in PBS-t before being mounted with Prolong Gold antifade reagent with DAPI (Life Technologies Corp., Carlsbad, CA, USA) and covered with a cover glass. The sections were visualised under a 10× / 0.30 NA air objective (UplanFL N; Olympus corp.; Tokyo, Japan) 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., Mississauga, ON, Canada). Fibre cross-sectional areas, fibre types and manual identification of capillaries were conducted and analysed using TEMA software (CheckVision, Denmark). The investigator was blinded for the subject and leg identity. A mean of 189 ± 41 (range: 118-251) fibres were analysed. Capillarisation was expressed as the capillary-to-fibre ratio, capillaries in contact with each fibre, and capillary density (capillaries per mm^2).

Protein immunoblot

For Western blotting analyses, ~60 mg of muscle tissue was homogenised in 1 ml T-PER (Tissue Protein Extraction Reagent, 78510; Thermo Fischer Scientific) and 20 µL Halt Protease & Phosphatase Inhibitor Cocktail (78440; Thermo Fischer Scientific). The tissue lysate was extracted, aliquoted and stored at -80^{o}C until further analyses. The protein concentration was measured using a commercial kit (BioRad DC Protein Assay, 5000116; Bio-Rad Laboratories, Hercules, CA, USA) and a FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany). 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.[32] 20µg of protein were 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, Darmstadt, Germany) 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 310005; GeneON, Ludwigshafen am Rhein, Germany) 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, Danvers, MA, USA) secondary antibody was applied for 1 hour at room temperature followed by visualisation using an HRP detection system (Super Signal West Dura Extended Duration Substrate; Thermo Fischer 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 (21059; Thermo Fischer 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 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 normalisation to allow for semi-quantitative comparisons across gels/subjects.[33]

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- and post-test were analysed 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-test were analysed 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, CA, USA) and Microsoft Office Excel 2013 (Microsoft Corporation, WA, USA) were used for statistical analysis.

Results

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 ($P < 0.001$; Table 1). This was accompanied by 20 ± 11% longer time to exhaustion ($P < 0.001$), 22 ± 17% higher $\dot{V}O_{2peak}$ ($P = 0.004$), 31 ± 21% higher peak ventilation ($P = 0.002$), 7 ± 8% higher peak heart rate ($P = 0.026$) and 28 ± 12% higher post-exercise blood lactate concentration ($P < 0.001$). $\dot{V}O_{2peak}$ during 2L-KE tended to increase (4 ± 6%; $P = 0.059$), but $\dot{V}O_{2max}$ during cycling was unchanged from pre- to post-training ($P = 0.890$; 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%; $P = 0.053$), but the quadriceps femoris muscle mass did not differ (2.48 ± 0.38 kg vs 2.43 ± 0.37 kg; difference: 2.0 ± 3.6%; $P = 0.138$).

<<Table 1 near here>>

O_2 extraction in the trained and the control leg

At rest, O_2 extraction and $\dot{V}O_2$ tended to be higher in TL compared to CON (Fig. 2-3; $P = 0.099$ and $P = 0.080$, respectively), while LBF was similar ($P = 0.611$). During 2L-KE with equal absolute power output produced by both legs, O_2 extraction was higher (difference: 1.7 ± 1.6%-points; $P = 0.010$; Fig. 3a), mass-specific LBF tended to be higher (difference: 7.1 ± 9.5%; $P = 0.051$; Fig. 2a), and mass-specific $\dot{V}O_2$ was slightly higher (difference: 9.8 ± 9.3%; $P = 0.016$; Fig. 2d) in TL compared to CON (the ANOVA main effects are given above; see Fig. 2-3 for post-hoc tests). Significant interactions (leg × power output) were found for O_2 extraction ($P = 0.032$) and arteriovenous O_2 difference (a-vO_2diff; $P = 0.029$), with the largest training-induced increase at the highest power output (3.2 ± 2.2%-points and 7.1 ± 4.8 mL · L^{-1}, respectively; both $P < 0.001$). 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 = -0.81; n = 9; P = 0.008), i.e., 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 = 0.005) due to a lower femoral venous pH (Table 2; P = 0.007).

<<Fig. 2-3 and Table 2 near here>>

Muscle O_2 conductance

The larger O_2 extraction resulted in lower femoral venous PO_2 (P = 0.004) and calculated mean capillary PO_2 (P = 0.003) 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 ± 8.3% larger in TL than in CON (P = 0.001; Fig. 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 mixed perfusion-diffusion limitation and Y > 3 indicates pure perfusion limitation)[28] was higher in TL (P = 0.003; Fig. 3c) and increased more with power output than in CON (interaction: P = 0.008; Fig. 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 < 0.001; Fig. 3d-e).

Skeletal muscle adaptations

Before training, none of the analysed mitochondrial enzymes differed between legs (P ≥ 0.56). After training, TL expressed a higher protein content of citrate synthase (45 ± 29%; P < 0.001), COX-IV (44 ± 46%; P = 0.044) and HAD (35 ± 28%; P = 0.007; Fig. 4). After training, the capillary-to-fibre ratio was 18 ± 23% (P = 0.031) higher in TL than in CON and the capillary density tended to be higher (12 ± 17%; P = 0.067; Table 3).

<<Fig. 4 and table 3 near here>>

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 ($r = 0.72$; $n = 9$; $P = 0.028$) and COX-IV ($r = 0.73$; $n = 9$; $P = 0.024$), as well did the two enzymes combined into a composite index ($P = 0.010$; $n = 9$; Fig 5a). When the mitochondrial protein contents were standardised to a human control sample, to allow for comparisons across subjects, COX-IV ($r = 0.57$; $n = 18$; $P = 0.013$), citrate synthase ($r = 0.47$; $n = 18$; $P = 0.049$) and their composite index correlated with leg O_2 extraction ($P = 0.011$; $n = 18$; Fig 5c). The composite index also showed a close relationship with the recruitment of D_MO_2 at 83% of $\dot{W}_{peak}$ ($r = 0.74$; $n = 18$; $P < 0.001$). Leg O_2 extraction correlated with the capillary-to-fibre ratio ($P = 0.029$; $n = 18$; Fig. 5d). However, no relationship was evident when the between-leg difference in capillary-to-fibre ratio was plotted against the between-leg difference in oxygen extraction ($P = 0.965$; $n = 9$; Fig. 5b); indicating a more important role of the increased citrate synthase and COX-IV protein content than increased capillary-to-fibre ratio on the improved O_2 extraction capacity.

<<Fig. 5 near here>>

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_MO_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.

Endurance training increases O_2 extraction

During maximal exercise with a small muscle mass (e.g., 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 hypothesised that elevating the muscle oxidative capacity with endurance training would enhance the recruitment of D_MO_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[9,10,34,35] or unchanged[13,36,37] 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.[13,37] 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 co-workers found an unchanged leg a-vO_2diff during submaximal exercise but found a ~7 mL $\cdot$ L^{-1} increase during maximal one-legged cycling after 8 weeks of one-legged endurance training.[38] The latter concurs with the study by Boushel and co-workers 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 large.[39] Thus, our results combined with previous studies strongly suggest that O_2 extraction is improved after endurance training, particularly when the recruited muscle mass is low and the exercise intensity close to maximal. However, the effect of endurance training on O_2 extraction during large muscle mass exercise remains controversial.[14,17,18,40]

During whole-body maximal exercise (e.g., running and cycling), the muscle oxidative capacity is far from being fully utilised and is therefore not considered a

limiting factor for muscle O_2 extraction and $\dot{V}O_{2max}$.[14] This also agrees with a recent meta-analysis reporting no significant change in systemic a-vO_2diff after endurance training, as calculated using the Fick equation from the measured $\dot{V}O_{2max}$ 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_2diff in the exercising limb (arterial and venous catheters). For example, Roca and co-workers[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 ($\dot{V}O_{2max}$ 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 ($\dot{V}O_{2max}$: 65-72 mL · kg^{-1} · min^{-1}).[26,41] Thus, even though conflicting evidence exists on whether systemic a-vO_2diff 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.[15,17,18,20,38,42]

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 with high muscle blood flow and O_2 delivery[19,43,44] 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 and COX-IV) 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.[45] However, new evidence suggests that the muscle possesses a functional reserve in D_MO_2 that is utilised when breathing hypoxic gas during incremental exercise to exhaustion on the cycle ergometer[27] and the 1L-KE ergometer.[2] These studies indicate that greater D_MO_2 can be

achieved with the same capillary-to-fibre ratio during exercise in hypoxia, meaning that the capillary-to-fibre 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-fibre ratio was not associated with the between leg difference in O_2 extraction (Fig. 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 what establishes the ceiling for O_2 extraction.[5,26,27]

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 is maintained during whole-body exercise even though the muscle oxidative capacity and $\dot{V}O_{2max}$ are substantially reduced.[46,47] Therefore, it is argued that the muscles have a substantial reserve of oxidative capacity and that $\dot{V}O_{2max}$ 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.[48,49] 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;[46,47] ultimately favouring 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.[50,51] Therefore, training-induced improvements in whole-body $\dot{V}O_{2max}$ 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 (i.e., oxidative capacity and MTT) is more critical for O_2 extraction than any of these factors alone.

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.[52] Hence, a close relationship between O_2 extraction and $P_{50}O_2$ has been found in humans during exercise.[26] Moreover, increased O_2 extraction and $\dot{V}O_{2max}$ 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.[53] 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 (Fig. 6), likely because of the enhanced muscle oxidative capacity and larger recruitment of D_MO_2.

<<Fig. 6 near here>>

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 (Fig. 7). In this context, by applying the Piiper and Scheid model, we calculated the equilibration index Y, which quantitatively describes diffusion vs perfusion limitations.[28] 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 (Fig. 3c), indicating that the training-induced reduction of 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 composite index of citrate synthase and COX-IV) and the recruitment of D_MO_2 ($r = 0.74$; $P<0.001$) and the equilibration index Y ($r = 0.50$; $n = 18$; $P = 0.034$) 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.

<<Fig. 7 near here>>

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.[54] It has been demonstrated that erythrocyte ATP release is inhibited in the presence of high lactate concentration[55] and accelerated by low PO_2.[56] Thus, the lower femoral venous PO_2 and lower lactate concentration during exercise in TL may have facilitated larger ATP-mediated vasodilation compared to CON, which could have facilitated the slightly higher LBF. Also, exercise training has previously been shown to improve functional sympatholysis,[13,57] also contributing to a higher vascular conductance after training. However, a study of 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.[9,13,38] In most of these studies, the effect of training has been assessed separately in the trained 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.[9,13,38] 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.[36,38] In the present study, both legs were exercised simultaneously and therefore had the same perfusion pressure and an essentially similar LBF in both legs. To our knowledge, only three previous investigations have assessed the haemodynamic response while exercising with one trained and one control leg simultaneously,[17,20,21] 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_2diff 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 through increased muscle oxidative capacity and larger recruitment of D_MO_2 that reduced the restrictions on O_2 diffusion from the capillaries to the mitochondria.

Perspective

A major aim of endurance training is to enhance exercise capacity by improving O_2 utilisation, 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-fibre ratio. Of particular interest, increased muscle oxidative capacity did not translate into improved O_2 extraction during low-intensity exercise, i.e., 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 drugs like metformin or statins, which may blunt the expected mitochondrial biogenesis.

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Additional information

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Conflict of interest

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

Author Contributions

Conception and design of the experiment: Ø.S., J.H., C.C., B.R.
Data collection: Ø.S., J.H., C.C., J.A.L.C., M.A.
Analysis of data: Ø.S., B.R., M.A.
Interpretation of data: Ø.S., J.H., C.C., J.A.L.C.
Writing the first draft: Ø.S.
Revised and approved the final version of the manuscript: all authors.

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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.

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 $\dot{V}O_{2peak}$ ($L \cdot min^{-1}$)	Peak power (watt)	Time to exhaustion (mm:ss)	VE_{peak} ($L \cdot min^{-1}$)	HR_{peak} ($beats \cdot min^{-1}$)	Lactate ($mmol \cdot L^{-1}$)
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

HR_{peak}, peak heart rate; VE_{peak}, peak ventilation; $\dot{V}O_{2peak}$, peak oxygen uptake (pulmonary); * Significantly ($P \leq 0.05$) or # trend towards ($0.05 < P \leq 0.10$) difference between legs (one-legged KE) or between pre- 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.

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) & interaction effects
Hb ($g \cdot dL^{-1}$)					
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=0.719
Femoral vein TL	15.7 ± 0.5	16.1 ± 0.6	16.4 ± 0.6	16.9 ± 0.6	Interaction: P=0.829
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=0.018
Femoral vein TL	57.3 ± 11.7	33.6 ± 4.1	27.2 ± 4.4#	21.6 ± 2.8*	Interaction: P=0.029
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=0.004
Femoral vein TL	31.2 ± 4.1	23.2 ± 2.0	21.3 ± 1.7*	20.1 ± 1.5*	Interaction: P=0.013
ctO_2 ($ml \cdot L^{-1}$)					
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=0.010
Femoral vein TL	121.5 ± 25.7#	73.3 ± 9.5	60.4 ± 10.2#	49.7 ± 7.4*	Interaction: P=0.030
$P_{50}O_2$ (mmHg)					
Femoral vein CON	27.5 ± 0.4	29.6 ± 0.7	30.8 ± 1.2	32.8 ± 1.7	Main: P=0.005
Femoral vein TL	27.5 ± 0.5	29.4 ± 0.7*	30.3 ± 1.1*	32.5 ± 1.6*	Interaction: P=0.251
Mean cap. PO_2 (mmHg)					
CON	57.4 ± 3.3	51.7 ± 2.2	50.9 ± 2.1	53.2 ± 2.5	Main: P=0.003
TL	56.1 ± 3.0#	51.3 ± 2.5	49.9 ± 2.0*	51.6 ± 2.1*	Interaction: P=0.008
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=0.007
Femoral vein TL	7.38 ± 0.02	7.32 ± 0.02*	7.29 ± 0.03*	7.22 ± 0.05*	Interaction: P=0.208
Lactate ($mmol \cdot L^{-1}$)					
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=0.005
Femoral vein TL	0.9 ± 0.2	1.7 ± 0.7*	3.9 ± 1.6*	10.4 ± 2.2*	Interaction: P=0.242

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 identifies significant (*$P \leq 0.05$) or trend towards (#$0.05 < P \leq 0.10$) differences between legs within each workload. A paired sample t-test analysed the difference between legs during rest.

Table 3. *Vastus lateralis* muscle composition in the trained leg and the control leg at post-test (mean ± SD).

Variable	Control leg	Trained leg	P-value
Fibre size (μm^2)			
All fibres	4954 ± 1063	5187 ± 1167	0.396
Type I	4510 ± 1156	4870 ± 1050	0.315
Type II	5245 ± 1321	5487 ± 1134	0.394
Fibre type (%)			
Type I	41 ± 7	45 ± 10	0.212
Type II	59 ± 7	55 ± 10	0.212
Capillaries around a fibre			
All fibres	3.1 ± 0.5	3.5 ± 0.8	0.122
Type I	3.3 ± 0.5	3.6 ± 0.7	0.170
Type II	3.1 ± 0.5	3.5 ± 0.8	0.138
Capillaries / mm^2	280 ± 59	311 ± 75	0.067
Capillary / fibre- ratio	1.35 ± 0.26	1.58 ± 0.33	0.031

n = 9.

