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The Role and Regulation of Hemoglobin Mass in Endurance Exercise

by

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Abstract

Endurance performance relies on sustained oxygen delivery to skeletal muscles. Consequently, the maximum capacity to deliver oxygen is closely related to the total amount of hemoglobin (Hb) in the circulation, hemoglobin mass (Hb_{mass}). Experimental reduction of Hb_{mass} results in lowered oxygen delivery in maximal exercise and hence a lower maximal oxygen uptake ($\dot{V}O_2max$). At submaximal intensities, compensatory adjustments can be employed in response to Hb_{mass} reduction to maintain oxygen delivery, potentially lessening the impact on intensities below $\dot{V}O_2max$; however, data relating to these effects are lacking. While females have a lower [Hb] and lower Hb_{mass} than males, the influence of sex on hematological properties has not been thoroughly investigated. In addition, the regulation of Hb turnover in endurance training is poorly understood, owing to methodological limitations. The purpose of this thesis was to expand on the current understanding about the role and regulation of Hb_{mass} in facilitating endurance exercise. In Study 1 (n=16), reducing effective [Hb] by ~5% using low-dose carbon monoxide inhalation destabilized the maximal lactate steady state. This effect was more prominent in trained individuals, suggesting higher sensitivity to impairments in oxygen delivery. In Study 2 (n=17), Hb_{mass} was reduced by withdrawal of 7% of blood volume through phlebotomy. In a 60-min exercise bout, ventilation, lactate, and heart rate were increased in a time-dependent manner, and time-to-task failure in a subsequent maximal task was reduced. Study 3 explored sex-differences in a pooled analysis (n=79) of four datasets. This cross-sectional study revealed that, due to sex differences in hematological variables, females must achieve a similar fat-free mass-normalized $\dot{V}O_2max$ with a lower Hb_{mass} than males. Study 4 (n=5) piloted the use of deuterated water as a metabolic label to quantify the fractional synthetic rate (FSR) of Hb *in vivo*. The preliminary data showed that this 28-day protocol was feasible and yielded expected FSR values ($<1\%\cdot day^{-1}$). Future research should explore the mechanisms behind impaired submaximal exercise performance after reduced oxygen delivery; how males and females employ specific strategies to reach a given oxygen uptake; and how Hb synthesis is influenced by sex and regulated in response to exercise training.

Preface

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Chapter III of this thesis has been published as H. Kontro et al. "Impairment in maximal lactate steady state after carbon monoxide inhalation is related to training status" Experimental Physiology, vol. 107, issue 11:1265-1282.

The remaining sections of this thesis are original, unpublished work by the author, Hilkka Kontro.

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List of Abbreviations

%COHb	Percent carboxyhemoglobin
^2H	Deuterium
^2H_2O	Heavy water
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
APD	Atom percent deuterium
APE	Atom percent excess
BV	Blood volume
BM	Body mass
CaO_2	Arterial oxygen content
CO	Carbon monoxide
CO_2	Carbon dioxide
CP	Critical power
CvO_2	Venous oxygen content
DXA	Dual-energy x-ray absorptiometry
f_B	Frequency of breathing
FFM	Fat-free mass
F_IO_2	Fraction of inspired oxygen
FS	Feeling scale
GET	Gas exchange threshold
Hb	Hemoglobin
Hb_{mass}	Total hemoglobin mass
Hct	Hematocrit
HR	Heart rate
ITT	Interpolated twitch technique
[La]	Concentration of blood lactate
LFF	Low-frequency fatigue
MLSS	Maximal lactate steady state
MMSS	Maximal metabolic steady state
MVC	Maximal voluntary contraction
NMF	Neuromuscular fatigue
PO_2	Partial pressure of oxygen
PPO	Peak power output
PT	Potentiated twitch
PV	Plasma volume
RBCV	Red blood cell volume
RCP	Respiratory compensation point
RPE	Rating of perceived exertion
RoF	Rating of fatigue
SaO_2	Arterial oxygen saturation
SpO_2	Peripheral oxygen saturation
SD	Standard deviation

SV	Stroke volume
VA	Voluntary activation
$\dot{V}CO_2$	Carbon dioxide production
$\dot{V}_E$	Ventilation
$\dot{V}_E/\dot{V}O_2$	Ventilatory equivalent of oxygen uptake
$\dot{V}O_2$	Rate of oxygen uptake
$\dot{V}O_2max$	Maximal rate of oxygen uptake
$\dot{V}O_2peak$	Peak rate of oxygen uptake
V_T	Tidal volume
$\dot{Q}$	Cardiac output
Δ	Change

Chapter I: Introduction

1.1 Background

Like all organisms, animals harvest chemical energy residing in molecular bonds to power their metabolism essential to their immediate and long-term survival. One of the most conspicuous qualities of animals is their bodily movements achieved by coordinated muscle contractions. Sustained muscle contractions, like most other ongoing energy-intensive processes, rely on aerobic metabolism. Only contractions of relatively short durations are possible with limited oxygen availability. Hence it is unsurprising that animals have evolved robust and reactive biological systems to ensure oxygen supply to tissues in changing conditions.

In vertebrates, including humans, oxygen is transported by blood in a closed circulation system from lungs to tissues where it enables ATP regeneration in the mitochondria by oxidative phosphorylation. The solubility of oxygen in plasma is low and therefore most of the oxygen in blood travels bound to the iron-containing heme of the hemoglobin protein (Hb). During intense exercise involving large muscle groups, the oxygen demand by skeletal muscles becomes greater than all other tissues combined, elevating the whole-body metabolic rate up to 20-fold relative to a resting state (Bassett & Howley, 2000). With increasing intensity, muscle oxygen supply eventually reaches a ceiling and cannot be increased further (Calbet et al., 2004). The upper limit for whole-body oxygen delivery in maximal exercise is set by maximal cardiac output ($\dot{Q}_{max}$) and the concentration of Hb ([Hb]). These two parameters combine to describe an individual's overall capacity to deliver oxygen, which, through mechanism discussed in later sections, is associated with total amount of hemoglobin protein in the circulation (i.e., hemoglobin mass (Hb_{mass})).