Figure legends

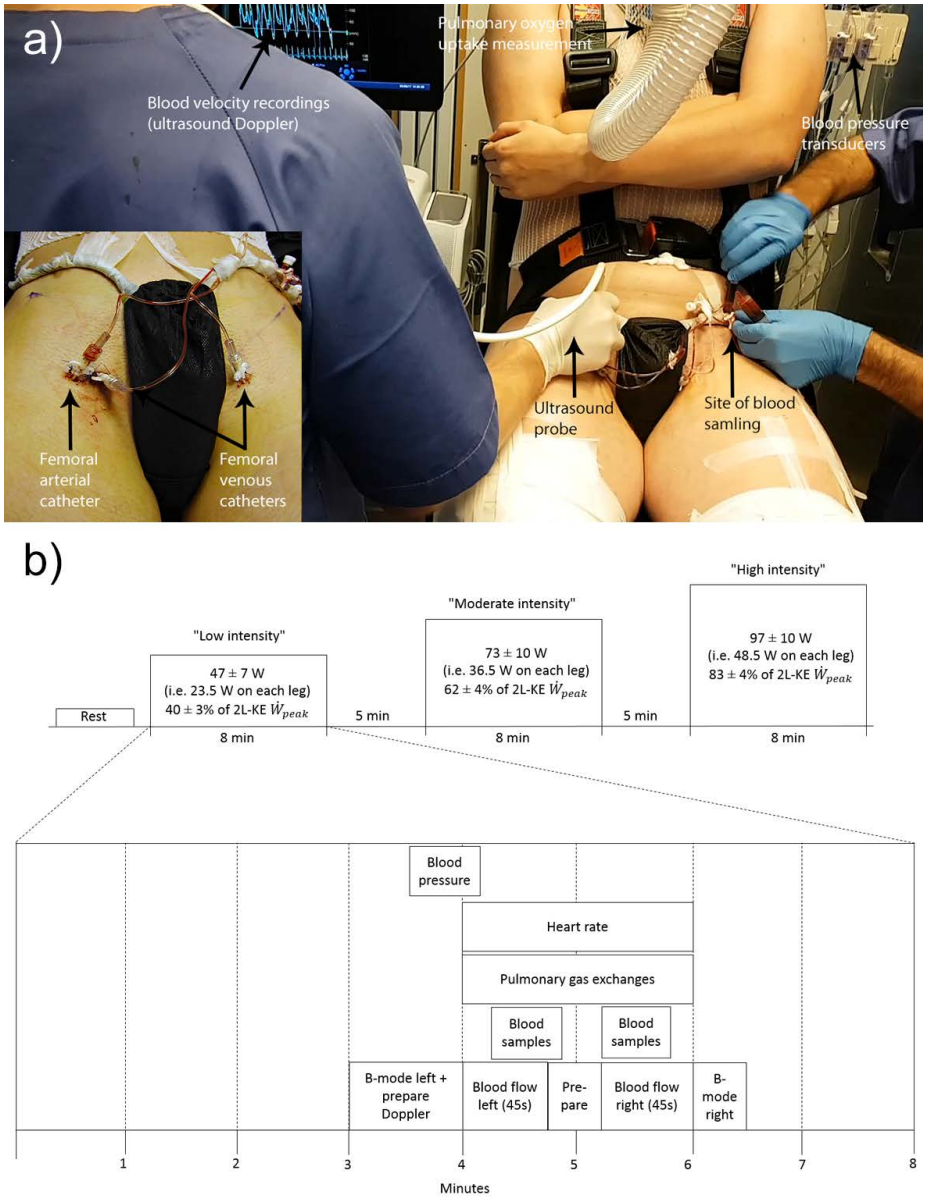

Fig. 1. The full setup of a subject seated in the knee extension ergometer (a); and the exercise protocol and timing of measurements (b).

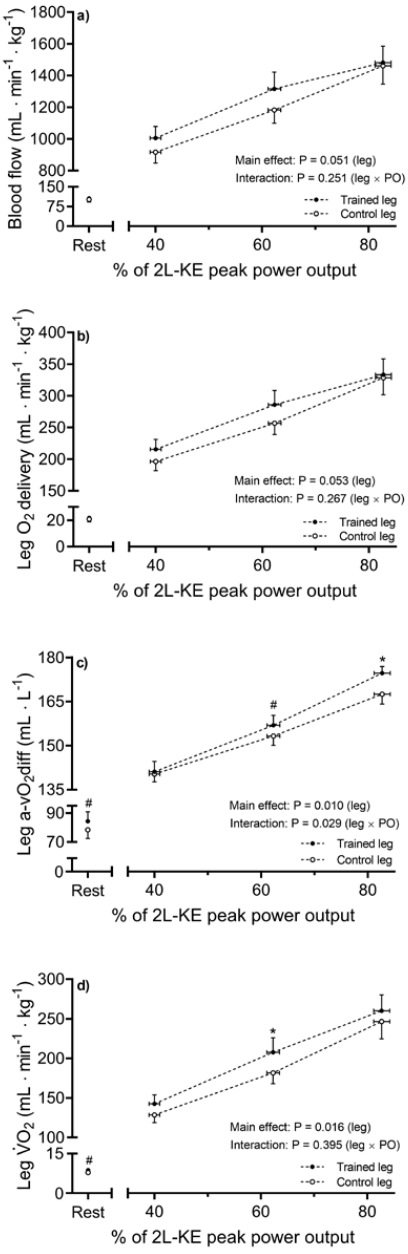

Fig. 2. Mass-specific leg blood flow (a); mass-specific O_2 delivery (b); arteriovenous O_2 difference (c); and mass-specific O_2 uptake (d) in the trained leg and the untrained control leg after six weeks of one-legged endurance training. Note that the trained leg and the control leg exercised simultaneously 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 ($P \leq 0.05$) and #trend towards ($0.05 < P \leq 0.10$) difference between legs (post-hoc comparisons).

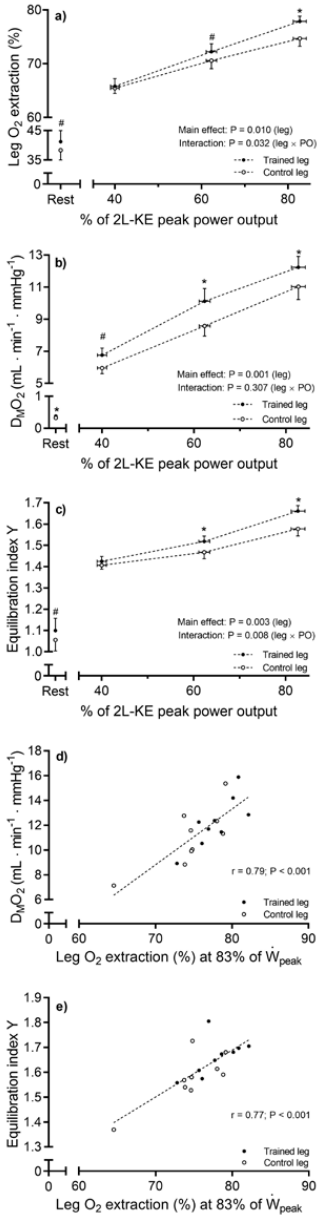

Fig. 3. Leg O_2 extraction (a); recruitment of muscle O_2 conductance (D_MO_2) (b); and the equilibration index Y (c) in the trained leg and the untrained control leg after 6 weeks of one-legged endurance training. The equilibration index Y quantitatively describes diffusion vs perfusion limitations (where $Y < 0.1$ indicates pure diffusion limitation, $0.1 < Y < 3$ indicates mixed perfusion-diffusion limitation and $Y > 3$ indicates pure perfusion limitation), i.e., the diffusion limitation is lower in the trained leg. The data are means, and error bars denote SEM ($n = 9$). *Significant ($P \leq 0.05$) and #trend towards ($0.05 < P \leq 0.10$) 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.

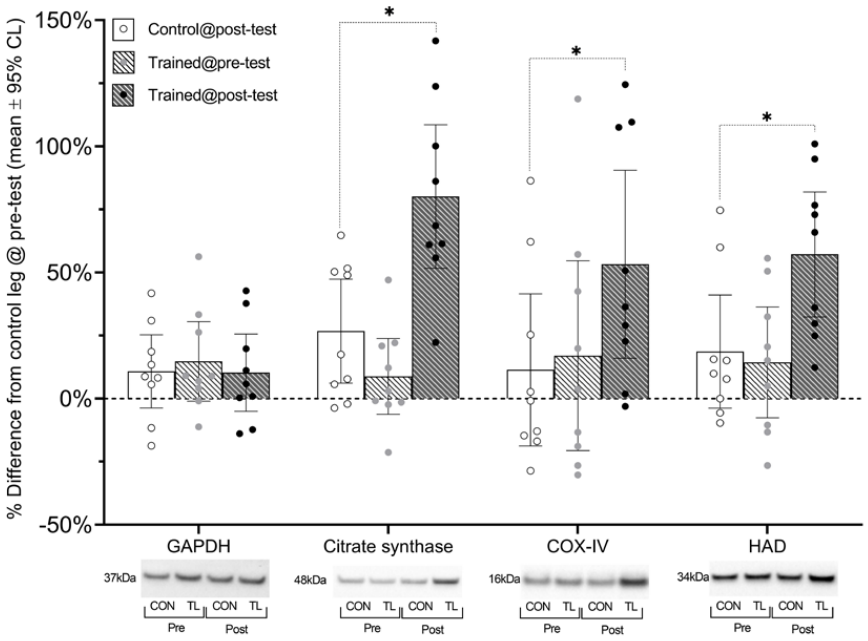

Fig. 4. The protein content of the mitochondrial enzymes citrate synthase, cytochrome-c-oxidase subunit 4 (COX-IV) and hydroxyacyl-CoA dehydrogenase (HAD) along with the loading control glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The individual and mean values are presented as percentage differences from the pre-test value of the control leg (n = 9). Error bars denote 95% confidence limits (CL). *Significant difference ($P \leq 0.05$) between legs at the post-test, i.e., at the time of the invasive experiment. Representative blots of the four proteins along with their observed molecular weights, are presented.

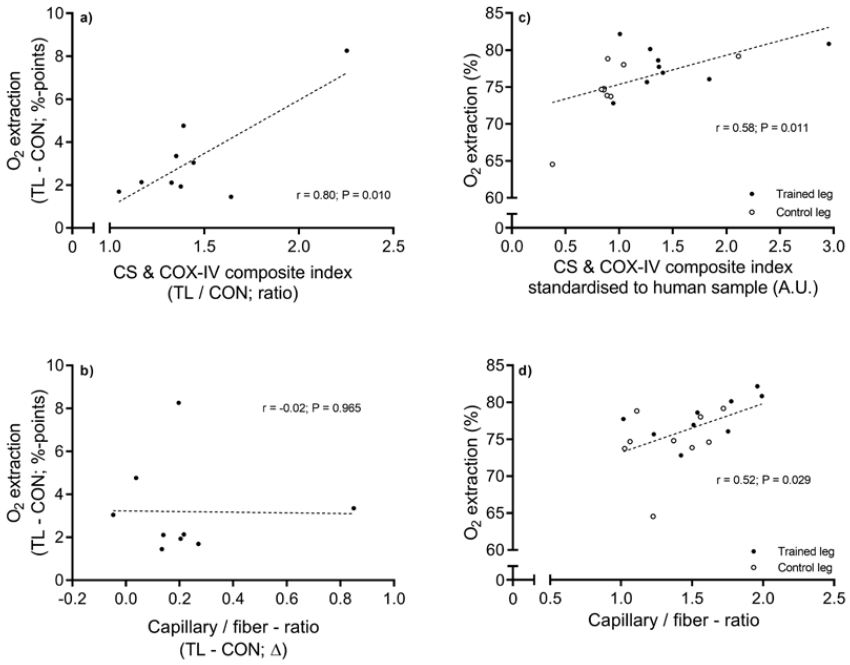

Fig. 5. Data are from the highest power output. The relationship between the between-leg difference in O_2 extraction and the between-leg ratio of mitochondrial protein content (mean value / composite index of CS and COX-IV; Fig. 5a) or the between-leg difference in the capillary-to-fibre ratio (Fig. 5b). In Fig. 5c, the mitochondrial protein content was standardised to a human control sample, which was loaded on each gel to allow for between-subject comparisons, and the individual levels (presented as arbitrary units; A.U.) was plotted against O_2 extraction. In Fig. 5d, the capillary-to-fibre ratio is plotted against O_2 extraction. CS, citrate synthase; COX-IV, cytochrome-c-oxidase subunit 4; TL, trained leg; CON, control leg.

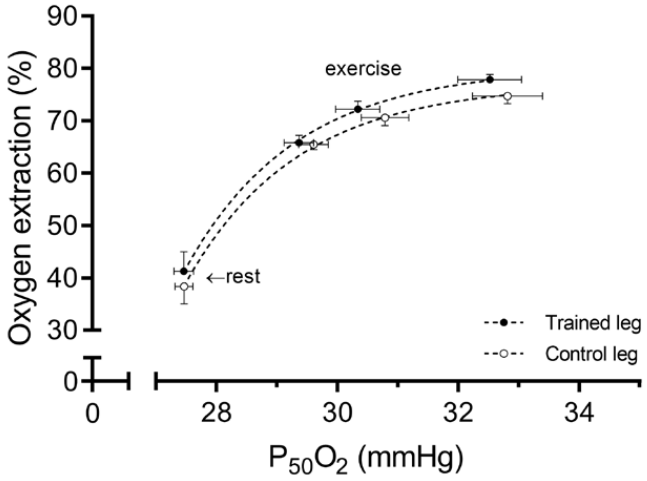

Fig. 6. O_2 extraction during rest and exercise as a function of the partial pressure of O_2 that causes haemoglobin to be 50% saturated ($P_{50}O_2$). Note that a high $P_{50}O_2$ is beneficial for O_2 off-loading and that the trained leg had the highest O_2 extraction despite having a lower $P_{50}O_2$ than the control leg.

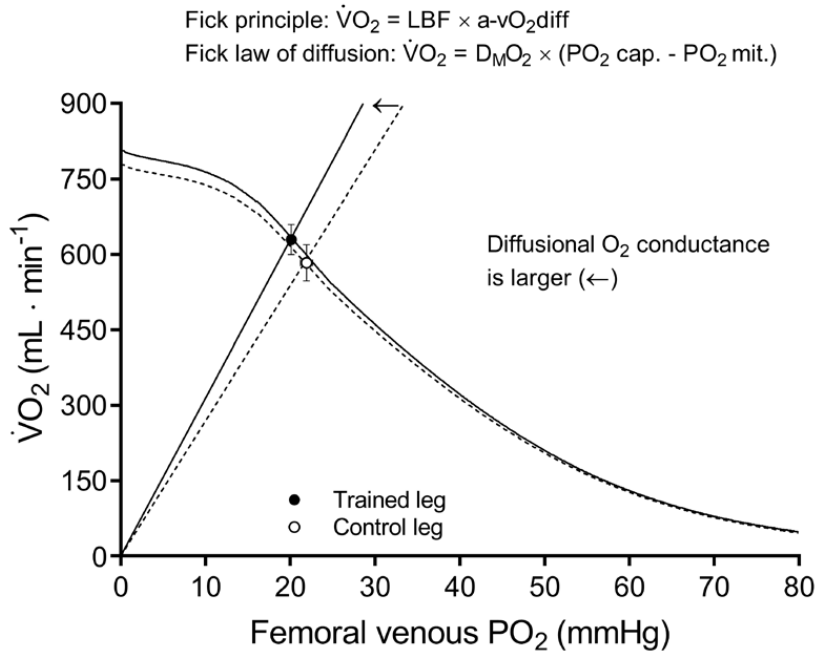

Fig. 7. The $\dot{V}O_2$ in the trained leg and the control leg are plotted as a function of femoral venous PO_2 at 83% of peak power output (filled symbols). The sigmoid curves represent the $\dot{V}O_2$ (the trained and the control leg are illustrated by a whole line or a dotted line, respectively) 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 (←) is recruited in the trained leg compared to 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.

Paper IV

Contribution of oxygen extraction fraction to maximal oxygen uptake in healthy young men

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Abstract

Aim: To analyse the importance of systemic and peripheral arteriovenous O_2 difference (a-$\bar{v}O_2$diff and a-$v_fO_2$diff, respectively) and O_2 extraction fraction for maximal oxygen uptake ($\dot{V}O_{2max}$). The Fick law of diffusion and the Piiper and Scheid model were applied to investigate whether diffusion vs perfusion limitations vary with $\dot{V}O_{2max}$.

Methods: Articles (n=17) publishing individual data (n=154) on $\dot{V}O_{2max}$, maximal cardiac output ($\dot{Q}_{max}$; indicator-dilution or Fick method), a-$\bar{v}O_2$diff (catheters or Fick equation) and systemic O_2 extraction fraction were identified. For the peripheral responses, group-mean data (articles: n=26; subjects: n=223) on leg blood flow (LBF; thermodilution), a-$v_fO_2$diff and O_2 extraction fraction (arterial and femoral venous catheters) were obtained.

Results: $\dot{Q}_{max}$ and two-LBF increased linearly by 4.9-6.0 $L \cdot min^{-1}$ per 1 $L \cdot min^{-1}$ increase in $\dot{V}O_{2max}$ (R^2=0.73 and R^2=0.67, respectively; both P<0.001). The a-$\bar{v}O_2$diff increased from 118-168 $mL \cdot L^{-1}$ from a $\dot{V}O_{2max}$ of 2-4.5 $L \cdot min^{-1}$ followed by a reduction (second-order polynomial: R^2=0.27). After accounting for a hypoxemia-induced decrease in arterial O_2 content with increasing $\dot{V}O_{2max}$ (R^2=0.17; P<0.001), systemic O_2 extraction fraction increased up to ~90% ($\dot{V}O_{2max}$: 4.5 $L \cdot min^{-1}$) with no further change (exponential decay model: R^2=0.42). Likewise, leg O_2 extraction fraction increased with $\dot{V}O_{2max}$ to approach a maximal value of ~90-95% (R^2=0.83). Muscle O_2 diffusing capacity and the equilibration index Y increased linearly with $\dot{V}O_{2max}$ (R^2=0.77 and R^2=0.31, respectively; both P<0.01), reflecting decreasing O_2 diffusional limitations and accentuating O_2 delivery limitations.

Conclusion: Although O_2 delivery is the main limiting factor to $\dot{V}O_{2max}$, enhanced O_2 extraction fraction (≥90%) contributes to the remarkably high $\dot{V}O_{2max}$ in endurance-trained individuals.

Keywords: Arteriovenous oxygen difference, Cardiac output, Exercise, Leg blood flow, Limiting factors, Maximal oxygen uptake, Oxygen diffusion, Stroke volume

Alphabetical list of abbreviations

a-$v_fO_2$diff: arterial to femoral venous oxygen difference

a-$\bar{v}O_2$diff: arterial to mixed venous oxygen difference

CaO_2: arterial oxygen content

Cv_fO_2: femoral venous oxygen content

$C\bar{v}O_2$: mixed venous oxygen content

CVP: central venous pressure

[Hb]: haemoglobin concentration

LBF: leg blood flow

Leg $\dot{V}O_{2max}$: maximal oxygen uptake of the leg

MAP: mean arterial blood pressure

MDO_2: muscle O_2 diffusing capacity

MTT: erythrocyte capillary mean transit time

$\bar{O}_2$ extraction: systemic oxygen extraction fraction

O_2 extraction: peripheral (leg) oxygen extraction fraction

OXPHOS: maximal mitochondrial respiratory capacity

PO_2: partial pressure of oxygen

$P_{50}O_2$: partial pressure of O_2 at 50% SO_2

$\dot{Q}_{max}$: maximal cardiac output

SO_2: oxygen saturation of haemoglobin

$\dot{V}O_2$: oxygen uptake

$\dot{V}O_{2max}$: pulmonary maximal oxygen uptake

Introduction

Under resting conditions in humans, the O_2 uptake ($\dot{V}O_2$) is 3-5 mL · kg^{-1} · min^{-1}, and only a small fraction is consumed within the skeletal muscles.[1] However, during incremental exercise, the pulmonary $\dot{V}O_2$ increases gradually and can reach a maximum ($\dot{V}O_{2max}$) of ~90 mL · kg^{-1} · min^{-1} depending on sex, age, body weight, genetics, training status and health.[1-3]

According to the Fick equation, $\dot{V}O_{2max}$ is determined by the product of the maximal cardiac output ($\dot{Q}_{max}$) and the arterial to mixed venous O_2 difference (a-$\bar{v}O_2$diff). $\dot{Q}_{max}$ multiplied by the arterial O_2 content (CaO_2) sets the upper limit of systemic O_2 delivery, which is the principal limitation to $\dot{V}O_{2max}$ during exercise recruiting a large muscle mass, at sea level.[4-6] Despite extensive research since the 1950s on the factors limiting $\dot{V}O_{2max}$, it is still debated whether peripheral O_2 extraction capacity contributes to limiting $\dot{V}O_{2max}$.[7,8] Several original studies[4,5,9-12] and review articles[6,13-15] have addressed this topic in recent decades, yet no study has aimed to statistically analyse all the existing data on the association between $\dot{V}O_{2max}$ and its limiting factors. This kind of analysis is warranted, as the original studies often used homogenous groups with a small number of subjects (< 10) since they applied costly and invasive techniques involving catheterisations to determine $\dot{Q}_{max}$ (indicator-dilution techniques or the direct Fick method), regional blood flows (thermodilution or indicator-dilution techniques) and O_2 extraction fraction (calculated by the Fick equation or directly measured through arterial and venous catheters). Consequently, the statistical power is often too low to detect small but meaningful differences between subjects, groups with different training status, and before and after training, thus precluding a definite conclusion.

It is documented that the a-$\bar{v}O_2$diff at $\dot{V}O_{2max}$ is only slightly different between untrained and endurance-trained individuals,[16,17] suggesting that peripheral adaptations to endurance training have a minor impact on $\dot{V}O_{2max}$. However, the a-$\bar{v}O_2$diff is determined not only by the peripheries' ability to extract O_2, reflected in the mixed venous O_2 content ($C\bar{v}O_2$), but also by the CaO_2, which sets the upper limit for the a-$\bar{v}O_2$diff during maximal exercise. The CaO_2 is set by the haemoglobin concentration ([Hb]) and the O_2 saturation of Hb (SO_2), which may change with training and is

acutely modified during exercise. For instance, endurance training causes plasma volume expansion[18] that can lead to haemodilution and a lower O_2 carrying capacity of the blood.[16] A high $\dot{Q}_{max}$ shortens the time for alveolar/capillary gas equilibration at the lung, causing exercise-induced arterial hypoxemia that further reduces the CaO_2.[19,20] Therefore, it may be that the a-$\bar{v}O_2$diff does not increase substantially after endurance training, due to a concurrent training-induced lowering of CaO_2, whereas the systemic O_2 extraction fraction may improve ($\bar{O}_2$ extraction: a-$\bar{v}O_2$diff / CaO_2).

Another aspect of this discussion is whether the measurement techniques are sensitive enough to detect meaningful differences in the a-$\bar{v}O_2$diff. Most studies have not measured a-$\bar{v}O_2$diff directly but calculated it using the Fick equation ($\dot{V}O_{2max}$ / $\dot{Q}_{max}$).[16,17,21-24] The reason why so few studies have measured a-$\bar{v}O_2$diff directly during maximal exercise is due to the need for right heart catheterisation. Therefore, studies measuring the arterial to femoral venous O_2 difference (a-$v_fO_2$diff) and leg O_2 extraction fraction directly using peripheral catheters may be more sensitive in evaluating whether the O_2 extraction capacity changes with endurance training.

It is important to note that the factors limiting $\dot{V}O_{2max}$ may change over the course of training. For instance, the maximal mitochondrial respiratory capacity (OXPHOS) measured in permeabilised muscle fibres *ex vivo* and $\dot{V}O_{2max}$ are associated in untrained, but not in trained individuals.[25] These and other data[26] suggest that peripheral factors contribute to limit $\dot{V}O_{2max}$ in the untrained state, but their influence may diminish with increased $\dot{V}O_{2max}$ and training status.

In the present study, we critically reviewed and statistically analysed the previously published data on the association between $\dot{V}O_{2max}$ and O_2 extraction fraction, in men, by focusing on catheterisation studies. Two approaches were used: 1) articles containing individual data on pulmonary $\dot{V}O_{2max}$, $\dot{Q}_{max}$ (indicator-dilution techniques or the Fick method), a-$\bar{v}O_2$diff (mostly calculated) and $\bar{O}_2$ extraction fraction measured during whole-body maximal exercise (running, cycling) were included; 2) to investigate the relationship between limb $\dot{V}O_2$ and peripheral O_2 extraction fraction, mean data from studies reporting leg blood flow (LBF), a-$v_fO_2$diff and leg O_2 extraction fraction (catheters) measured during whole-body maximal exercise (running, cycling, cross-country skiing) were included. To investigate whether the limiting factors vary with $\dot{V}O_{2max}$, we employed the Fick law of diffusion to calculate the muscle O_2 diffusing

capacity (MDO_2) and subsequently used the Piiper and Scheid model to calculate the relative roles of perfusion vs diffusion limitations to $\dot{V}O_{2max}$.[27] Finally, we discuss the potential mechanisms behind the elevated O_2 extraction fraction observed after endurance training.

Results

Part 1: Systemic responses during maximal exercise (individual data)

$\dot{Q}_{max}$ increased by 4.9 L · min^{-1} for each L · min^{-1} increase in $\dot{V}O_{2max}$ (Fig. 1a; $P < 0.001$), explained by a linear increase in stroke volume (Fig. 1b; $P < 0.001$).

The calculated a-$\bar{v}O_2$diff ($\dot{V}O_{2max}$ / $\dot{Q}_{max}$) showed an inverse J-shaped curve, reaching the highest level between 4.5-5.0 L · min^{-1} before declining at higher $\dot{V}O_{2max}$ (Fig. 1c). After accounting for the decrease in CaO_2 with increasing $\dot{V}O_{2max}$ (Fig. 1e; $P < 0.001$), the calculated $\bar{O}_2$ extraction fraction increased up to a $\dot{V}O_{2max}$ of ~4.5-5.0 L · min^{-1} and then approached a maximal value at ~90% (Fig. 1d) when restricting the exponential decay model to plausible physiological limits ($\dot{V}O_{2max}$: 6-7 L · min^{-1}). The linear decrease in CaO_2 was explained by arterial hypoxemia (decreased arterial SO_2; Fig. 1f; $P < 0.001$) and a non-significant negative relationship between [Hb] and $\dot{V}O_{2max}$ (Fig. 1g; $P = 0.232$). The calculated $C\bar{v}O_2$ gradually decayed and approached a minimum at ~10-15 mL · L^{-1} in the subjects with the highest $\dot{V}O_{2max}$ (Fig. 1h).

<<Fig. 1 near here>>

Systemic vascular conductance was strongly positively correlated with $\dot{V}O_{2max}$ (Fig. 2b; $P < 0.001$). There were no significant associations between mean arterial pressure (MAP) and $\dot{V}O_{2max}$ (Fig. 2a; $P = 0.289$) nor $\dot{Q}_{max}$ and $\dot{V}O_{2max}$ ($y = -0.2x + 125$; $R^2 = 0.004$; $n = 119$; $P = 0.475$).

<<Fig. 2 near here>>

Whether controlling the regression between the individual data of $\dot{V}O_{2max}$ and the calculated $\bar{O}_2$ extraction fraction with mean values from studies measuring $\bar{O}_2$ extraction fraction directly using the Fick method (right heart catheterisation), or

indirectly using the Fick equation ($\dot{Q}_{max}$: indicator-dilution or transpulmonary thermodilution), most values fell close to the regression curve (Fig. 3).

<<Fig. 3 near here

Part 2: Peripheral responses during maximal exercise (mean data)

LBF and two-LBF rose by 4.6 and 6.0 L · min^{-1} for each L · min^{-1} increase in leg and pulmonary $\dot{V}O_{2max}$, respectively (Fig. 4a & 4d; both $P < 0.001$). Leg and pulmonary $\dot{V}O_{2max}$ displayed a linear relationship ($y = 1.33x – 2.25$; $R^2 = 0.84$; $n = 27$; $P < 0.001$). The directly measured leg a-$v_fO_2$diff and leg O_2 extraction fraction were best explained by exponential decay models and increased gradually with the increase of leg and pulmonary $\dot{V}O_{2max}$ to approach a maximum at ~180-190 mL · L^{-1} and ~90-95%, respectively (Fig. 4b-c & 4e-f). These relationships were equally strong when $\dot{V}O_{2max}$ was standardised to body weight (Supporting material Fig. 1). Note that leg a-$v_fO_2$diff was not lower for the subjects with the highest $\dot{V}O_{2max}$, as observed for the systemic a-$\bar{v}O_2$diff (Fig. 1c), possibly since only one subject group exceeded a $\dot{V}O_{2max}$ of 4.7 L · min^{-1}, where this occurred for the systemic responses (see Fig. 1c). In connection, no association was evident between pulmonary $\dot{V}O_{2max}$ and CaO_2 for these data ($y = 3.1x + 186$; $R^2 = 0.05$; $n = 29$; $P = 0.252$).

<<Fig. 4 near here>>

Like the systemic responses, the measured femoral venous O_2 content (Cv_fO_2) decreased gradually with increasing pulmonary $\dot{V}O_{2max}$ until reaching a minimum of ~10 mL · L^{-1} (Fig. 5a). Likewise, the femoral venous SO_2 and partial pressure of O_2 (PO_2) decreased gradually to approach ~5% and ~10 mmHg at the highest $\dot{V}O_{2max}$, respectively (Fig. 5b-c).

<<Fig. 5 near here>>

MDO_2 was positively correlated with leg $\dot{V}O_{2max}$ ($y = 27x – 6$; $R^2 = 0.92$; $n = 21$; $P < 0.001$), pulmonary $\dot{V}O_{2max}$ (Fig. 6a; $P < 0.001$) and leg O_2 extraction fraction ($y = 1.7x – 110$; $R^2 = 0.80$; $n = 21$; $P < 0.001$). Interestingly, the equilibration index Y, which quantitatively describes diffusion vs perfusion limitations to muscle $\dot{V}O_2$ (where $Y < 0.1$ indicates pure diffusion limitation, $0.1 < Y < 3$ indicates mixed perfusion-diffusion

limitation and Y > 3 indicates pure perfusion limitation),[27] was well above 1.0 for all subject groups (Fig. 6b) and increased progressively with leg $\dot{V}O_{2max}$ (y = 0.28x + 1.40; R^2 = 0.37; n = 21; P = 0.003), pulmonary $\dot{V}O_{2max}$ (Fig. 6b; P = 0.008) and leg O_2 extraction fraction (y = 0.023x – 0.129; R^2 = 0.53; n = 21; P < 0.001). The equilibration index Y was also correlated with pulmonary $\dot{V}O_{2max}$ standardised to body weight (R^2 = 0.38; P = 0.003; Supporting material Fig. 2). Therefore, the leg muscles were more perfusion than diffusion limited, even for subjects with the lowest $\dot{V}O_{2max}$, and were progressively more perfusion/O_2 delivery limited with a gradually higher $\dot{V}O_{2max}$. This can also be illustrated by applying the Piiper and Scheid model to calculate the fractional extent to which $\dot{V}O_{2max}$ is expected to change if MDO_2 or LBF are modified;[27] Fig. 6c shows that an individual's $\dot{V}O_{2max}$ is less sensitive to any change in MDO_2 if the $\dot{V}O_{2max}$ is already high, which is caused by the little remaining O_2 available for extraction in the femoral venous (i.e. end-capillary) blood. For instance, according to this theoretical model and using the relationship in Fig 6c; if a subject with a $\dot{V}O_{2max}$ of 5 L · min^{-1} changed his MDO_2 by 20%, he would only change his $\dot{V}O_{2max}$ by ~6% (20% × 0.3). Conversely, the same subject would increase $\dot{V}O_{2max}$ by ~14% after a 20% increase in LBF (20% × 0.7).

<<Fig. 6 near here>>

Discussion

To our knowledge, the present investigation is the first to critically review the existing research on the association between $\dot{V}O_{2max}$ and systemic and peripheral O_2 extraction fractions in healthy young men. Our findings are:

1) Pulmonary and leg $\dot{V}O_{2max}$ were best explained by $\dot{Q}_{max}$ and LBF, respectively, agreeing with most previous studies where these variables have been directly manipulated.
2) The systemic $\overline{O}_2$ extraction fraction increased with $\dot{V}O_{2max}$ until approximately 4.5-5.0 L · min^{-1}. Above this value, the $\overline{O}_2$ extraction fraction was typically around ~90%.
3) The measured leg O_2 extraction fraction increased with leg and pulmonary $\dot{V}O_{2max}$ to approach a maximal value at ~90-95%, strengthening the findings

from the calculated systemic $\bar{O}_2$ extraction fraction. This strongly suggests that O_2 extraction increases after endurance training and contributes to a high $\dot{V}O_{2max}$.

4) The calculated $C\bar{v}O_2$ and the measured Cv_fO_2 indicate a minimum value at ~15 and ~10 mL · L^{-1}, respectively, associated with a femoral venous SO_2 and PO_2 of ~5% and ~10 mmHg, respectively. At this point, further peripheral O_2 extraction may no longer be possible due to diffusional limitations and/or because the remaining O_2 represents blood perfusing the least active muscle regions of the leg, connective tissue, bone marrow, adipose tissue, and skin, which are characterised by a lower O_2 extraction.
5) The progressive increase in the equilibration index Y with pulmonary and leg $\dot{V}O_{2max}$ indicates that the muscles become gradually more perfusion/O_2 delivery limited with increasing $\dot{V}O_{2max}$.