As a product of [Hb] and total blood volume (BV), Hb_{mass} is closely correlated with BV. BV is a dynamic component of the cardiovascular system in healthy humans. To maintain homeostasis at rest and in physical exercise, BV is under constant regulation and fluctuates both short-term and long-term in response to internal and external stimuli (Sawka et al., 2000). Day-to-day changes in BV normally do not affect Hb_{mass}, but changes in BV sustained for weeks or months are typically accompanied by parallel changes in Hb_{mass} (Convertino, 1991; Schmidt et al., 1988). As an example of this response, commencing a demanding endurance training regimen leads to an increase in both BV (in days due to plasma volume [PV] expansion) and Hb_{mass} (in weeks) in previously untrained individuals (Montero et al., 2017; Schmidt & Prommer, 2010). A high BV is required to achieve a high $\dot{Q}_{max}$, which in turn predicts whole-body maximal oxygen uptake ($\dot{V}O_2max$) across different populations (Lundby et al., 2017; Oberholzer et al., 2023).

Several potential bottlenecks to oxygen consumption exist (Bassett & Howley, 2000). Whether oxygen delivery, rather than muscles' capacity to use oxygen, limits $\dot{V}O_2max$ has been investigated by manipulating BV and [Hb] prior to maximal exercise (Calbet et al., 2006). These experiments have demonstrated that oxygen supply limits $\dot{V}O_2max$ more often than oxygen extraction, particularly in well-conditioned individuals. While every step of the oxygen transport and utilization cascade affects $\dot{V}O_2$ (Wagner, 1996), apparently a 'central' delivery limitation rather than a 'peripheral' uptake limitation to maximum whole-body exercise capacity exists in endurance-trained humans (Saltin & Calbet, 2006).

While reaching $\dot{Q}_{max}$ elicits $\dot{V}O_2max$, most endurance training and race efforts take place at lower intensities, and these intensities are relevant to overall athletic performance. Intensities associated with the attainment of $\dot{V}O_2max$ are not sustainable beyond ~15 minutes in healthy people (Hill et al., 1997; Poole et al., 2016). Instead, a threshold describing the intensity at which a metabolic steady state is still achievable may be better predictive of prolonged exercise performance. This threshold is often referred to as the maximal metabolic steady state (MMSS) and can be estimated by determining the maximal lactate steady state (MLSS) or critical power (CP) (Jones et al., 2019; Poole et al., 2016). Above this boundary, aerobic energy production is unable to fully meet the ATP demand and the contracting muscles' finite, immediate energy stores are depleted until exercise must be terminated or the intensity reduced to MMSS or lower.

On the other hand, reductions in the maximal capacity to deliver oxygen also affect responses during submaximal exercise when $\dot{Q}_{max}$ has not yet been reached. To some degree, adjustments can be made to keep oxygen delivery sufficient for continuous exercise despite compromised stroke volume or arterial oxygen content (CaO_2) (Celsing et al., 1986; Gonzalez-Alonso et al., 2001). However, compensatory mechanisms will become less feasible with increasing intensity when the cardiovascular system is approaching its maximum capacity and critical organs need to remain perfused (Calbet et al., 2004). These adjustments to maintain homeostasis place additional strain on the cardiorespiratory system which may affect oxygen delivery to the limbs and thereby alter muscle metabolism, accelerating fatigue development (Sheel et al., 2018).

Despite the evidence for oxygen delivery limiting supra-threshold exercise capacity, the effects of altered maximal oxygen delivery on the sustainability of submaximal intensities are not well understood. Thresholds are often purported to depend on peripheral qualities, like mitochondrial density and capillarization, rather than central factors (Gollnick & Saltin, 1982; Ivy et al., 1980). However, if $\dot{V}O_2max$ is reduced to a large enough extent, it is obvious that MMSS will be compromised, too. For example, if the individual MMSS occurs at 80% of $\dot{V}O_2max$ and $\dot{V}O_2max$ is reduced by >20%, MMSS would have to occur at >100% of the new $\dot{V}O_2max$ if it remained unchanged. Whether minor decreases (e.g., 5%) in $\dot{V}O_2max$ following the manipulation of blood are sufficient to affect the MMSS intensity has not been demonstrated. Additionally, it is unclear whether individual characteristics, such as training status or sex, affect the extent to which endurance performance relies on oxygen delivery. Understanding the effects of altered capacity to deliver oxygen on the achievement of MMSS is important to help pinpoint the physiological determinants of this threshold, the existence of which has been well characterized in numerous populations while its limiters remain incompletely explained. Even intensities below the MMSS may be impacted by changes in hematological parameters. While compensation for reduced SV or CaO_2 is possible by increasing blood flow, either by elevating heart rate or by vasodilation in the locomotor muscles and blood flow redistribution, whether these adjustments affect the sustainability of prolonged submaximal exercise by accelerated fatigue development is poorly understood.

Well-established physiological sex-differences exist, affecting hematological variables and $\dot{V}O_2max$ (Joyner, 2017; Murphy, 2014). However, the literature provides conflicting conclusions for the existence and magnitude of differences in Hb_{mass} and BV, which may be a result of improper matching (Cureton, 1981) of aerobic fitness between the male and female participants in the studies. Since both aerobic fitness and sex may affect these variables (Falz et al., 2019; Stevenson et al., 1994), it is paramount that appropriate matching is performed to isolate the effect of sex.

Finally, while endurance training is associated with higher Hb_{mass} and BV, the regulation of erythropoiesis (the maturation of new red blood cells) in athletes is not well understood.

Although methods exist to measure Hb synthesis rates, they have not been used to study potential exercise-induced changes in erythropoiesis, possibly due to the cost and risks associated with using isotope-labelled and/or radioactive substances (Garby et al., 1962; Hibbert et al., 2001). A better understanding could be achieved with more practical and safe methods that could quantify the rate of erythropoiesis. One such method could be metabolic labelling of Hb during its maturation process. Using orally administered deuterated water, as has been successfully done to measure synthetic rates of other proteins, is a novel low-risk technique with potential for use in studies of erythropoiesis *in vivo*.

1.2 Thesis outline

This thesis is comprised by 6 chapters (including the present introductory chapter).

- Chapter II includes a review of literature on the topic and highlights some gaps in the scientific literature that are addressed in this current thesis.
- Chapter III examines the metabolic and cardiorespiratory consequences of exercising at the MLSS after inhalation of carbon monoxide to reduce the available [Hb].
- Chapter IV investigates the metabolic and cardiorespiratory consequences of prolonged exercise after a blood donation equal to 7% of blood volume.
- Chapter V reports sex differences in hemoglobin mass and vascular volumes in males and females of similar aerobic capacity normalized to fat-free mass.
- Chapter VI describes a novel stable isotope method to quantify the rate of hemoglobin synthesis in humans.
- Chapter VII: Discussion and conclusion

Chapter II: Review of the literature

2.1 Role of oxygen delivery in endurance exercise

For sustained human locomotion, oxygen supply to contracting skeletal muscles must be continuous and equal to or greater than its demand. Oxygen supply depends largely on the cardiovascular system, which, through the pumping action of the heart, circulates oxygenated blood from the lungs to the tissues (Pittman, 2011a). The energy released from the stepwise oxidation of substrates is harvested in the mitochondria through the synthesis of ATP, the energy currency of the cell, needed for the cross-bridge cycling that shortens muscle sarcomeres and produces forces (Lai et al., 2008). In the absence of sufficient oxygen, synthesis of ATP through non-oxidative pathways is required, but the capacities of these energy systems are severely limited in comparison to the aerobic system, and their use has consequences for muscle function. Perpetual production of ATP, albeit at lower rates, is achieved by the mitochondrial machinery given a continuous availability of electrons to the electron transport system (ETS), provided by nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$). These electron carriers are reduced in the citric acid cycle via oxidation of substrates, derived mainly from the breakdown of carbohydrates and lipids, and release their electrons to the ETS, where molecular oxygen is the final electron acceptor, forming water. The potential energy of a proton gradient between the mitochondrial matrix and the intermembrane space, generated through energy released by electron transfers, powers the enzyme complex, ATP synthase, which produces ATP from ADP and inorganic phosphate (Jonckheere et al., 2012). As the continuous operation of the ETS relies on oxygen to accept electrons, the rate at which blood can deliver oxygen to contracting skeletal muscles is vital for endurance performance.

One of the primary functions of blood is oxygen delivery. Blood is composed of red and white blood cells and a water-based component (plasma), in which proteins, lipids, and small soluble molecules are carried to and from tissues. The volume percentage of blood containing red blood cells (RBC) is termed hematocrit (Hct) and is typically 40-50%. Less than 1% of blood consists of white blood cells and platelets, and the remaining fraction is plasma. The components of blood are depicted in **Figure 2.1**.

The main function of RBCs is to transport oxygen. They achieve this task by their high (>96% of all protein) hemoglobin (Hb) content, a protein with an iron-containing functional domain that has high affinity for molecular oxygen in the chemical environment of the alveoli but lower affinity in the peripheral tissues (Barcroft & Uyeno, 1923; Shepherd et al., 2019). The [Hb] of blood reflects blood's oxygen carrying capacity and is expressed as g/dL or g/L of whole blood. The total mass of Hb in circulation (Hb_{mass}) is given in grams, and it is an integrative parameter of BV and [Hb]. Changes in Hb_{mass} relate to changes in $\dot{V}O_2max$, such that a 1 g increase in Hb_{mass} is associated with a ~4 mL/min increase in $\dot{V}O_2max$ (Schmidt & Prommer, 2010).