Oxygen delivery

To match O_2 delivery to O_2 consumption, $\dot{Q}_{max}$ and two-LBF increased by ~5-6 L · min^{-1} per 1 L · min^{-1} increase in pulmonary $\dot{V}O_{2max}$. These relationships were strong and complied with previous research and the 'classic' view that O_2 delivery is the primary determinant of whole-body $\dot{V}O_{2max}$.[4,7,11] As maximal heart rate showed no apparent relationship with $\dot{V}O_{2max}$, the high stroke volumes (> 180 mL · $beat^{-1}$) explained the large $\dot{Q}_{max}$ in the athletes included in the present analysis (> 35 L · min^{-1}), in agreement with previous knowledge.[13,16,28]

Despite increased $\dot{Q}_{max}$, MAP was unchanged with increasing $\dot{V}O_{2max}$ due to increased vascular conductance. Although untrained individuals typically display a rise in MAP from rest to maximal exercise,[29] well-trained athletes can display an unchanged MAP or even a small reduction due to profound peripheral vasodilation.[30] Consequently, vasodilation of a well-developed peripheral vascular network likely contributed to the extremely high stroke volumes by minimising afterload in the subjects with the highest $\dot{V}O_{2max}$. To substantiate, endurance training of each leg separately, to evoke extensive peripheral adaptations without stimulating the central circulation substantially, has been shown to decrease MAP and the total peripheral resistance during two-legged maximal exercise that likely contributed to the elevated stroke volume and $\dot{Q}_{max}$ after training.[28] The high stroke volumes are probably achieved

through the combined effect of a large left ventricular mass,[31] compliant cardiac chambers[32,33] and an expanded blood volume[34,35] that facilitates a high end-diastolic volume and preload combined with the relatively low afterload.

Oxygen extraction

The calculated systemic a-$\bar{v}O_2$diff showed a large variability for a given $\dot{V}O_{2max}$ and was, if anything, lower in those subjects displaying the highest $\dot{V}O_{2max}$ (> 5 L · min^{-1}) compared to those being moderately- to well-trained ($\dot{V}O_{2max}$: 4-5 L · min^{-1}). This agrees with previous studies showing only a small difference between non-endurance-trained and active individuals[16,17] and no apparent difference between well-trained individuals and elite athletes.[16] This has led previous investigators to argue that improved O_2 extraction does not contribute or only minimally contributes to the remarkably high $\dot{V}O_{2max}$ observed in elite athletes.[14,36] However, these papers may not have considered that endurance training causes plasma volume expansion,[18] which often leads to haemodilution and a lower O_2 carrying capacity of the arterial blood.[16] Combined with the below-average haemoconcentration from rest to maximal exercise that occurs in well-trained individuals[16] and the exercise-induced arterial hypoxemia that often accompanies a high $\dot{Q}_{max}$,[19,20] individuals with the highest $\dot{V}O_{2max}$ displayed a substantially lower CaO_2 (~10%) than those with a low $\dot{V}O_{2max}$ (< 180 mL · L^{-1} vs > 200 mL · L^{-1}; Fig. 1e). Therefore, the lower CaO_2 may explain why moderately- and well-trained individuals can have a similar a-$\bar{v}O_2$diff, despite differing markedly in MDO_2, mitochondrial mass and capillary density.[37,38] Actually, parts of this mechanism are demonstrated experimentally since acute plasma volume expansion increases $\dot{Q}_{max}$ but lowers the CaO_2 and, hence, reduces the a-$\bar{v}O_2$diff during maximal exercise.[39,40]

Conversely, the systemic $\bar{O}_2$ extraction fraction – i.e., the fraction of O_2 that is taken up with respect to the amount available for utilisation (a-$\bar{v}O_2$diff / CaO_2) – increased with $\dot{V}O_{2max}$ until reaching ~90%. This pattern was confirmed in the leg when measured using catheters, with the O_2 extraction fraction increasing progressively with leg and pulmonary $\dot{V}O_{2max}$ until reaching ~90 to 95%. Therefore, the calculated systemic $\bar{O}_2$ extraction fraction (Fick equation) is supported by direct measurements via arterial and femoral venous blood sampling and strongly indicates that the $\bar{O}_2$ extraction fraction is improved with elevated $\dot{V}O_{2max}$ to a certain level.

In most previous endurance training studies aiming to investigate the importance of central vs peripheral adaptations to $\dot{V}O_{2max}$, $\dot{Q}_{max}$ was measured by non-invasive methods (such as inert-gas rebreathing techniques, impedance cardiography, and bioreactance) and the Fick equation was used to derive the a-$\bar{v}O_2$diff (for references, see the meta-analysis by Montero et al., 2015b).[41] Consequently, these studies may have failed to detect actual improvements in systemic $\bar{O}_2$ extraction fraction when the a-$\bar{v}O_2$diff was mostly unchanged, as endurance training may have evoked an accompanying reduction in CaO_2. Therefore, future studies should strive to measure peripheral or systemic O_2 extraction fraction directly, or at least combine the calculations of a-$\bar{v}O_2$diff with measurement of CaO_2 (arterial catheter). Actually, in the endurance training studies where peripheral O_2 extraction fraction was measured directly during maximal exercise, the vast majority found an increased O_2 extraction fraction after training.[12,28,42-44]

A particular case, concerning the relationship between one-leg $\dot{V}O_{2max}$ and O_2 extraction fraction (Fig. 4c) and between pulmonary $\dot{V}O_{2max}$ and two-LBF (Fig. 4d) deserves some attention (the white squares). These data were collected during combined upper- and lower-body exercise (diagonal cross-country skiing) and 6.6 $L \cdot min^{-1}$ of $\dot{Q}_{max}$ was distributed to the two arms.[30] Hence, when combining the locomotor blood flow (arms + legs), the data falls perfectly on the regression line between blood flow and pulmonary $\dot{V}O_{2max}$. When redistributing LBF towards other exercising musculature, the erythrocyte capillary mean transit time (MTT) is increased. Therefore, the conditions for Hb-O_2 off-loading are improved, resulting in a slightly higher O_2 extraction fraction for a given leg $\dot{V}O_2$. The same phenomenon can be seen when adding arm cycling to ongoing leg cycling[45] or vice versa,[46] which increases the O_2 extraction fraction that compensates for some of the reduction in blood flow.

Limitations to $\dot{V}O_2max$ by O_2 delivery and O_2 extraction varies with training status

The equilibration index Y was positively correlated with $\dot{V}O_{2max}$. Therefore, endurance training leads to a situation where the muscles become gradually more O_2-delivery limited. Thus, individuals with the highest $\dot{V}O_{2max}$ can only achieve a further substantial improvement in $\dot{V}O_{2max}$ by increasing O_2 delivery, a conclusion supported by the extremely low levels of Cv_fO_2 and $C\bar{v}O_2$ in these subjects. Therefore, the limiting

factors to $\dot{V}O_{2max}$ change with training status and $\dot{V}O_{2max}$: i) untrained, but healthy individuals display mixed perfusion-diffusion limitations; and ii) this diffusional limitation reduces as $\dot{V}O_{2max}$ is increased.[26] These conclusions are similar to those of Gifford et al. (2016),[25] who found a clear relationship between OXPHOS measured in permeabilised muscle fibres *ex vivo* and $\dot{V}O_{2max}$ in untrained but not in trained individuals.

Why is not all the O_2 extracted from the blood?

The entire $\dot{Q}_{max}$ cannot be directed to the skeletal muscles during exercise. Other organs like the brain, heart, splanchnic organs and skin need perfusion and O_2 delivery to maintain homeostasis. $\dot{Q}_{max}$ must also serve the O_2 demand of the respiratory muscles and the muscles in the trunk and the arms that stabilise the subject's position on the cycle ergometer, and these tissues are characterised by a substantially lower O_2 extraction than the legs during maximal exercise.[5,47] As a mean of those investigations measuring $\dot{Q}_{max}$ and LBF simultaneously (Table 1), the non-leg blood flow was 6.4 L · min^{-1} and was unaffected by the level of $\dot{Q}_{max}$ ($y = 0.002x + 6.4$; $R^2 < 0.001$; $n = 12$; $P > 0.999$).[4,5,9,11,29,48-54] The O_2 extraction was calculated to be 68% on average for all non-leg tissues (Table 1; head, trunk and arms), explaining why the $\overline{O}_2$ extraction fraction of the central circulation was slightly lower than in the legs (79% vs 84%, respectively; Table 1). A mean difference of 5 percentage points might be a small underestimation since the studies using right heart catheterisation[4,9,48,52,55,56] combined with arterial and femoral venous catheters indicated a mean difference of 8 percentage points. A difference of 5-8%-points fits well, since the O_2 extraction fraction of the arms, myocardium, brain and trunk range from 40-80% during exercise.[5,47,57-59] Therefore, the $C\bar{v}O_2$ can never reach the same level as the Cv_fO_2 during exercise involving the legs and was calculated to reach a minimum of ~15 ml · L^{-1} in subjects having a $\dot{V}O_{2max}$ of 6 L · min^{-1} (Fig. 1h). To our knowledge, the lowest $C\bar{v}O_2$ measured at sea level using right heart (atrium) catheterisation is 20.1 ml · L^{-1} (group mean) in athletes with a $\dot{V}O_{2max}$ of 5.1 L · min^{-1}.[55] A slightly lower value was measured in one of these cross-country skiers (15.5 ml · L^{-1}), and a mean value of 18.6 ml · L^{-1} has been measured in moderately trained individuals after acclimatising to 6500 metres above sea level;[60] indicating that 15 ml · L^{-1} or lower is approachable.

<<Table 1 near here>>

The highest recorded leg O_2 extraction fraction was 93% (group mean)[55] and the regression models indicated a plateau at ~95% within physiological limits for pulmonary $\dot{V}O_{2max}$. Hence, a minimum of ~10 mL O_2 remains in each litre of femoral venous blood associated with a PO_2 of ~10 mmHg, even for the best-trained individuals. In this situation, a PO_2 gradient persists between the blood and myoglobin (myoglobin/intracellular PO_2: ~1-2 mmHg),[61] where myoglobin-facilitated diffusion should proceed given the high myoglobin O_2 affinity (myoglobin $P_{50}O_2$: ~5 mmHg) and the low myoglobin SO_2 at maximal exercise.[61] However, it has been shown that the primary site of resistance to O_2 diffusion is between the capillaries and the sarcoplasm and it has been estimated that the 'critical capillary PO_2' needed to overcome this resistance may be as high as 10-20 mmHg.[61-64] The remaining O_2 may, therefore, represent diffusional limitations across the combined capillary wall, interstitium and sarcolemma barriers together with a MTT that is too short for complete Hb-O_2 off-loading. This is supported by the need for an infinitesimal PO_2 gradient for O_2 to diffuse from the sarcoplasm to cytochrome c oxidase[65] and the estimate that a mitochondrial PO_2 of ~1 mmHg may be sufficient to support maximal mitochondrial respiration.[66,67] The remaining O_2 may also represent muscle metabolism-perfusion mismatch[68,69] and an inevitable lower O_2 extraction from the blood perfusing the skin, connective tissue, fat and bone marrow of the leg causing venous admixture. In this context, the end-capillary PO_2, assessed using video microscopy, was found to be lower than the PO_2 both in the venule (O_2 microelectrode) and vein (blood gas) draining the muscle region of interest.[70] Hence, the lowest femoral venous PO_2 values of ~10 mmHg indicates an even lower end-capillary PO_2 in the capillaries adjacent to the most metabolically active muscle regions during maximal exercise, possibly approaching ~5 mmHg. Therefore, no matter which kind of limitation prevails, it is highly unlikely that leg O_2 extraction fraction can improve much further, and that a theoretical threshold of ~95% exists due the above diffusional and distributional limitations and barriers.

What mechanisms explain the improvement of O_2 extraction with training?

The peripheral O_2 extraction depends on the interaction between several factors: i) the kinetics of O_2 off-loading from Hb; ii) the erythrocyte MTT, which is determined by the blood flow, the capillary density and the degree of matching of blood flow distribution to the metabolic demand; iii) the diffusional O_2 conductance over the combined capillary wall, interstitium and sarcolemma barriers; and iv) the muscle oxidative capacity.[55]

A right-shifted O_2-Hb dissociation curve (elevated $P_{50}O_2$) increases the O_2 extraction fraction in pump-perfused dog muscle.[71] A close relationship has also been demonstrated between O_2 extraction fraction and in vivo $P_{50}O_2$ in humans during exercise.[55] Very few of the studies included in the present analysis reported the in vivo $P_{50}O_2$, but it was possible to calculate it from the other blood gas parameters using Kelman's equation[72] after assuming a femoral venous blood temperature of 39.0°C at maximal exercise.[9,52,73] Based on 15 of the studies presented in Table 3, the $P_{50}O_2$ was linearly associated with leg O_2 extraction fraction ($R^2 = 0.27$; n = 15; P = 0.048). Despite this relationship, the high $P_{50}O_2$ does not seem to be a limiting factor for O_2 extraction during whole-body maximal exercise, as demonstrated in experiments using carbon monoxide, which left-shifts the ODC without a negative impact on O_2 extraction fraction.[53]

Increased MTT has the potential to increase O_2 extraction, but whether this occurs after endurance training is determined by the balance between the changes in blood flow and the capillary blood volume. Capillary density typically improves by 10-30% after 4-24 weeks of endurance training,[74-76] which is similar to the changes in $\dot{V}O_{2max}$ for this training duration.[75-77] Moreover, cross-sectional data indicate a similar difference in capillary density to that of $\dot{V}O_{2max}$ between untrained and endurance trained men.[38] Therefore, the capillary growth probably maintains the MTT despite elevated $\dot{Q}_{max}$ and peripheral blood flow after training. In support, similar improvements in arm blood flow and capillary density have been observed after a period of arm training, causing no change in the calculated MTT.[44] The arm O_2 extraction fraction was increased in the same study, suggesting that elevated MTT is not the primary mechanism by which O_2 extraction is improved after training. An increased capillary to fibre ratio, on the other hand, increases the number of contact points between the capillary and the muscle fibre. This increases the diffusional surface, which is one of the

most critical determinants of O_2 diffusion from the erythrocytes to the cytoplasm,[78,79] and is proposed as an explanation for the higher O_2 extraction fraction in the legs than in the arms during exercise.[55]

During whole-body maximal exercise, the oxidative capacity of skeletal muscle exceeds the O_2 delivery, as illustrated by the two-fold higher mass-specific $\dot{V}O_2$ during dynamic one-legged knee-extension (1L-KE) compared to cycling exercise (approximately 2.5 vs 20 kg active muscle mass, respectively).[10,80] Therefore, the leg muscles possess an oxidative reserve capacity at $\dot{V}O_{2max}$ during whole-body exercise, which has frequently been used as an argument to indicate that the large improvements in mitochondrial and capillary networks after endurance training are likely only crucial for improvements in endurance performance and do not affect the limiting factors to $\dot{V}O_{2max}$.[81] In support of this view, the calculated $\bar{O}_2$ extraction fraction is maintained or increases after prolonged bed rest, although a substantial reduction in mitochondrial volume density occurs.[82,83] However, the $\bar{O}_2$ extraction fraction depends on the interactions between several factors. For instance, by acutely decreasing $\dot{Q}_{max}$ and LBF using β-adrenergic blockade, a-$\bar{v}O_2$diff and a-$v_fO_2$diff increase during sub-maximal and maximal exercise, facilitated by increased erythrocyte MTT.[84,85] This is substantiated by the positive relationship between the ratio of OXPHOS / O_2 delivery and the leg O_2 extraction fraction,[10] meaning that the balance between muscle oxidative capacity and blood flow (i.e., oxidative capacity and MTT) is more critical for O_2 extraction than any of these factors alone. Therefore, as bed rest reduces $\dot{Q}_{max}$ dramatically but causes only a minor change in capillary density,[82,83] the MTT is elevated, and the ratio of OXPHOS / O_2 delivery is probably the same, in favour of increased or maintained $\bar{O}_2$ extraction fractions. In contrast, by changing the exercise mode from upright to supine cycling after bed rest, which preserves $\dot{Q}_{max}$ at the pre-bed rest level, the calculated a-$\bar{v}O_2$diff is decreased (154 vs 120 mL $\cdot$ L^{-1}).[86] Similarly, after a dog gastrocnemius muscle was immobilised for 3 weeks, followed by electrical stimulation to $\dot{V}O_{2max}$ while being pump-perfused to receive a similar O_2 delivery as a control muscle, the O_2 extraction fraction was dramatically reduced.[79] Therefore, muscle oxidative capacity seems to play a role in determining O_2 extraction, and the bed rest studies need to be evaluated carefully due to the consequences for peripheral MTT.