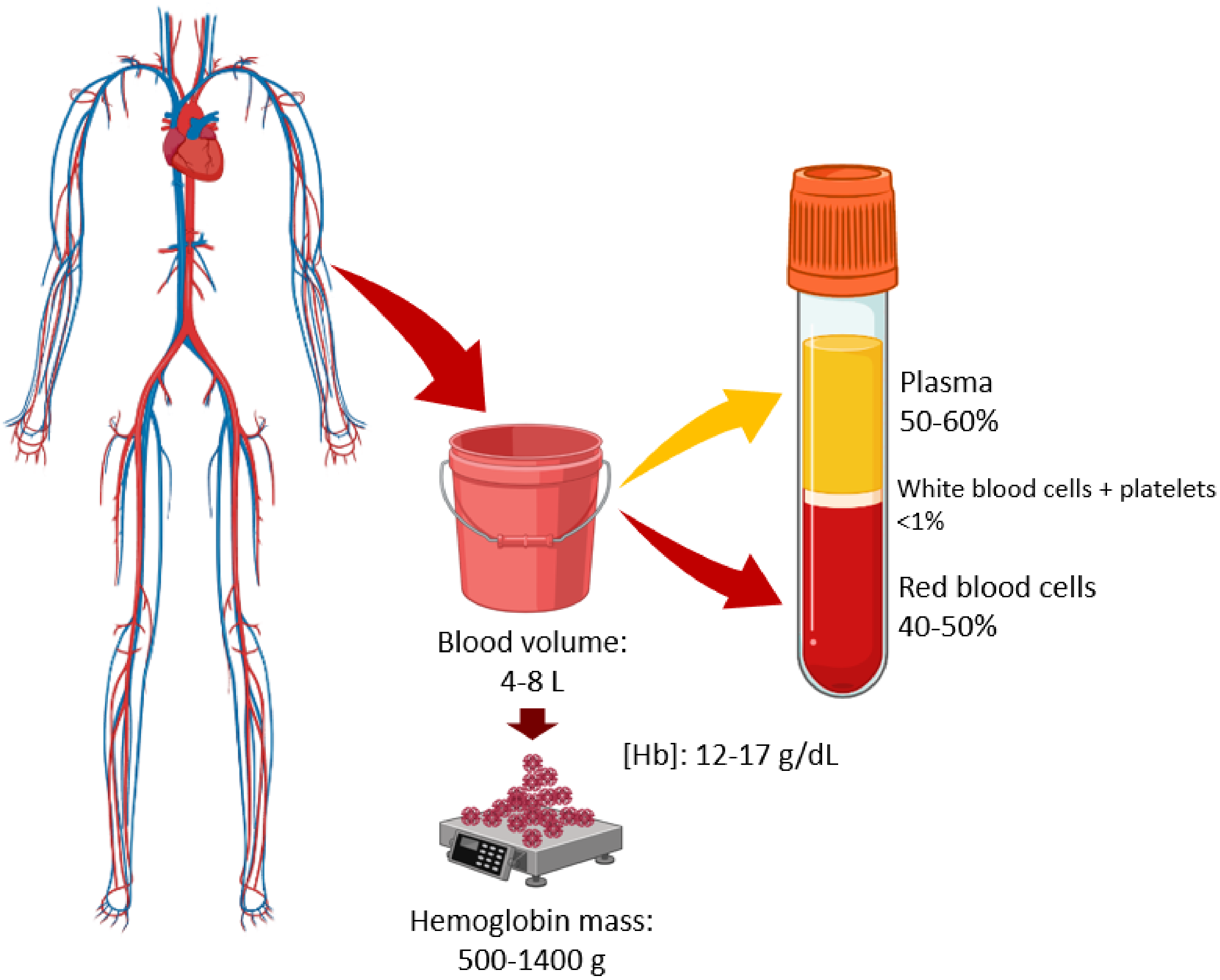

Figure 2.1. The human circulatory system with approximate ranges for values typically measured in healthy adults for blood volume, hemoglobin mass, and the fractions of plasma and red blood cells. Values vary with factors including body size, sex, and fitness status. Created with BioRender.com

Oxygen delivery at a given moment is a function of both arterial oxygen content (CaO_2) of the blood and its flow rate (Pittman, 2011b). The most important determinant of CaO_2 is [Hb], but it also depends on factors affecting its saturation level (SaO_2; % of Hb binding sites bound to oxygen), including the partial pressure of inspired oxygen, the effectiveness of ventilation, and oxygen affinity of Hb. A small amount of oxygen (~2%) is unbound to Hb and its dissolved concentration is determined by the partial pressure of oxygen in arterial blood (PaO_2). The maximum level of CaO_2 achievable when Hb is fully saturated is referred to as the oxygen carrying capacity. CaO_2 is expressed in volume of oxygen per given volume of blood and is approximately 20 mL oxygen per 100 mL in healthy individuals at sea level at rest (or 200 mL per 1 L). The maximal rate of whole-body oxygen delivery is thus limited by i) CaO_2 and ii) cardiac output ($\dot{Q}$). Adjustments in $\dot{Q}$ appear to follow oxygen demand closely and to be sensitive to changes in [Hb], increasing when [Hb] decreases and vice versa (Ferretti et al., 1992; Freedson, 1981).

While oxygen delivery sets a ceiling for oxygen uptake in tissues, not all of the oxygen delivered can be extracted: oxygen uptake ($\dot{V}O_2$) is determined by both its delivery and extraction. The Fick equation of $\dot{V}O_2$ describes this as:

$$\dot{V}O_2 = \dot{Q} \times (CaO_2 - CvO_2) \qquad \text{(Eq. 2.1)}$$

In Equation 1, the two factors affecting oxygen delivery ($\dot{Q}$ and CaO_2) can also be separated further:

$$\dot{V}O_2 = (HR \times SV) \times ([Hb] \times SaO_2 \times 1.34 + (PaO_2 \times 0.003) - CvO_2) \qquad \text{(Eq. 2.2)}$$

where HR is heart rate, SV is stroke volume and CvO_2 is mixed venous oxygen content (which may also be expanded like CaO_2). 1.34 is the volume (mL) of oxygen bound by 1 g of Hb, PaO_2 is the partial pressure of dissolved O_2, and 0.003 is the solubility coefficient of oxygen in plasma (in units of $ml \cdot dL^{-1} \cdot mmHg^{-1}$). While all the factors presented in equation (2.2) may affect the maximal rate of oxygen uptake ($\dot{V}O_2max$), only two of them can be markedly improved with endurance training: SV and CvO_2 (Blomqvist & Saltin, 1983; Skattebo, Calbet, et al., 2020). [Hb] does not increase with improved training status. In fact, [Hb] may decrease with chronic hypervolemia, i.e., elevated plasma volume (Mairbäurl, 2013). SaO_2 and PaO_2 can also not be improved with training and may even decrease at high intensities in more trained athletes due to exercise-induced arterial hypoxemia (Dempsey & Wagner, 1999). Thus, the only variable responsible for improved central oxygen delivery after sea-level exercise training is SV (Blomqvist & Saltin, 1983; Hellsten & Nyberg, 2015).

SV may increase through two main mechanisms: 1) an enhancement in venous return, which improves ventricular filling increasing the end-diastolic volume, and a consequent increase in SV through the Frank-Starling mechanism (Katz, 2002; Young, 2010); and 2) cardiac remodelling, leading to increases in cardiac dimensions and compliance (Pluim et al., 2000; Shave et al., 2019). The former mechanism can explain acute or short-term changes in stroke volume due to changes in BV, or movement or position-induced changes in venous return (Berger et al., 2006; González-Alonso et al., 1999; Kanstrup & Ekblom, 1982; Krip et al., 1997; Sejersen et al., 2021). The latter mechanism can explain chronic changes in stroke volume, typically not observed until after several weeks or months of endurance training (Arbab-Zadeh et al., 2014; Vogelsang et al., 2008). Both the BV-mediated enhancement of SV, and cardiac remodelling are responsible for the elevated SV and $\dot{Q}_{max}$ observed in endurance-trained individuals. For example, equalizing BV between two groups of trained and untrained men by phlebotomy and PV expansion, respectively, led to an increase in SV in the untrained and a reduction in SV in the trained (Krip et al., 1997). The changes in SV were mostly attributable to changes in diastolic filling rate and less to the systolic emptying rate of the left ventricle, which indicates that a difference remained in cardiac contractile properties even after BV matching.

2.2 Association between Hbmass and SV

[Hb] is not correlated with maximal aerobic capacity at least within a normal range of [Hb] (14 to 18 g/dl in males; 12 to 16 g/dl in females) observed in healthy humans (Schmidt & Prommer, 2008) and tends to decrease rather than increase in the first weeks of exercise training due to hemodilution (Convertino, 1991; Schmidt et al., 1988) or with chronic endurance training (Mairbäurl, 2013); however, Hb_{mass} correlates with $\dot{V}O_2max$ (Lundby et al., 2017; Schmidt & Prommer, 2008). This correlation is due to greater Hb_{mass} being an indicator of greater BV, which enhances oxygen delivery by facilitating a high SV (Bonne et al., 2014; Krip et al., 1997). The experimental observation about Hb_{mass} and BV being coupled is also supported by cross-sectional and longitudinal evidence showing parallel long-term changes in Hb_{mass} and BV in both athletic and non-athletic populations (Eastwood et al., 2012; Houtman et al., 2000). While specific circumstances, like iron deficiency or relocation to altitude may change the coupling of Hb_{mass} with BV (by lowering and elevating [Hb], respectively), it appears that [Hb] is tightly regulated within a specific individual range determined mostly (50-90%) by genetics in healthy individuals (Okada & Kamatani, 2012). Trained endurance athletes have more blood overall when expressed as absolute BV, BV per kg body mass, or BV per kg fat-free mass (Brotherhood et al., 1975; Schmidt & Prommer, 2008; Ulrich et al., 2011) but a similar or often slightly lower [Hb] and hematocrit than their untrained peers (Hellsten & Nyberg, 2015). The observed lower [Hb] and Hct of athletes may be due to chronic hypervolemia but also result from the timing of the measurement: athletes training daily are likely to be less than 24 hours from their last training session. An acute effect of PV expansion post-exercise may last more than 48 hours (Fellmann, 1992). This means that blood data from athletes may potentially be collected in a state of acute hypervolemia unlike the tests taken from inactive individuals, distorting the interpretation of "non-exercised" [Hb] and Hct. In summary, experimental evidence indicates a causal relationship between Hb_{mass} and maximal endurance performance, making it an important variable to consider when investigating determinants of $\dot{V}O_2max$.

2.3 Determinants of endurance performance

Despite the well-established correlation between Hb_{mass} and $\dot{V}O_2max$, it is apparent that endurance performance is better predicted by the highest intensity at which the body can achieve a metabolic steady state than $\dot{V}O_2max$ alone (Bassett & Howley, 2000; Coyle, 1995). The maximal metabolic steady state (MMSS) can be estimated using stable blood lactate ([La]) as a surrogate (giving the maximal lactate steady state, MLSS) (Billat et al., 2003), derived mathematically from the power-duration relationship using the critical power (CP) concept (Jones et al., 2019), or estimated from an incremental test using ventilatory and/or lactate thresholds (Faude et al., 2009; Keir et al., 2015). The extent to which oxygen delivery determines the MMSS intensity has not been a popular target of research in spite of its importance to understanding fatigue resistance.

Since [La] reaches a steady state in the circulation only if lactate production and lactate removal are in equilibrium, the highest intensity at which this balance may occur, the MLSS, has been considered a surrogate for the MMSS (Iannetta, Inglis, et al., 2018). While the absolute values of [La] at which the steady state is achieved vary greatly between individuals and modes of exercise

(Beneke & von Duvillard, 1996; Quinn et al., 2024), MLSS shows good agreement with the second ventilatory threshold or respiratory compensation point (Inglis et al., 2019; Pallarés et al., 2016). The MLSS has been shown to correlate well with half-marathon running race speed (Legaz-Arrese et al., 2011), rowing performance (Beneke, 1995), and 40-km time trial performance in cycling (Harnish et al., 2001). It also shows demonstrable improvement with aerobic training (Bergman et al., 1999; Billat et al., 2004), owing to increased lactate clearance and decreased lactate production at a given work rate (Bergman et al., 1999).

As another popular approach to differentiate aerobically sustainable intensities from unsustainable intensities, the CP is a non-invasive method of estimating the MMSS. It relies on mathematical analysis of the individual's power-duration curve in the severe intensity domain. The time for which a given speed or power can be sustained is increased in a hyperbolic fashion with reducing speed/power. The asymptote of this hyperbolic relationship is CP and it is thought to represent the highest intensity at which ATP can be produced fully aerobically, i.e., without gradual depletion of the anaerobic energy systems (Poole et al., 2016). Note that substrate level phosphorylation still occurs in glycolysis for aerobic metabolism, but this anaerobic contribution to ATP synthesis is relatively minor. If the power-duration data points are plotted by taking the inverse of time (1/t) against power, or as work completed against time, the resulting relationship is linear, in which case critical power and work prime (W′, which represents finite work capacity above CP) can be derived from the parameters of the linear regression.

The definition of MLSS has traditionally been the highest constant load intensity (power or speed) at which [La] rises by less than 1 mmol/L between minutes 10 and 30. When defined this way, the MLSS is typically found slightly lower than CP, whether it is estimated by linear or nonlinear methods (Dekerle et al., 2003; Keir et al., 2015; Mattioni Maturana et al., 2016). Debate surrounding the most appropriate surrogate for the MMSS has been intensified after observations of $\dot{V}O_2$ reaching a steady state at an intensity slightly (i.e., 10 W) above MLSS (Iannetta, Inglis, et al., 2018; Nixon et al., 2021), and some researchers argue that the highest intensity of exercise yielding a $\dot{V}O_2$ steady state, which better coincides with CP, should be the gold standard for MMSS and define the boundary between the heavy and severe exercise domains (Jones et al., 2019). On the other hand, studies measuring CP rarely measure $\dot{V}O_2$ at CP, and it has been suggested that CP is not a sudden transition but a phase transition (Pethick et al., 2020).

2.4 Effects of the manipulation of oxygen delivery on exercise performance

An association of Hb_{mass} with $\dot{V}O_2max$ alone cannot be considered sufficient evidence for a causal relationship between the two variables. Nevertheless, experimental interventions involving manipulation of convective oxygen transport, such as changes in BV or [Hb], have resulted in extensive literature demonstrating a direct link between blood-carrying capacity and $\dot{V}O_2max$. For example, a standard blood donation of 450 mL has been shown to reduce $\dot{V}O_2max$ for 3 weeks in healthy women (Stangerup et al., 2017) and for at least 1 week in competitive male cyclists (Panebianco et al., 1995). Likewise, an autologous blood transfusion increases $\dot{V}O_2max$ in trained males (Buick et al., 1980; Ekblom et al., 1976). Another example of an acute manipulation is reducing effective [Hb] by carbon monoxide (CO). CO inhalation acutely reduces $\dot{V}O_2max$ at low (6-7% COHb) (Ekblom & Huot, 1972; Schmidt & Prommer, 2005), and high (20-24% COHb) (Ekblom & Huot, 1972; Vogel & Gleser, 1972) exposure levels in healthy individuals, with the magnitude of impairment approximating the COHb level achieved. **Tables 2.1, 2.2** and **2.3** summarize studies in which oxygen delivery has been manipulated to assess changes in maximal exercise capacity using blood withdrawal or transfusions, carbon monoxide, or other techniques, respectively. For extensive reviews on how acute manipulations affect $\dot{V}O_2max$, see Calbet et al. (2006) and Lundby et al. (2017).