If $\overline{O}_2$ extraction fraction improves after endurance training, is probably affected by the balance between central and peripheral adaptations. For instance, after 2 weeks of high-intensity interval training that elevated the cytochrome c oxidase activity by 20% but caused no change in $\dot{Q}_{max}$, $\dot{V}O_{2max}$ was increased by 8% and was entirely attributed to the improved systemic (calculated a-$\bar{v}O_2$diff) and leg (increased deoxyhaemoglobin and decreased tissue oxygenation index in Vastus Lateralis, assessed using NIRS) O_2 extraction.[87] However, after 3 to 8 weeks of endurance training, improvements in $\dot{Q}_{max}$ explain almost the entire increase in $\dot{V}O_{2max}$, as indicated by meta-regression.[41] If the training lasts longer (> 8 weeks), enhancements of $\dot{Q}_{max}$ decelerate and improvements in a-$\bar{v}O_2$diff is again evident.[41,88] Therefore, the peripheral adaptations are probably just sufficient to counteract the 'negative influence' of elevated $\dot{Q}_{max}$ and LBF on MTT in periods with large central adaptations, and improvements in $\overline{O}_2$ extraction fraction is likely only evident when the peripheral adaptations largely surpass those of the central circulation. This can be substantiated by findings from one-legged endurance training, which induces robust peripheral adaptations without stimulating the central circulation substantially, commonly improving leg a-$v_fO_2$diff by 5-10 ml $\cdot$ L^{-1}.[28,42]

The mitochondrial volume density can differ by as much as 150% between untrained and well-trained individuals in extreme cases (e.g., ~4 vs ~10 vol. %)[89,90] and can improve by as much as ~40-55% after 6 weeks of endurance training in previously sedentary individuals.[35,91,92] Why does this disproportionate adaptation occur when the muscle already possesses an oxidative reserve capacity? Does it have any physiological meaning for $\dot{V}O_{2max}$ or is it only important for improvements in, for example, fat oxidation[93] and the lactate threshold,[94] thus improving endurance?

Although an impressive increase in leg O_2 extraction fraction from 72% to 82% has been reported after only 9 weeks of intense endurance training in previously sedentary subjects,[12] we propose that remarkable increases in muscle oxidative capacity are needed to achieve the outstanding leg O_2 extraction fraction observed in elite athletes (close to 95%).[55,95] By analogy, the oxidative reserve capacity may act as a 'bottomless pit', keeping the myoglobin SO_2 and intracellular PO_2 low. This, in turn, maintains the PO_2 gradient between the capillary and the muscle cell, promoting O_2 diffusion and O_2 extraction even at a very low capillary PO_2. As shown in Fig. 6c, a

subject's $\dot{V}O_{2max}$ becomes gradually less sensitive to adaptations improving diffusion when $\dot{V}O_{2max}$ is already high. Therefore, to raise the O_2 extraction fraction even slightly (e.g., 2%), it is likely that more substantial improvement in peripheral adaptations is needed. However, a change in leg O_2 extraction fraction from, for example, 93-95% would only have a small impact on whole-body $\dot{V}O_{2max}$: for an athlete with a $\dot{V}O_{2max}$ of 5 L · min^{-1}, a two-LBF of 24 L · min^{-1} ($\dot{Q}_{max}$: ~31 L · min^{-1}) and an CaO_2 of 190 mL · L^{-1}, the $\dot{V}O_{2max}$ would only increase by ~90 mL · min^{-1} (1.8%). In comparison, an increase of 1 L · min^{-1} in two-LBF would increase $\dot{V}O_{2max}$ by ~170 mL · min^{-1} (3.4%) if all other factors remained the same.

Study considerations

The data were collected from several research groups and published over six decades (1958-2017) using a variety of gas analysers, flow sensors, methods to determine blood O_2 content and PO_2, and several procedures to analyse the indicator-dilution and blood temperature curves for $\dot{Q}_{max}$ and LBF measurements, respectively. Therefore, for a given $\dot{V}O_{2max}$, the between-subject variability presented here may be overestimated. Moreover, several different averaging strategies for $\dot{V}O_2$ and the associated variables have likely been applied (rarely stated in the manuscripts). Despite these potential sources of noise, in general, the studies' mean values converged to similar values. The fact that, despite the combination of several measurements with distinct methods (such as pulmonary gas exchange, thermodilution, and blood gas analyses), the integrations of the obtained values fitted into the physiological range and agreed between studies, demonstrates the quality of these studies and the robustness of the analysis presented here.

Conclusion and perspective

In conclusion, measurements of $\dot{Q}_{max}$ and LBF show that O_2 delivery is the primary determinant of whole-body and limb $\dot{V}O_{2max}$. However, we also show that a very high O_2 extraction fraction contributes to the remarkably high $\dot{V}O_{2max}$ in well-trained individuals and elite endurance athletes. To reinforce this conclusion we can, using the regression lines established in the present investigation, compare a typically sedentary subject and an elite endurance athlete with a large difference in $\dot{V}O_{2max}$ (3.0 vs 5.5 L ·

min^{-1}): the elite athlete has a 1.83-fold higher $\dot{V}O_{2max}$, a 1.60-fold higher $\dot{Q}_{max}$ and a 1.26-fold higher $\overline{O}_2$ extraction fraction (Fig. 7). However, due to the lower CaO_2, the a-$\bar{v}O_2$diff is only 1.13-fold higher in the elite athlete. This also stresses that a-$\bar{v}O_2$diff and $\overline{O}_2$ extraction fraction cannot be used interchangeably when evaluating central vs peripheral limitations to $\dot{V}O_{2max}$. Finally, the limitations for whole-body $\dot{V}O_{2max}$ change with training status, with an accentuated O_2 delivery limitation and conversely a decreasing O_2 diffusional limitation with increasing $\dot{V}O_{2max}$.

<<Fig. 7 near here>>

Materials and methods

The data used in this statistical analysis were collected using two approaches:

1) Articles containing individual data on pulmonary $\dot{V}O_{2max}$, $\dot{Q}_{max}$, a-$\bar{v}O_2$diff and systemic O_2 extraction ($\overline{O}_2$ extraction) fraction measured during whole-body maximal exercise (running, cycling) were included (Part 1).
2) To investigate the relationship between limb $\dot{V}O_2$ and peripheral O_2 extraction fraction, mean data from studies reporting leg $\dot{V}O_{2max}$, LBF, a-$v_fO_2$diff and leg O_2 extraction fraction measured during whole-body maximal exercise (running, cycling, cross-country skiing) were included (Part 2).

This strategy was chosen since a large amount of individual data has been published on systemic responses, whereas we were unable to identify other than mean values in studies investigating peripheral haemodynamics and O_2 extraction fraction. The data were identified through searches conducted in the PubMed database using several combinations of the following search terms: circulation, circulatory, hemodynamic(s), cardiac output, leg blood flow, arteriovenous oxygen difference, oxygen extraction and exercise. Cross-reference checks were also conducted, in addition to separate searches on authors with articles already included in the database. Only exercise modes engaging a large muscle mass that could elicit $\dot{V}O_{2max}$ were included (cycling, running and cross-country skiing using the diagonal technique). Data from cross-sectional studies or before and after training interventions that were collected in normoxia on young (< 40 years old) and healthy individuals were included. Data collected in hypoxia, after

acclimatisation to altitude, in altitude natives, in hyperthermia, with atrial pacing, after bed rest and after blood volume manipulations were excluded. The control condition was used when the above forms of manipulations of the cardiovascular system were conducted. Only catheterisation studies that used invasive methods to measure $\dot{Q}_{max}$ (indicator-dilution techniques or the Fick method) and LBF (bolus or continuous infusion thermodilution and indicator-dilution techniques) were included. Only individual data from men are used (Part 1). In Part 2, studies that had a sample with a majority of men were used (≥ 50%). When several papers reported data from the same data collection, only one of the articles was included. If an article used some of the same subjects as previously reported, but with supplementation with new subjects, the data was included. The included articles are presented in Tables 2 and 3 for Parts 1 and 2, respectively.

<< ***Table 2 and 3 near here***>>

Calculations

When the data were published in graphs and not in tables or text, ImageJ (v1.50b; National Institutes of Health, USA) was used for data extraction. If not all variables were reported in the articles, the reported data were used to derive the missing values via the following formulas or combination of formulas if possible:

$$\text{Pulmonary } \dot{V}O_{2max} = \dot{Q}_{max} \times a\text{-}\bar{v}O_2diff \quad (1)$$

$$\text{Leg } \dot{V}O_{2max} = LBF \times a\text{-}v_fO_2diff \quad (2)$$

$$\text{Stroke volume} = \dot{Q}_{max} / \text{heart rate} \quad (3)$$

$$\text{Blood } O_2 \text{ content (e.g., } CaO_2) = 1.39 \times [Hb] \times SO_2 + 0.003 \times PO_2 \quad (4)$$

$$\text{Leg } O_2 \text{ delivery} = LBF \times CaO_2 \quad (5)$$

$$\overline{O}_2 \text{ extraction} = a\text{-}\bar{v}O_2diff / CaO_2 \quad (6)$$

$$O_2 \text{ extraction} = a\text{-}v_fO_2diff / CaO_2 \quad (7)$$

$$\text{Systemic vascular conductance} = \dot{Q}_{max} / (MAP\text{-}CVP) \quad (8)$$

If no arterial PO_2 was reported, 100 mmHg was assumed for the calculation of CaO_2 (i.e., 3mL O_2 freely dissolved in blood plasma per 1 L of blood). Central venous pressure (CVP) at $\dot{V}O_{2max}$ was taken as 5 mmHg[4] when calculating systemic vascular

conductance. MDO_2 and mean capillary PO_2 were calculated as previously described,[55,56] using the measured arterial and femoral venous PO_2. MDO_2 [$MDO_2 = \dot{V}O_2$ / (mean capillary PO_2 – mitochondrial PO_2)] is recognised as a compound variable integrating several steps in the O_2 cascade, including the 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 utilisation by the cytochromes. The equilibration index Y, which quantitatively describes perfusion vs diffusion limitations to $\dot{V}O_2$, was calculated according to Piiper and Scheid.[27]

Statistical analyses

Data are presented as mean ± standard deviation, if not otherwise stated. Regression was analysed using simple linear regression, second-order polynomials and exponential decay models ($Y = a \cdot e^{-K \cdot X} + plateau$), all using least squares as the fitting method. Regression lines/curves are presented with 95% confidence bands representing the likely location of the true curve. 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, CA, USA) and Microsoft Office Excel 2013 (Microsoft Corporation, WA, USA) were used for statistical analysis.

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Author Contributions

Conception and design of the investigation: Ø.S., J.H., C.C., J.A.L.C.
Literature search and analysis of data: Ø.S.
Interpretation of data: Ø.S., J.H., C.C., J.A.L.C., B.R.
Writing the first draft of the manuscript: Ø.S.
Revising and approving the final version: Ø.S., J.H., C.C., J.A.L.C., B.R.

Conflict of interest

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

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Data availability statement

Data sharing is not applicable to this article, as no new data were created in this study.

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Tables

Table 1. Data from studies measuring pulmonary O_2 uptake, cardiac output (indicator-dilution, Fick method or transpulmonary thermodilution), leg blood flow (thermodilution) and leg arteriovenous O_2 difference (a-vO_2diff; catheters) simultaneously during maximal exercise. From these measurements, O_2 extraction fraction was calculated for the central circulation and the non-leg tissue (combined trunk, arms and head).

	Central circulation (mean ± SD)	Two-leg circulation (mean ± SD)	Non-leg tissue circulation (mean ± SD)
Blood flow ($L \cdot min^{-1}$)	25.0 ± 2.4	18.6 ± 3.0	6.4 ± 1.7
Arterial O_2 content ($mL \cdot L^{-1}$)	203 ± 10	203 ± 10	203 ± 10
O_2 delivery ($L \cdot min^{-1}$)	5.03 ± 0.60	3.77 ± 0.63	1.26 ± 0.32
O_2 uptake ($L \cdot min^{-1}$)	4.02 ± 0.65	3.19 ± 0.65	0.83 ± 0.24
a-vO_2diff ($mL \cdot L^{-1}$)	160 ± 17	172 ± 14	137 ± 48
O_2 extraction fraction (%)	79 ± 8	84 ± 5	68 ± 26
Venous O_2 content ($mL \cdot L^{-1}$)	42 ± 18	31 ± 10	66 ± 52
O_2 not utilised ($L \cdot min^{-1}$)	1.01 ± 0.36	0.58 ± 0.07	0.43 ± 0.32

n = 12 (articles)[4,5,9,11,29,48-54] or n = 117 (subjects).

Table 2. Articles reporting individual values of maximal oxygen uptake ($\dot{V}O_{2max}$), maximal cardiac output ($\dot{Q}_{max}$) and arterial to mixed venous O_2 difference (a-$\bar{v}O_2$diff). In studies reporting arterial O_2 content, the systemic O_2 extraction fraction ($\bar{O}_2$ extraction) was calculated.

				Method used to measure:			Reported or can be calculated	
Article	n	Exercise	Age	$\dot{Q}_{max}$	$\dot{V}O_{2max}$	a-$\bar{v}O_2$diff	$\bar{O}_2$ extraction	MAP
Blomqvist et al. (1970)[110]	4	Cycling	23-33	ID	DB		-	Yes
Ekblom & Hermansen (1968)[16]	14	Run	22-34	ID	DB	Calculated	Yes	Yes
Ekblom et al. (1968)[17]	8	Cycling	19-27	ID	DB	using Fick	Yes	Yes
Ekblom (1970)[106]	7	Cycling	22-26	ID	DB	equation	Yes	Yes
Epstein et al. (1965)[111]	2	Run	21	ID	Custom		-	Yes
Epstein et al. (1967)[112]	4	Run	18-30	Fick	Custom	Measured	-	Yes
Gleser (1973)[22]	6	Cycling	20-23	ID	DB		Yes	Yes
Hermansen et al. (1970)[21]	13	C / Run	19-34	ID	DB		Yes	Yes
Mitchell et al. (1958)[23]	6	Run	-	ID	DB		-	-
Robinson et al. (1966)[113]	5	Run	19-31	ID	Custom		-	Yes
Saltin (1964)[104]	4	Cycling	23-26	ID	DB	Calculated	-	-
Saltin & Stenberg (1964)[103]	4	Cycling	23-25	ID	DB	using Fick	-	Yes
Saltin et al. (1968a)[83]	5	Cycling	19-21	ID	DB	equation	Yes	Yes
Saltin et al. (1968b)[105]	4	Cycling	20-21	ID	DB		-	-
Stenberg et al. (1966)[108]	6	Cycling	20-36	ID	DB		Yes	Yes
Stenberg et al. (1967)[102]	5	Cycling	20-39	ID	DB		-	Yes
Åstrand et al. (1964)[24]	12	Cycling	21-30	ID	DB		Yes	Yes

n: number of subjects meeting the inclusion criteria. Note that some subjects were investigated on more than one occasion (before/after training, running/cycling); ID: indicator-dilution method using indocyanine green or Evans blue dye (only used in Mitchell et al., 1958); DB: Douglas bag technique; MAP: mean arterial pressure

Table 3. Articles reporting mean values of leg oxygen uptake (leg $\dot{V}O_{2max}$), leg blood flow (LBF) and arterial to femoral venous O_2 difference (a-$v_fO_2$diff) during maximal exercise (cycling and cross-country skiing).

		Age	Method used to measure:			Reported or can be calculated
Article	n	($\bar{x}$)	Pulmonary $\dot{V}O_2$	LBF	a-$v_fO_2$diff	O_2 extraction
Bender et al. (1988)[96]	7♂	22	Custom	TD-B	Measured via arterial and femoral venous blood sampling	Yes
Calbet et al. (2003)[54]	4♂3♀	24	Med. Graph. CPX	TD-C		Yes
Calbet et al. (2004; 2005)[30,55]	3♂	24	Amis 2001	TD-C		Yes
Calbet et al. (2006)[49]	10♂	24	Quark b2	TD-C		Yes
Calbet et al. (2007)[5]	9♂	33	Quark b2	TD-C		Yes
Calbet et al. (2015a)[29]	9♂	31	Quark b2	TD-C		Yes
Calbet et al. (2015b)[53]	11♂	22	Vmax 29	TD-C		Yes
Cardinale et al. (2018)[10]	4♂3♀	33	Oxycon Pro	TD-C		Yes
Cardus et al. (1998)	13♂5♀	23	Custom	TD-C		Yes
Gonzalez-Alonso et al. (2003)[11]	8♂	24	OCM-2	TD-C		Yes
Harms et al. (1997)[99]	7♂	29	Custom	TD-C		Yes
Klausen et al. (1982)[28]	6♂	23	Douglas bag tech.	ID-B		-
Knight et al. (1992)[97]	7♂	29	Custom	TD-C		Yes
Knight et al. (1993)[95]	12♂	29	Custom	TD-C		Yes
Lundby et al. (2008a)[50]	8♂	26	Quark b2	TD-C		Yes
Lundby et al. (2008b)[51]	8♂	27	Quark b2	TD-C		Yes
Lundby et al. (2004; 2006)[101,107]	6♂	26	Custom	TD-C		Yes
Mortensen et al. (2008)[9]	13♂	28	Quark b2	TD-C		Yes
Mortensen et al. (2005)[4]	10♂	27	Quark b2	TD-C		Yes
Munch et al. (2014)[52]	10♂	27	Quark CPET	TD-C		Yes
Poole et al. (1991)[109]	6♂	26	Custom	TD-C		Yes
Roca et al. (1989)[56]	6♂	24	Custom	-		Yes
Roca et al. (1992)[12]	8♂4♀	22	Custom	TD-C		Yes
Rud et al. (2012)[42]	4♂4♀	23	Douglas bag tech.	TD-C		Yes
Trangmar et al. (2017)[73]	9♂	26	Not reported	TD-C		Yes
van Hall et al. (2001)[100]	5♂1♀	26	Med. Graph. CPX	TD-C		Yes

n: number of subjects; TD-B: bolus-infusion thermodilution method; TD-C: continuous-infusion TD; ID-B; bolus indicator-dilution method (I-labelled human albumin).