Fewer data are available on the effect of oxygen delivery on submaximal exercise performance, particularly the MMSS. Alterations in oxygen delivery in normoxia can arise from changes to either limb blood flow (by changes in $\dot{Q}$ or blood flow distribution) or CaO_2 (by altered SpO_2 and/or [Hb]). As per Equation 2.2, at submaximal exercise intensities, a decrease in one parameter may often be compensated for by an increase in another, resulting in maintained oxygen delivery (Ekblom et al., 1975; Ferretti et al., 1992; Gonzalez-Alonso et al., 2001; Koskolou et al., 1997). Therefore, submaximal exercise performance may be affected by a lesser extent than maximal exercise if oxygen delivery is impaired, but still elicit an increase in indicators of exercise-induced stress, such as increased [La] or ventilation, or a greater rate of neuromuscular fatigue (NMF) development.

As mentioned, two commonly applied techniques to acutely manipulate the oxygen carrying capacity include CO inhalation and blood withdrawals or transfusions (Calbet et al., 2006; Celsing et al., 1986; Ekblom et al., 1976; Johnson et al., 2019). Most studies have focused on short-duration high-intensity exercise while few studies report effects on prolonged exercise capacity. However, the available studies demonstrate the detrimental effects of reduced oxygen carrying capacity on $\dot{V}O_2max$ and describe negative changes to indicators of exercise-induced stress at submaximal intensities. As an example of indicators of exercise-induced stress, [La] has been shown to increase at submaximal intensities after blood loss. While no individual thresholds were studied, at a fixed [La] concentration of 4.0 mmol/L, $\dot{V}O_2$ was increased and running speed reduced after a series of blood withdrawals over nine weeks to induce and maintain anemia (Celsing & Ekblom, 1986). These effects were diminished after RBC reinfusion with no difference between baseline and reinfusion. In a second study from the same authors, blood [La] at fixed submaximal work intensities responded to blood withdrawal and reinfusion by increasing and decreasing, respectively (Celsing et al., 1986). A third study demonstrated a stepwise increase in SV, and a reduction in [La] and $\dot{V}_E$, at a fixed workload with consecutive RBC reinfusions after an anemic period achieved by five blood donations in five weeks (Celsing et al., 1987); however, the change in oxygen carrying capacity in these three studies was relatively severe, as [Hb] was reduced from 14.5 to 11.0 g/dL (Celsing & Ekblom, 1986) or increased from 13.7 to 17.0 g/dL (Celsing et al., 1987), a change of ~25%. This change corresponded to a change of ~19% in $\dot{V}O_2max$ in either direction according to the intervention (phlebotomy or transfusion). Smaller decreases in oxygen carrying capacity may not manifest as lower $\dot{V}O_2max$ (Skattebo, Bjerring, et al., 2020), perhaps due to the ability to maintain oxygen delivery by unchanged stroke volume after rapid PV restoration together with slightly improved extraction. **Table 2.4** summarizes the studies in which BV or RBCV has been manipulated to investigate the effects on submaximal exercise responses.

Low-dose CO inhalation is a non-invasive strategy to transiently alter the [Hb] available for oxygen-binding. While submaximal responses to mild COHb elevation have been reported (Ekblom & Huot, 1972; Gonzalez-Alonso et al., 2001; Vogel & Gleser, 1972), no studies have demonstrated whether these changes are sufficient to affect performance at intensities at or below the MMSS and/or in prolonged exercise. **Table 2.5** summarizes the studies in which %COHb has been manipulated to investigate the effects on submaximal exercise responses.

Other interventions that may be considered manipulating limb oxygen delivery include blood flow restriction (BFR) studies and studies using beta-blockers. BFR is achieved by applying inflatable cuffs around the upper and lower limbs to reduce blood flow into and out of the extremities. Willis et al. (Willis et al., 2020) studied the effects of BFR on leg and arm cycling in normoxia and hypoxia by measuring [La], HR, RPE, pulmonary gas exchange variables, and muscle Hb deoxygenation (deoxy-Hb) during a 6-min submaximal constant load exercise bout. The observed changes induced by BFR within a given FiO_2 condition were generally non-significant; however, the small sample size (n=7) might not have provided enough statistical power to detect effects on variables like [La], and V_E, which often possess high test-retest variability (Faude et al., 2017). Others have found increased $\dot{V}O_2$ for a given work rate, indicating impaired exercise efficiency when BFR is applied (Silva et al., 2021). Beta-blockers affect maximal heart rate and despite an increase in SV, a lower Q_{max} typically results from higher doses; therefore, it is unsurprising that $\dot{V}O_2max$ has been found to be compromised after beta-blockage (Wilmore et al., 1985). Despite the compensation of reduced HR by increased SV, beta-blockage also affects endurance capacity at submaximal intensities of 60% (Galbo et al., 1976) and 80% of $\dot{V}O_2max$ (van Baak et al., 1986). Methods manipulating oxygen delivery without involving manipulation of effective [Hb] or Hb_{mass} are summarized in **Table 2.6**.

Whether oxygen delivery directly impacts the boundary between heavy and severe exercise has been investigated by hypoxia and hyperoxia interventions but not by manipulating oxygen delivery in normoxia. The fraction of inspired oxygen (FiO_2) has been varied to measure the impact of altered oxygen delivery on exercise thresholds (Friedmann et al., 2004; Koistinen et al., 1995; Simpson et al., 2015; Vanhatalo et al., 2010), and to demonstrate its effect on NMF (Amann & Calbet, 2008), but the FiO_2 used in these studies was relatively severe. Additionally, a change in PaO_2 and SaO_2 consequent to hypoxia and hyperoxia have effects which are independent of oxygen carrying capacity [Hb] and SV (Calbet et al., 2003). While hypoxia has been a popular target of research, these data are mainly applicable in an altitude-related context, and a gap in the literature exists with regards to the impact of a mild reduction in oxygen carrying capacity of blood with normal FiO_2. It will therefore be the focus of this literature review.

Table 2.1. Key studies of maximal exercise performance after blood withdrawal or blood transfusions.

Reference	Type of intervention	Relevant outcome variables	Subjects (n, M/F, training status)	Main findings	Double-blind?	Cross-over?
Bonne et al., 2014	Phlebotomy (382 mL; volume equal to BV increase during training intervention)	PPO, $\dot{V}O_2max$, $\dot{Q}max$	9 M, moderately trained	$\dot{Q}max$ ↓ by 12%, $\dot{V}O_2max$ ↓ by 8%, PPO ↓ by 7%	No	No
Buick et al. 1980	Blood transfusion (900 mL)	Running $\dot{V}O_2max$, TTE	11 M, highly trained runners	$\dot{V}O_2max$ ↑ at 24h, 7d, and 16w, TTE ↑ at 24h, 7d	Yes	No
Burnley et al. 2006	Phlebotomy (450 mL)	Cycling $\dot{V}O_2max$, TTE	11 M, highly trained runners	$\dot{V}O_2max$ ↓ by 4%, TTE ↓ by 14%	Yes	No
Celsing & Ekblom 1986	Repeated phlebotomies (25% of Hb_{mass}) + blood reinfusion	Running $\dot{V}O_2max$	8 M, moderately trained	$\dot{V}O_2max$ ↓ after phlebotomy, ↑ after reinfusion	No	No
Ekblom et al. 1972	Phlebotomy (800/1200 mL) + blood reinfusion	Running $\dot{V}O_2max$, TTE	4 M	$\dot{V}O_2max$ ↓ by 10%, TTE ↓ by 30% at 2d	No	No
Ekblom et al. 1976	Phlebotomy (800 mL) + blood reinfusion 30-35d post	Cycling $\dot{V}O_2max$	9 M, moderately trained	$\dot{V}O_2max$ ↓ by 6% after phlebotomy, ↑ by 7% after reinfusion	No	No
Gordon et al., 2013	Phlebotomy (450 mL)	Cycling $\dot{V}O_2max$, TTE	15 M, trained	$\dot{V}O_2max$ ↓ after phlebotomy, TTE ↔	No	No
Krip et al., 1997	Phlebotomy in trained (490 mL), PV expansion in untrained (500 mL) to equalize BV	$\dot{V}O_2max$, $\dot{Q}max$	6 trained, 6 untrained M	$\dot{V}O_2max$ ↓ by 13% in trained, ↑ by 7% in untrained. $\dot{V}O_2max$, $\dot{Q}max$ ↓ by 15% in trained, ↑ by 9% in untrained	No	No
Montero et al., 2015	Phlebotomy (362 mL; volume equal to BV increase during training intervention)	$\dot{V}O_2max$, $\dot{Q}max$, PPO	16 M, moderately trained	$\dot{Q}max$ ↓ by 10%, $\dot{V}O_2max$ ↓ by 7%, PPO ↓ by 5%	No	No
Panebianco et al. 1997	Phlebotomy (450 mL)	Cycling $\dot{V}O_2max$	10 M, trained cyclists	$\dot{V}O_2max$ ↓	No	No
Skattebo et al., 2021	Phlebotomy (450 mL or 150 mL)	Cycling $\dot{V}O_2max$	12 M, moderately trained	$\dot{V}O_2max$ ↓ by 7% in 450 mL, ↔ in 150 mL trial	No	No
Skattebo et al., 2020	Phlebotomy (181 mL; volume equal to BV increase during training intervention)	Cycling $\dot{V}O_2max$, $\dot{Q}max$	12 M, moderately trained	$\dot{V}O_2max$ ↔ , $\dot{Q}max$ ↔	No	No

Note: PPO: Peak power output; $\dot{Q}max$: Maximal cardiac output; TTE: Time to exhaustion

Table 2.2. Key studies of maximal exercise performance after carbon monoxide inhalation.

Reference	Type of intervention	Outcome variables	Subjects (n, M/F, training status)	Main findings	Double-blind?	Cross-over?
Ekblom & Huot 1972	CO inhalation (7% and 20% COHb)	Cycling, running $\dot{V}O_2max$, V_Emax, [La]	5 N/A, well-trained	$\dot{V}O_2max$ ↓ 9%, 22% with low and high dose CO, respectively. V_Emax, [La] ↔	No	No
Ekblom et al. 1975	CO inhalation (13% COHb)	Cycling $\dot{V}O_2max$	9 M, moderately trained	$\dot{V}O_2max$ ↓ 14%	No	No
Koike et al. 1991	CO inhalation (11 and 20% COHb)	Cycling $\dot{V}O_2max$, PPO	9 M, 1 F, untrained and trained	$\dot{V}O_2max$ ↓ 12% and 20%, PPO ↓ 11% and 16% for the two CO doses, respectively	No	Yes
Schmidt & Prommer, 2005	CO inhalation (~6% (COHb)	$\dot{V}O_2max$, V_Emax,	8 M, 5 F moderately trained	$\dot{V}O_2max$ ↓ 3.0% V_Emax ↑	No	Yes
Sutehall et al. 2018	CO inhalation (4.7% (COHb)	Cycling $\dot{V}O_2max$	11 M, moderately trained	$\dot{V}O_2max$ ↓ 4.8%	No	Yes
Vogel & Gleser, 1972	CO inhalation (20% COHb)	Cycling $\dot{V}O_2max$, $\dot{Q}_{max}$, blood pH	8 M, untrained	$\dot{V}O_2max$ ↓ 22% $\dot{Q}_{max}$ ↔, pH ↔	No	No

Note: PPO: Peak power output; $\dot{Q}max$: Maximal cardiac output; V_Emax: Maximal ventilation

Table 2.3. Key studies of maximal exercise performance following other interventions to alter oxygen delivery.