Legends to figures

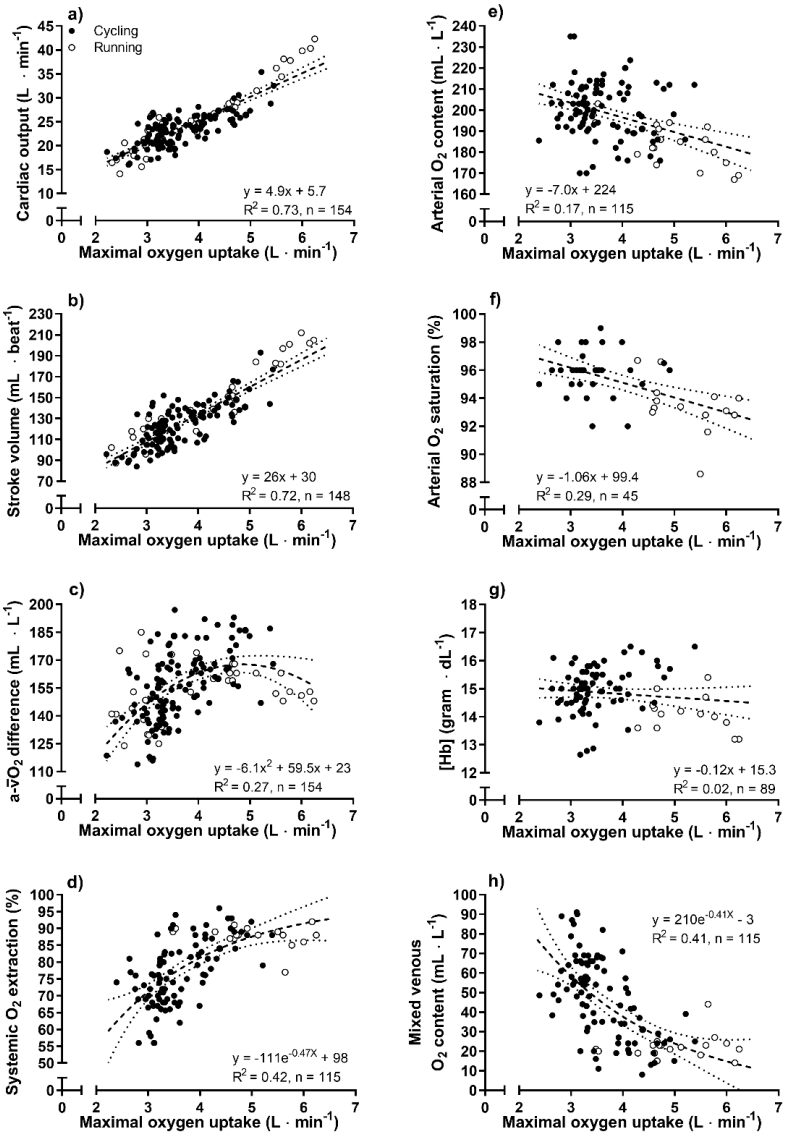


Fig. 1: The relationship between individual values (from studies reported in Table 2) of pulmonary maximal oxygen uptake and cardiac output (a), stroke volume (b), arterial to mixed venous oxygen difference (a-$\bar{v}O_2$ difference; c), systemic oxygen extraction fraction (d), arterial oxygen content (e), arterial oxygen saturation (f), haemoglobin concentration ([Hb]; g) and the calculated mixed venous oxygen content (h). All data were obtained during maximal exercise. Inserted in each graph are the formulas for the regression equations along with the goodness of fit (R^2) and the number of data pairs (n).

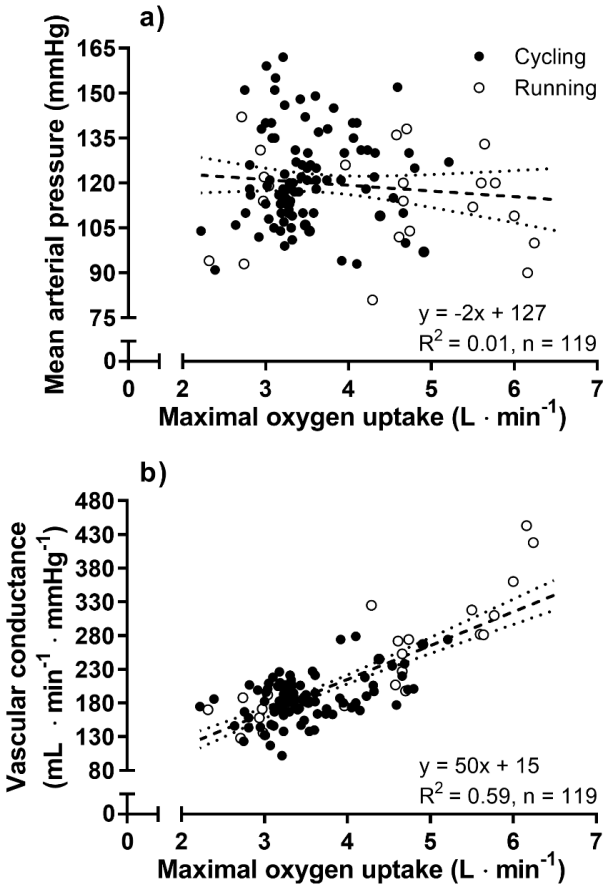


Fig. 2: The relationship between individual values (from studies reported in Table 2) of pulmonary maximal oxygen uptake and mean arterial pressure (a) and systemic vascular conductance (b). Inserted in each graph are the formulas for the linear regression along with the goodness of fit (R^2) and the number of data pairs (n).

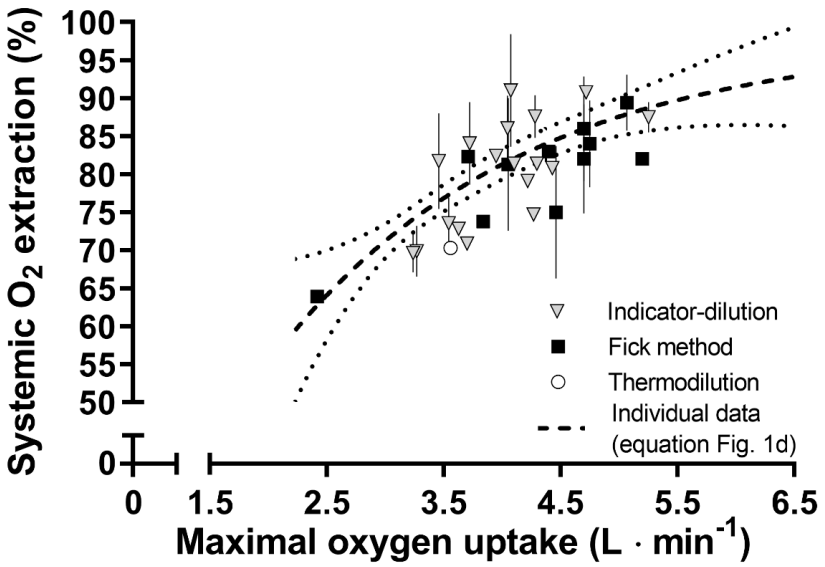


Fig. 3: Mean values (± 95% confidence limits, where available) of systemic oxygen extraction fraction vs maximal oxygen uptake from studies using the direct (pulmonary artery catheter) or the modified (right atrium catheter) Fick method,[4,9,30,48,52,56,60,114-117] the indicator dilution method[5,11,16,17,21,22,24,29,39,49-51,54,83,106,108,118,119] and the transpulmonary thermodilution method.[53] Broken line is the regression equation obtained from Fig. 1d.

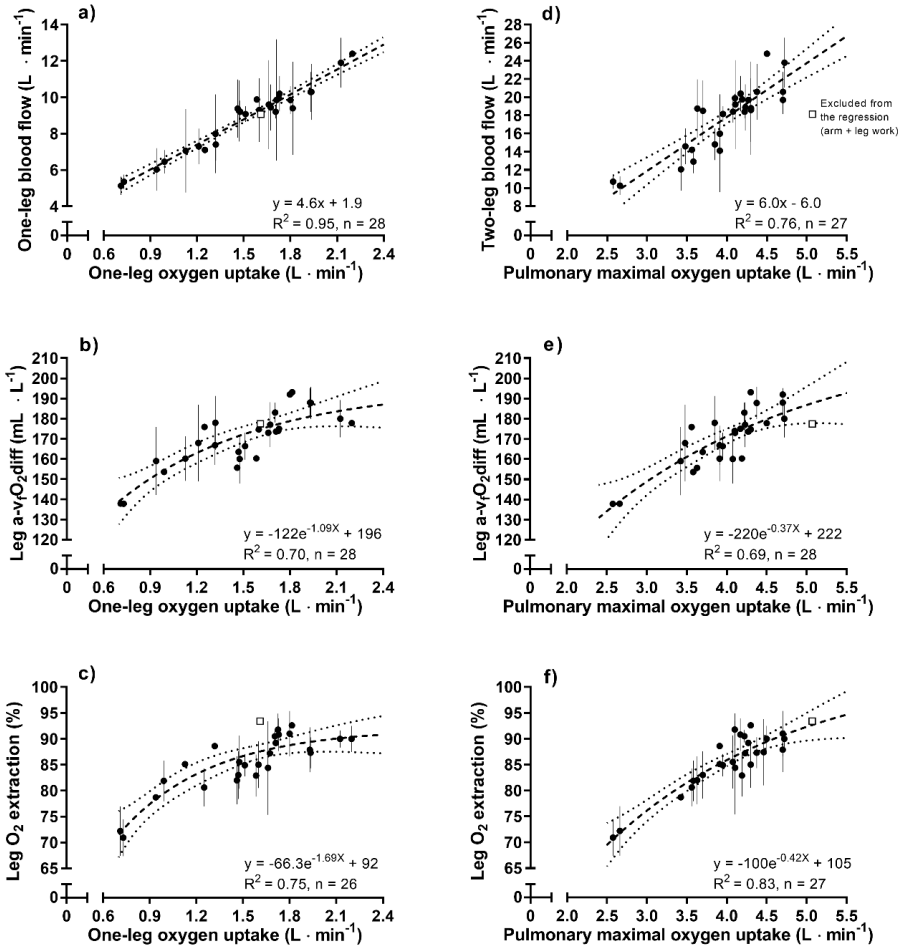


Fig. 4: The relationship between one-leg or pulmonary maximal oxygen uptake and leg blood flow (Fig. 4a and 4d, respectively), arterial to femoral venous oxygen difference (a-$v_fO_2$diff; Fig. 4b and 4e, respectively), and leg oxygen extraction fraction (Fig. 4c and 4f, respectively). Black circles and white squares denote cycling and diagonal cross-country skiing, respectively. The skiers are excluded from the regression in Fig. 4d due to the combined leg and arm use for locomotion that distributed 6.6 L · min^{-1} blood flow to the exercising arms (see the discussion). Data are mean values (± 95% confidence limits, where available) from studies reported in Table 3.

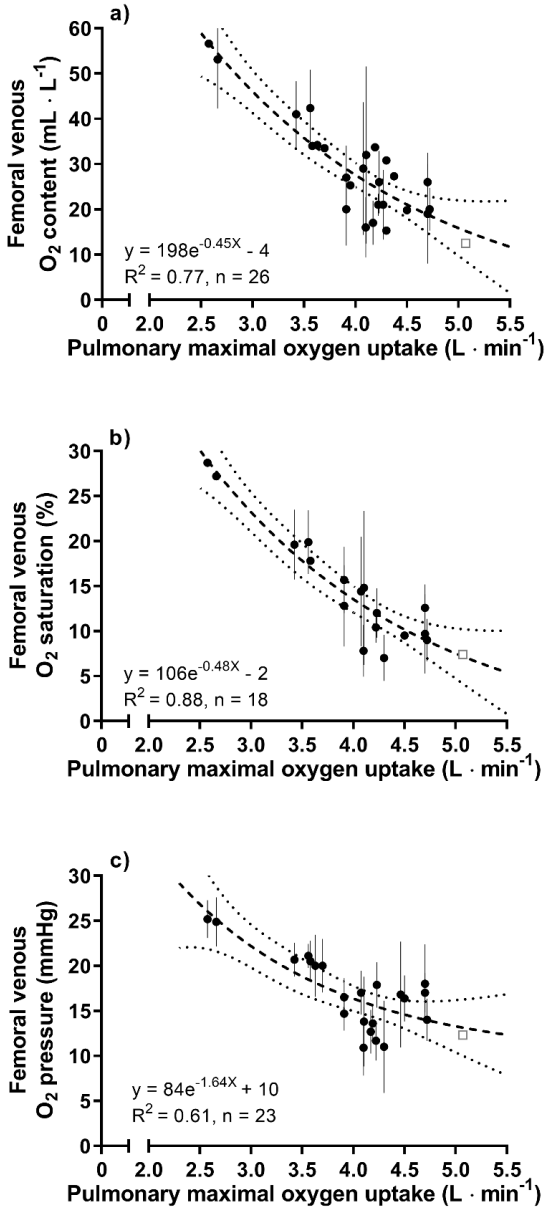


Fig. 5: The relationship between pulmonary maximal oxygen uptake and the associated femoral venous O_2 content (a), femoral venous O_2 saturation (b) and femoral venous O_2 pressure (c). Black and white symbols denote cycling and diagonal skiing, respectively. Data are mean values (± 95% confidence limits, where available) from studies reported in Table 3.

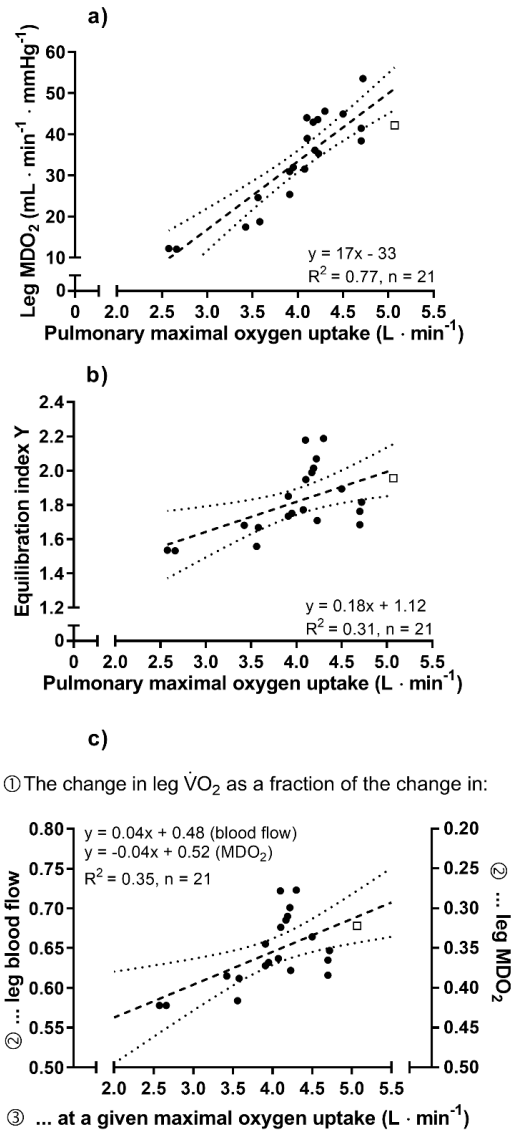


Fig. 6: The relationship between pulmonary maximal oxygen uptake ($\dot{V}O_{2max}$) and one-leg muscle O_2 diffusing capacity (MDO_2; a), the equilibration index Y (b), calculated using the Piiper and Scheid model, and the fractional extent to which $\dot{V}O_{2max}$ is expected to change if MDO_2 or leg blood flow (LBF) is changed alone (c).[27] Data are mean values from studies reported in Table 3.

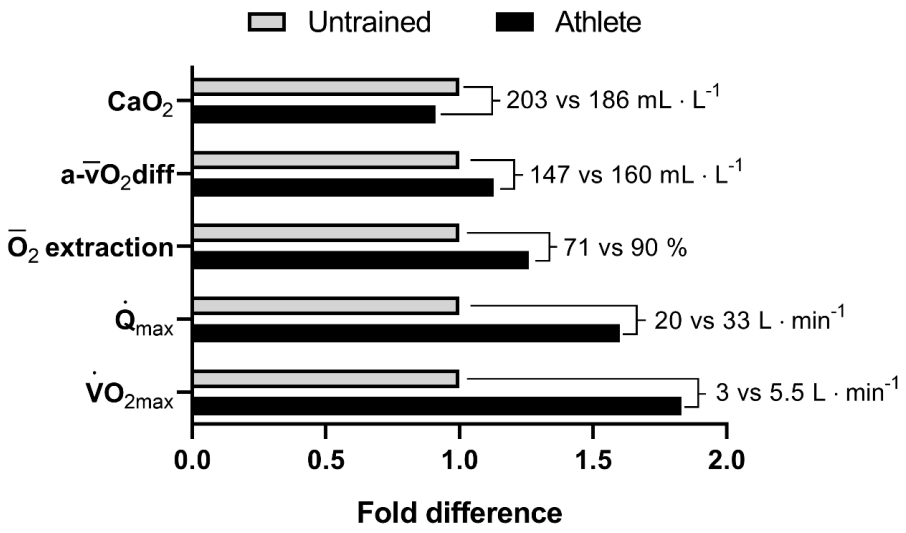
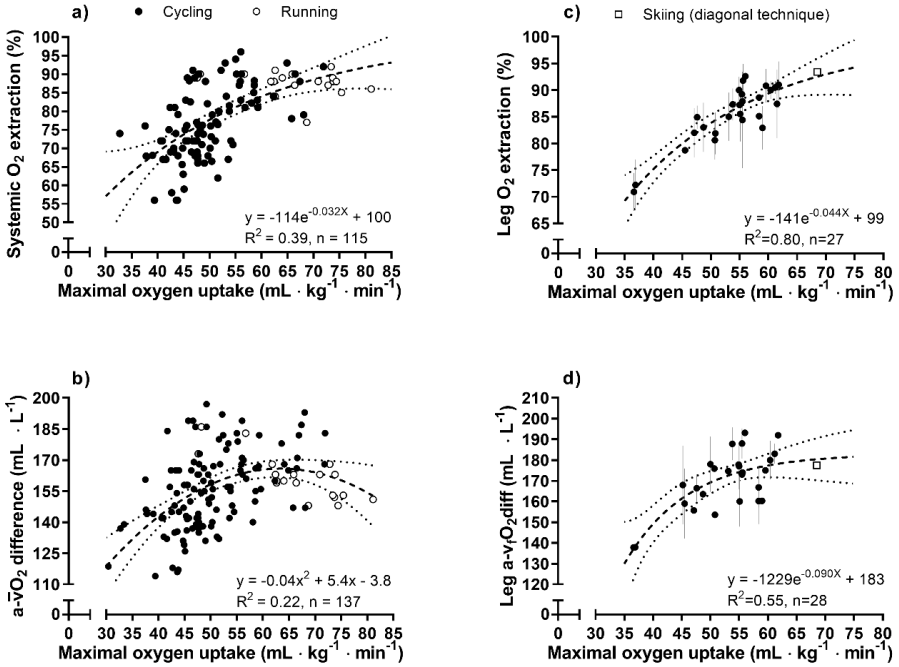
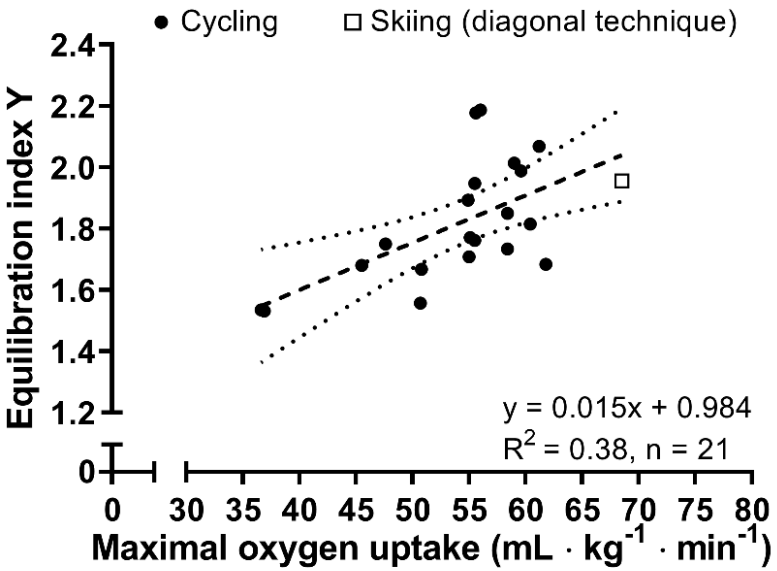


Fig. 7: A comparison of an untrained individual and an elite endurance athlete with maximal oxygen uptakes ($\dot{V}O_{2max}$) of 3.0 and 5.5 L · min^{-1}, respectively. The maximal cardiac output ($\dot{Q}_{max}$), systemic O_2 extraction fraction ($\bar{O}_2$ extraction), arterial to mixed venous O_2 difference (a-$\bar{v}O_2$diff) and arterial O_2 content (CaO_2) were calculated using the regression equations presented in Fig. 1.



Supplements Fig. 1: The relationships between pulmonary maximal oxygen uptake standardised to body weight and systemic oxygen extraction fraction (a), leg oxygen extraction fraction (c), arterial to mixed venous oxygen difference (a-$\bar{v}O_2$diff; c) and arterial to femoral venous oxygen difference (a-$v_fO_2$diff; d). Black and white symbols denote cycling and diagonal skiing, respectively. Data are mean values (± 95% confidence limits, where available) from studies reported in Table 2 (systemic responses) and Table 3 (peripheral responses) of the manuscript.



Supplements Fig. 2: The relationship between pulmonary maximal oxygen uptake standardised to body weight and the equilibration index Y. Black and white symbols denote cycling and diagonal skiing, respectively. Data are from studies reported in Table 3 of the manuscript.

Appendix I

Approval letter from the Ethical Committee study I

Jostein Hallen
Seksjon for fysisk prestasjon

OSLO 28. august 2017

Søknad 13-220817 – Hvordan påvirker kondisjonstrening maksimalt oksygenopptak

Vi viser til søknad, prosjektbeskrivelse, informasjonsskriv og innsendt og godkjent søknad til NSD.

I henhold til retningslinjer for behandling av søknad til etisk komite for idrettsvitenskapelig forskning på mennesker, ble det i komiteens møte av 22. august 2017 konkludert med følgende:

Vedtak

På bakgrunn av forelagte dokumentasjon finner komiteen at prosjektet er forsvarlig og at det kan gjennomføres innenfor rammene av anerkjente etiske forskningsetiske normer nedfelt i NIHs retningslinjer. Til vedtaket har komiteen lagt følgende forutsetning til grunn:

- *At eventuelle vilkår fra NSD følges*
- *At oversikt og registering av biobank skjer etter gjeldende nasjonale retningslinjer*

Avdeling for forskning og bibliotek vil utrede eventuelle nasjonale krav for registrering av forskningsbiobank ved NIH, for deretter å melde tilbake til prosjektleder så snart dette er klart. For øvrig skal materiale i biobank behandles i tråd vilkår fra NSD.

Komiteen gjør oppmerksom på at vedtaket er avgrenset i tråd med fremlagte dokumentasjon. Dersom det gjøres vesentlige endringer i prosjektet som kan ha betydning for deltakernes helse og sikkerhet, skal dette legges fram for komiteen før eventuelle endringer kan iverksettes.

Med vennlig hilsen
Professor Sigmund Loland
Leder, Etisk komite, Norges idrettshøgskole

Appendix II

Approval letter from the Ethical Committee study II

Jostein Hallen
Seksjon for fysisk prestasjonsevne

OSLO 05. september 2019

Søknad 100-290819 – Effekten av blodvolum på maks O2-opptak

Vi viser til søknad, prosjektbeskrivelse, informasjonsskriv, innsendt melding til NSD og innhentet tilleggsinformasjon mottatt i mail datert 29. august 2019.

I henhold til retningslinjer for behandling av søknad til etisk komite for idrettsvitenskapelig forskning på mennesker, ble det i komiteens møte av 29. august 2019 konkludert med følgende:

Vurdering

I prosjektets utvalg er det kun menn som skal inkluderes. På bakgrunn av en noe uklar begrunnelse, ble det innhentet tilleggsinformasjon om dette. Komiteen vil bemerke at det er prosjektleders ansvar å tydelig begrunne sitt valg for utelukkelse av et kjønn i utvalget. Etter å ha mottatt en grundigere redegjørelse for hvorfor bare menn vil bli inkludert, vurderer komiteen prosjektet som etisk forsvarlig. Det presiseres imidlertid at det for fremtidige prosjekter skal legges vekt på å inkludere begge kjønn, og at eventuell avvik fra hovedregelen må begrunnes tydelig og underbygges faglig.

Vedtak

På bakgrunn av forelagte dokumentasjon finner komiteen at prosjektet er forsvarlig og at det kan gjennomføres innenfor rammene av anerkjente etiske forskningsetiske normer nedfelt i NIHs retningslinjer. Til vedtaket har komiteen lagt følgende forutsetning til grunn:

- *Vilkår fra NSD følges*

Komiteen gjør oppmerksom på at vedtaket er avgrenset i tråd med fremlagte dokumentasjon. Dersom det gjøres vesentlige endringer i prosjektet som kan ha betydning for deltakernes helse og sikkerhet, skal dette legges fram for komiteen før eventuelle endringer kan iverksettes.

Med vennlig hilsen
Professor Sigmund Loland
Leder, Etisk komite, Norges idrettshøgskole

NIH NORGES IDRETTSHØGSKOLE

Besøksadresse: Sognsveien 220, Oslo
Postadresse: Pb 4014 Ullevål Stadion, 0806 Oslo
Telefon: +47 23 26 20 00, postmottak@nih.no
www.nih.no

Appendix III

Approval letter from the Ethical Committee study III

Jostein Hallen
Seksjon for fysisk prestasjon

OSLO 08. mai 2017

Søknad 03-020517 – Betydningen av muskulære tilpasninger til utholdenhetstrening for oksygenleveranse og oksygenopptak

Vi viser til søknad, prosjektbeskrivelse, informasjonsskriv og innsendt søknad til NSD.

I henhold til retningslinjer for behandling av søknad til etisk komite for idrettsvitenskapelig forskning på mennesker, ble det i komiteens møte av 2. mai 2017 konkludert med følgende:

Vedtak

På bakgrunn av forelagte dokumentasjon finner komiteen at prosjektet er forsvarlig og at det kan gjennomføres innenfor rammene av anerkjente forskningsetiske normer nedfelt i NIHs retningslinjer.

Til vedtaket har komiteen lagt følgende forutsetning til grunn:

- *At NSD godkjenner prosjektet, og at eventuelle vilkår fra NSD følges*
- *At setningen på side 5 i informasjonsskrivet "Her vil det bli oppbevart muskelvev og blodprøver for senere analyse" enten tas ut eller at "senere" strykes*

Komiteen gjør oppmerksom på at vedtaket er avgrenset i tråd med fremlagte dokumentasjon. Dersom det gjøres vesentlige endringer i prosjektet som kan ha betydning for deltakernes helse og sikkerhet, skal dette legges fram for komiteen før eventuelle endringer kan iverksettes.

Med vennlig hilsen
Professor Sigmund Loland
Leder, Etisk komite, Norges idrettshøgskole

Appendix IV

Approval letter from The Norwegian Centre for Research Data study I

Øyvind Skattebo
Postboks 4014
0806 OSLO

Vår dato: 17.08.2017 Vår ref: 55151 / 3 / AMS Deres dato: Deres ref:

Tilbakemelding på melding om behandling av personopplysninger

Vi viser til melding om behandling av personopplysninger, mottatt 18.07.2017.
Meldingen gjelder prosjektet:

55151	*Hvordan påvirker kondisjonstrening maksimalt oksygenopptak?*
Behandlingsansvarlig	*Norges idrettshøgskole, ved institusjonens øverste leder*
Daglig ansvarlig	*Øyvind Skattebo*
Student	*Marius Auensen*

Personvernombudet har vurdert prosjektet, og finner at behandlingen av personopplysninger vil være regulert av § 7-27 i personopplysningsforskriften. Personvernombudet tilrår at prosjektet gjennomføres.

Personvernombudets tilråding forutsetter at prosjektet gjennomføres i tråd med opplysningene gitt i meldeskjemaet, korrespondanse med ombudet, ombudets kommentarer samt personopplysningsloven og helseregisterloven med forskrifter. Behandlingen av personopplysninger kan settes i gang.

Det gjøres oppmerksom på at det skal gis ny melding dersom behandlingen endres i forhold til de opplysninger som ligger til grunn for personvernombudets vurdering. Endringsmeldinger gis via et eget skjema. Det skal også gis melding etter tre år dersom prosjektet fortsatt pågår. Meldinger skal skje skriftlig til ombudet.

Personvernombudet har lagt ut opplysninger om prosjektet i en offentlig database.

Personvernombudet vil ved prosjektets avslutning, 01.03.2020, rette en henvendelse angående status for behandlingen av personopplysninger.

Dersom noe er uklart ta gjerne kontakt over telefon.

Vennlig hilsen

Dokumentet er elektronisk produsert og godkjent ved NSDs rutiner for elektronisk godkjenning.

Marianne Høgetveit Myhren

Anne-Mette Somby

Kontaktperson: Anne-Mette Somby tlf: 55 58 24 10 / anne-mette.somby@nsd.no
Vedlegg: Prosjektvurdering
Kopi: Marius Auensen, mariusa@student.nih.no

Personvernombudet for forskning

Prosjektvurdering - Kommentar

Prosjektnr: 55151

FORMÅL
Det er stor enighet om at økt kapasitet hos hjertet til å pumpe blod (økt minuttvolum) er den viktigste årsaken til økt maksimalt oksygenopptak som følger utholdenhetstrening. Imidlertid er det stor uenighet tilknyttet betydningen av de muskulære tilpasningene til utholdenhetstrening. I dette prosjektet skal vi undersøke hvorvidt treningsstatusen til muskulaturen er av betydning for det maksimale oksygenopptaket.

ANDRE TILLATELSER
Det vil søkes om godkjenning av prosjektet av Norges idrettshøgskole sin interne etiske komite. Vi legger til grunn at prosjektet godkjennes, og at gjennomføringen skjer i tråd med eventuelle vilkår satt av komiteen.

UTVALG OG REKRUTTERING
Utrente, men skadefrie og friske menn og kvinner i alderen 18-40 år.

Deltakere rekrutteres gjennom informasjonsplakater på ulike høgskoler i Oslo-området, samt oppslag om studien på hjemmesider og Facebook-sider for ulike miljøer som kan tenkes å være interessert i tematikken i denne studien. Interesserte tar selv kontakt med forsker om de ønsker å delta.

INFORMASJON OG SAMTYKKE
Utvalget informeres skriftlig og muntlig om prosjektet og samtykker til deltakelse. Informasjonsskrivet er godt utformet, men dere må legge til at student Marius Aunesen skal bruke data i sitt masterprosjekt og ha tilgang til personopplysninger.

DATAMATERIALET
I denne studien skal deltakerne gjennomføre et 10-uker langt treningsprogram med fysiske tester og andre tester før og etter. Data som samles inn er:
- arbeidsbelastning og hjertefrekvens på treningsøkter
- blodvolum, hemoglobinmasse, hematokrit og hemoglobinkonsentrasjon før og etter trening
- oksygenopptak før og etter trening
- hjertefrekvens på tester før og etter trening
- laktatkonsentrasjon i blod på tester før og etter trening (fingerstikk)
- minuttvolum på tester før og etter trening
- høyde, vekt og alder
- kroppssammensetning før og etter trening
- konsentrasjon av blodårer (kapillærer) og aerobe enzymer i muskulatur før og etter trening. Dette innhentes ved å ta 1stk muskelbiopsi fra m.vastus lateralis (muskel i låret) før og etter trening

Det behandles sensitive personopplysninger om helseforhold.

Personvernombudet legger til grunn at forskerne innehar kompetansen som er nødvendig for å sikre forsvarlig gjennomføring av treningsopplegget og testene, eventuelt at en studielege er tilknyttet prosjektet.

FORSKNINGSBIOBANK
REK sin tilbakemelding om at slike prosjekter faller utenfor helseforskningslovens virkeområde får betydning for forskningsbiobanken som opprettes i prosjektet. Dette er en spesifikk forskningsbiobank tilknyttet inneværende forskningsprosjekt. I biobanken oppbevares muskelvev og blodprøver for analyser. Prøvene lagres i fem år og oppbevares avidentifisert. Ansvarshavende for forskningsbiobanken er stipendiatens veileder ved Norges idrettshøgskole, Jostein Hallén.

Ettersom REK ikke tar stilling til opprettelse av biobanker som faller utenfor helseforskningslovens virkeområde, må biobanken vurderes etter personopplysningsloven, jf. tilbakemelding fra NEM i 2014 (vår ref. 41030).

Personvernombudet har vurdert at prosjektet med den tilhørende forskningsbiobanken ikke krever konsesjon fra Datatilsynet da den kan opprettes med hjemmel i personopplysningsforskriften§ 7-27. Følgende ligger til grunn for vår vurdering:
Opplysningene som samles inn skal benyttes til et spesifikt forskningsprosjekt, all behandlingen er basert på samtykke, omfanget av opplysninger lite, og prosjektet er kortvarig.

Prosjektet og opprettelsen av den forskningspesifikke biobanken kan derfor gjennomføres med hjemmel i personopplysningsforskriften § 7-27.

VIKTIG: Vi forstår det slik at alle biobanker skal registreres i Biobankregisteret ved Folkehelseinstituttet. Norges idrettshøgskole må selv sørge for at biobanken meldes til Biobankregisteret.

INFORMASJONSSIKKERHET
Personvernombudet legger til grunn at forskerne etterfølger Norges idrettshøgskole sine interne rutiner for datasikkerhet.

For å sikre at forskningsdata ikke kommer på avveie eller blir utsatt for bevisst eller ubevisst ødeleggelse, er fysisk sikring av data og biologisk materiale viktig. Blodprøver og annet materiale må oppbevares innelåst i rom, frysebokser, eller låste skap med begrenset tilgang. Blodprøver og andre biologiske prøver må merkes slik at prøven kan spores tilbake til rett person, men det er mulig å gjøre tilbakesporing for uvedkommende vanskelig ved å unngå navn, og heller bruke strekkode eller løpenummer. Prøvene kan siden kobles til rett person ved hjelp av navneliste som oppbevares på et annet sted (https://www.etikkom.no/FBIB/Temaer/Forskning-pa-menneskelig-materiale/Forskningsbiobanker/#_Toc225585706).

I prosjektperioden lagres avidentifisert datamateriale på en passordbeskyttet datamaskin med kryptert harddisk.

Etter prosjektperioden overføres datamaterialet til en sikker intern server der det oppbevares i 5 år etter at dataene ble samlet inn. Koblingsnøkkel oppbevares adskilt fra datamaterialet. Kun Jostein Hallén, stipendiat Øyvind Skattebo og student Marius Auensen har tilgang til koblingsnøkkelen.

PROSJEKTSLUTT

Forventet prosjektslutt er 01.03.2020. Ifølge prosjektmeldingen skal innsamlede opplysninger inkludert biologisk materiale oppbevares med personidentifikasjon frem til 01.03.2025 for etterprøvbarhet/kontroll, i tråd med retningslinjer vedtatt av Styret ved Norges idrettshøgskole. Deretter skal datamaterialet anonymiseres. Dette innebærer at direkte personopplysninger (navn/koblingsnøkkel) slettes, indirekte personopplysninger (identifiserende sammenstilling av bakgrunnsopplysninger som f.eks. bosted/arbeidssted, alder og kjønn) omskrives/fjernes. Videre skal prøver/biologisk materiale i forskningsbiobanken slettes.

Appendix V

Approval letter from The Norwegian Centre for Research Data study II

NSD NORSK SENTER FOR FORSKNINGSDATA

NSD sin vurdering

Prosjekttittel

Effekten av blodvolum på maksimalt oksygenopptak

Referansenummer

418763

Registrert

01.07.2019 av Øyvind Skattebo - oyvind.skattebo@nih.no

Behandlingsansvarlig institusjon

Norges idrettshøgskole / Seksjon for fysisk prestasjonsevne

Prosjektansvarlig (vitenskapelig ansatt/veileder eller stipendiat)

Øyvind Skattebo, oyvind.skattebo@nih.no, tlf: 97105742

Type prosjekt

Forskerprosjekt

Prosjektperiode

02.09.2019 - 01.06.2020

Status

15.07.2019 - Vurdert

Vurdering (1)

15.07.2019 - Vurdert

Det er vår vurdering at behandlingen av personopplysninger i prosjektet vil være i samsvar med personvernlovgivningen så fremt den gjennomføres i tråd med det som er dokumentert i meldeskjemaet 15.07.2019 med vedlegg, samt i meldingsdialogen mellom innmelder og NSD. Behandlingen kan starte.

MELD VESENTLIGE ENDRINGER
Dersom det skjer vesentlige endringer i behandlingen av personopplysninger, kan det være nødvendig å melde dette til NSD ved å oppdatere meldeskjemaet. Før du melder inn en endring, oppfordrer vi deg til å lese om hvilke type endringer det er nødvendig å melde: https://nsd.no/personvernombud/meld_prosjekt/meld_endringer.html
Du må vente på svar fra NSD før endringen gjennomføres.

TYPE OPPLYSNINGER OG VARIGHET
Prosjektet vil behandle særlige kategorier av personopplysninger om helse og alminnelige kategorier av personopplysninger frem til 01.062020. Forskningsdataene oppbevares i 5 år etter prosjektslutt for etterprøvbarhet og kontroll.

LOVLIG GRUNNLAG
Prosjektet vil innhente samtykke fra de registrerte til behandlingen av personopplysninger. Vår vurdering er at prosjektet legger opp til et samtykke i samsvar med kravene i art. 4 nr. 11 og art. 7, ved at det er en frivillig, spesifikk, informert og utvetydig bekreftelse, som kan dokumenteres, og som den registrerte kan trekke tilbake.

Lovlig grunnlag for behandlingen vil dermed være den registrertes uttrykkelige samtykke, jf. personvernforordningen art. 6 nr. 1 a), jf. art. 9 nr. 2 bokstav a, jf. personopplysningsloven § 10, jf. § 9 (2).

PERSONVERNPRINSIPPER
NSD vurderer at den planlagte behandlingen av personopplysninger vil følge prinsippene i personvernforordningen om:

- lovlighet, rettferdighet og åpenhet (art. 5.1 a), ved at de registrerte får tilfredsstillende informasjon om og samtykker til behandlingen
- formålsbegrensning (art. 5.1 b), ved at personopplysninger samles inn for spesifikke, uttrykkelig angitte og berettigede formål, og ikke viderebehandles til nye uforenlige formål
- dataminimering (art. 5.1 c), ved at det kun behandles opplysninger som er adekvate, relevante og nødvendige for formålet med prosjektet
- lagringsbegrensning (art. 5.1 e), ved at personopplysningene ikke lagres lengre enn nødvendig for å oppfylle formålet

DE REGISTRERTES RETTIGHETER
Så lenge de registrerte kan identifiseres i datamaterialet vil de ha følgende rettigheter: åpenhet (art. 12), informasjon (art. 13), innsyn (art. 15), retting (art. 16), sletting (art. 17), begrensning (art. 18), underretning (art. 19), dataportabilitet (art. 20).

NSD vurderer at informasjonen som de registrerte vil motta oppfyller lovens krav til form og innhold, jf. art. 12.1 og art. 13.

Vi minner om at hvis en registrert tar kontakt om sine rettigheter, har behandlingsansvarlig institusjon plikt til å svare innen en måned.

FØLG DIN INSTITUSJONS RETNINGSLINJER
NSD legger til grunn at behandlingen oppfyller kravene i personvernforordningen om riktighet (art. 5.1 d), integritet og konfidensialitet (art. 5.1. f) og sikkerhet (art. 32).

For å forsikre dere om at kravene oppfylles, må dere følge interne retningslinjer og eventuelt rådføre dere med behandlingsansvarlig institusjon.

OPPFØLGING AV PROSJEKTET
NSD vil følge opp underveis (hvert annet år) og ved planlagt avslutning for å avklare om behandlingen av personopplysningene er avsluttet/ pågår i tråd med den behandlingen som er dokumentert.

Lykke til med prosjektet!

Kontaktperson hos NSD: Kajsa Amundsen
Tlf. Personverntjenester: 55 58 21 17 (tast 1)

Appendix VI

Approval letter from The Norwegian Centre for Research Data study III

Øyvind Skattebo
Seksjon for fysisk prestasjonsevne Norges idrettshøgskole
Postboks 4014
0806 OSLO

Vår dato: 02.05.2017 Vår ref: 53552 / 3 / STM Deres dato: Deres ref:

TILBAKEMELDING PÅ MELDING OM BEHANDLING AV PERSONOPPLYSNINGER

Vi viser til melding om behandling av personopplysninger, mottatt 10.03.2017. Meldingen gjelder prosjektet:

53552	*Betydningen av muskulære tilpasninger til utholdenhetstrening for oksygenleveranse og oksygenopptak*
Behandlingsansvarlig	*Norges idrettshøgskole, ved institusjonens øverste leder*
Daglig ansvarlig	*Øyvind Skattebo*

Personvernombudet har vurdert prosjektet, og finner at behandlingen av personopplysninger vil være regulert av § 7-27 i personopplysningsforskriften. Personvernombudet tilrår at prosjektet gjennomføres.

Personvernombudets tilråding forutsetter at prosjektet gjennomføres i tråd med opplysningene gitt i meldeskjemaet, korrespondanse med ombudet, ombudets kommentarer samt personopplysningsloven og helseregisterloven med forskrifter. Behandlingen av personopplysninger kan settes i gang.

Det gjøres oppmerksom på at det skal gis ny melding dersom behandlingen endres i forhold til de opplysninger som ligger til grunn for personvernombudets vurdering. Endringsmeldinger gis via et eget skjema, http://www.nsd.uib.no/personvernombud/meld_prosjekt/meld_endringer.html. Det skal også gis melding etter tre år dersom prosjektet fortsatt pågår. Meldinger skal skje skriftlig til ombudet.

Personvernombudet har lagt ut opplysninger om prosjektet i en offentlig database, http://pvo.nsd.no/prosjekt.

Personvernombudet vil ved prosjektets avslutning, 01.03.2020, rette en henvendelse angående status for behandlingen av personopplysninger.

Vennlig hilsen

Katrine Utaaker Segadal

Siri Tenden Myklebust

Kontaktperson: Siri Tenden Myklebust tlf: 55 58 22 68
Vedlegg: Prosjektvurdering

Dokumentet er elektronisk produsert og godkjent ved NSDs rutiner for elektronisk godkjenning.

Personvernombudet for forskning

Prosjektvurdering - Kommentar

Prosjektnr: 53552

FORMÅL
«Det er stor enighet om at økt minuttvolum er den viktigste årsaken til økt maksimalt oksygenopptak som følger utholdenhetstrening. Imidlertid er det stor uenighet tilknyttet betydningen av de muskulære tilpasningene. I dette prosjektet skal vi undersøke hvorvidt treningsstatusen til muskulaturen er av betydning for oksygenleveranse, oksygenekstraksjon og oksygenopptak lokalt i muskulaturen.»

REK har vurdert at prosjektet faller utenfor helseforskningslovens virkeområde (2017/171 REK sør-øst B).

ANDRE TILLATELSER
Det vil søkes om godkjenning av prosjektet av Norges idrettshøgskole sin interne etiske komite. Vi legger til grunn at prosjektet godkjennes, og at gjennomføringen skjer i tråd med eventuelle vilkår satt av komiteen.

UTVALG OG REKRUTTERING
Utvalget består av skadefrie, friske og moderat til godt trente menn i alderen 18-40 år, anslagsvis 12 personer.

Deltakere rekrutteres gjennom informasjonsplakater på ulike høgskoler i Oslo-området, samt oppslag om studien på hjemmesider og Facebook-sider for ulike miljøer som kan tenkes å være interessert i tematikken i denne studien. Interesserte tar selv kontakt med forsker om de ønsker å delta.

INFORMASJON OG SAMTYKKE
Utvalget informeres skriftlig og muntlig om prosjektet og samtykker til deltakelse. Informasjonsskrivet er godt utformet.

DATAMATERIALET
I denne studien skal deltakerne gjennomføre et 6 uker langt treningsprogram med fysiske tester før og etter. Data som samles inn er:
- arbeidsbelastning (effektutvikling målt på ergometeret), oksygenopptak (indirekte kalorimetri), hjertefrekvens (pulsklokke) og blodlaktat (fingerstikk og laktatanalysator) registreres ved submaksimale og maksimale belastninger på sykkel og ved dynamiske kneekstensjoner.
- høyde, vekt, alder og kroppssammensetning (dual-energy x-ray absorptiometry)
- blodstrøm i begge bein målt med ultralyd Doppler teknikk.
- blodgasser, hematokrit og hemoglobinkonsentrasjon målt med blodgassapparat
- konsentrasjon av aerobe enzymer og kapillærertetthet i muskelvev analysert fra en muskelbiopsi fra m. vastus lateralis (muskel i låret)

Det behandles sensitive personopplysninger om helseforhold.

Personvernombudet legger til grunn at forskerne innehar kompetansen som er nødvendig for å sikre forsvarlig gjennomføring av treningsopplegget og testene, eventuelt at en studielege er tilknyttet prosjektet.

FORSKNINGSBIOBANK
REK sin tilbakemelding om at prosjektet faller utenfor helseforskningslovens virkeområde får betydning for forskningsbiobanken som opprettes i prosjektet. Dette er en spesifikk forskningsbiobank tilknyttet inneværende forskningsprosjekt. I biobanken oppbevares muskelvev og blodprøver for analyser. Prøvene lagres i fem år og oppbevares avidentifisert. Ansvarshavende for forskningsbiobankener stipendiatens veileder ved Norges idrettshøgskole, Jostein Hallén.

Ettersom REK ikke tar stilling til opprettelse av biobanker som faller utenfor helseforskningslovens virkeområde, må biobanken vurderes etter personopplysningsloven, jf. tilbakemelding fra NEM i 2014 (vår ref. 41030).

Personvernombudet har vurdert at prosjektet med den tilhørende forskningsbiobanken ikke krever konsesjon fra Datatilsynet da den kan opprettes med hjemmel i personopplysningsforskriften§ 7-27. Følgende ligger til grunn for vår vurdering:
Opplysningene som samles inn skal benyttes til et spesifikt forskningsprosjekt, all behandlingen er basert på samtykke, omfanget av opplysninger lite, og prosjektet er kortvarig.

Prosjektet og opprettelsen av den forskningspesifikke biobanken kan derfor gjennomføres med hjemmel i personopplysningsforskriften § 7-27.

VIKTIG: Vi forstår det slik at alle biobanker skal registreres i Biobankregisteret ved Folkehelseinstituttet. Norges idrettshøgskole må selv sørge for at biobanken meldes til Biobankregisteret.

INFORMASJONSSIKKERHET
Personvernombudet legger til grunn at forskerne etterfølger Norges idrettshøgskole sine interne rutiner for datasikkerhet.

For å sikre at forskningsdata ikke kommer på avveie eller blir utsatt for bevisst eller ubevisst ødeleggelse, er fysisk sikring av data og biologisk materiale viktig. Blodprøver og annet materiale må oppbevares innelåst i rom, frysebokser, eller låste skap med begrenset tilgang. Blodprøver og andre biologiske prøver må merkes slik at prøven kan spores tilbake til rett person, men det er mulig å gjøre tilbakesporing for uvedkommende vanskelig ved å unngå navn, og heller bruke strekkode eller løpenummer. Prøvene kan siden kobles til rett person ved hjelp av navneliste som oppbevares på et annet sted (https://www.etikkom.no/FBIB/Temaer/Forskning-pa-menneskelig-materiale/Forskningsbiobanker/#_Toc225585706).

I prosjektperioden lagres avidentifisert datamateriale på en passordbeskyttet datamaskin med kryptert harddisk. Etter prosjektperioden overføres datamaterialet til en sikker intern server der det oppbevares i 5 år etter at dataene ble samlet inn (2022). Koblingsnøkkel oppbevares adskilt fra datamaterialet. Kun Jostein Hallén og stipendiat Øyvind Skattebo har tilgang til koblingsnøkkelen.

PROSJEKTSLUTT

Forventet prosjektslutt er 01.03.2020. Ifølge prosjektmeldingen skal innsamlede opplysninger inkludert biologisk materiale oppbevares med personidentifikasjon frem til 30.06.2022 for etterprøvbarhet/kontroll, i tråd med retningslinjer vedtatt av Styret ved Norges idrettshøgskole. Deretter skal datamaterialet anonymiseres. Dette innebærer at direkte personopplysninger (navn/koblingsnøkkel) slettes, indirekte personopplysninger (identifiserende sammenstilling av bakgrunnsopplysninger som f.eks. bosted/arbeidssted, alder og kjønn) omskrives/fjernes. Videre skal prøver/biologisk materiale i forskningsbiobanken slettes.