Reference	Type of intervention	Outcome variables	Subjects (n, M/F, training status)	Main findings	Double-blind?	Cross-over?
Ashenden et al. 2006	Infusion of Hemopure™ (artificial oxygen carrier)	Cycling $\dot{V}O_2max$, HRmax, V_Emax	12 M, trained	VO_2max ↔, HRmax ↓, V_Emax ↔	No	Yes
Durussel et al. 2013	rhEPO treatment	3km running TT, $\dot{V}O_2max$, Hb_{mass}	19 M, trained	VO_2max ↑ 8%, TT time ↓ 6%, Hbmass ↑ 19%	No	No No control group
Pollock et al. 1991	Beta-blocker (propranolol) treatment, different doses	HRmax, BP, $\dot{V}O_2max$, RPE	12 M, 1 F moderately trained	HRmax ↓, Sys BPmax ↓, VO_2max ↓, O_2 pulse ↑, RPE ↔	Yes	Yes
Sutehall et al. 2018	Microdose rHuEPO treatment	Cycling $\dot{V}O_2max$, Hb_{mass}, Repeated Sprint Ability (RSA)	14 M, trained	VO_2max ↑ 3.9%, Hbmass ↑ 14%, RSA ↔	No	No
Wilmore et al. 1985	Beta-blocker (sotalol, propranolol, atenolol) treatment	HRmax, SV, $\dot{V}O_2max$	93 (sex N/A) Combined data from four studies	HRmax ↓ in all, SV ↑ in all, VO_2max ↓ in trained (~10%) but not untrained	Yes	Yes

Note: PPO: Peak power output; SV: Stroke volume; $\dot{Q}max$: Maximal cardiac output; V_Emax: Maximal ventilation; TT: Time trial; BP: Blood pressure

Table 2.4. Key studies measuring submaximal exercise responses after blood withdrawal or blood transfusions.

Reference	Type of intervention	Outcome variables	Subjects (n, M/F, training status)	Main findings	Double-blind?	Cross-over?
Bejder et al. 2019	RBC transfusion (135 mL)	Cycling time trial (650 kcal)	9 M, trained cyclists and triathletes	Time in TT (~40min) ↓ 5.1% Mean power in TT ↑ 5.6%	Yes	Yes
Brien et al. 1987	RBC transfusion (400 mL)	Running race (10 km)	6 M, well-trained runners	Time in 10 km race (~33min) ↓ 3.5%	Yes	Yes
Buick et al. 1980	Blood transfusion (900 mL)	HR at fixed running speed	11 M, highly trained runners	HR ↓ at 24h, 7d, and 16w (-7, -6, -6 b/min)	Yes	No
Celsing & Ekblom 1986	Repeated phlebotomies (25% of Hbmass) + blood reinfusion	$\dot{V}O_2$ at [La-] 4.0 mmol/L in cycling	8 M, moderately trained	VO_2 at [La] 4.0 mmol/L ↓ 13%, %VO_2 ↑	No	No
Celsing et al. 1986	Repeated phlebotomies (25% of Hbmass) + blood reinfusion	[La] at 150 or 175 W cycling	9 M, moderately trained	[La] ↑ after phlebotomy (2.5 to 4.3), ↓ after reinfusion (to 2.9)	No	No
Celsing et al. 1987	Repeated phlebotomies (2250 mL) + repeated reinfusions	V_E, [La], HR in fixed running speed	8 M, healthy untrained to well-trained	V_E ↓, [La] ↓, HR ↓ after reinfusions	No	No
Ekblom et al. 1972	Phlebotomy (800/1200 mL) + blood reinfusion	[La], HR in two fixed cycling loads	4 M / 3 M, moderately trained	[La] and HR ↑, RPE ↔ during higher load	No	No
Ekblom et al. 1976	Phlebotomy (800 mL) + blood reinfusion	$\dot{Q}$, SV, HR, [La] at two submax cycling loads	9 M, moderately trained	Q ↔, SV ↓, HR ↑, [La] ↑ post-phlebotomy, Q ↔, SV ↑, HR, [La] ↓ post-reinfusion	No	No
Janetzko et al. 1998	Phlebotomy (450 mL)	Treadmill workload at HR 130	38 M / 16 F, healthy 55-69y	WL ↔ (in older) WL ↑ (in younger)	No	No
Kantsrup & Ekblom 1984	PV exp., phlebotomy (900 mL), phlebotomy + PV exp., blood reinfusion, and blood reinfusion + PV exp.	$\dot{V}O_2$, HR, [La], O_2 pulse in submax cycling (50 and 75% of $\dot{V}O_2$max)	5 M / 3 M, healthy	VO_2 ↔ in all conditions; HR, ↓ post PV exp and ↑ post-phlebotomy, O_2 pulse ↓ post-phlebotomy and ↑ blood reinfusion + PV exp.	No	No

Koskolou et al. 1997	Phlebotomy (20% of BV) + PV exp.	$\dot{V}O_2$, $\dot{Q}$, LBF in 2-legged knee extension	7 M	Q ↔, LBF ↑	No	No
Panebianco et al. 1997	Phlebotomy (450 mL)	$\dot{V}O_2$ at HR 130, 150, 170, and VT2	10 M, trained cyclists	VO_2 ↔ in all conditions	No	No
Robertson et al. 1984	Reinfusion of 334 mL packed RBCs	$\dot{V}O_2$, V_E, HR, a-vO_2diff, SV, $\dot{Q}$, [La] at 70% of baseline $\dot{V}O_2$max	9 F, active	$\dot{V}O_2$ ↔, V_E ↓, HR ↓, SV ↔, $\dot{Q}$ ↓, [La] ↓, a-vO_2diff ↑	No	No
Williams et al. 1978	Phlebotomy (450 mL) + blood reinfusion	TTE (~41 min)	16 M, trained runners	Time in TTE ↔ in both conditions	No	No
Williams et al. 1981	Blood transfusion (920 mL)	Running time trial (8 km)	12 M, trained runners	Time in TT ↓ 2.7%	Yes	Yes

Note: SV: Stroke volume; $\dot{Q}$: Cardiac output; V_E: Ventilation; TTE: Time to exhaustion; TT: Time trial; LBF: Leg blood flow; WL: Workload

Table 2.5. Key studies of submaximal exercise with carbon monoxide inhalation.

Reference	Type of intervention	Outcome variables	Subjects (n, M/F, training status)	Main findings	Double-blind?	Cross-over?
Ekblom & Huot 1972	CO inhalation (7% and 20% COHb)	$\dot{V}O_2$, HR, [La] at two submax cycling loads	5 N/A, well-trained	With 20% COHb $\dot{V}O_2$ ↔, [La] and HR ↑; non-sign. changes with 7%	No	No
Ekblom et al. 1975	CO inhalation (13% COHb)	$\dot{Q}$, HR, SV, BP, a-vO2 diff at two submax cycling loads	9 M, moderately trained	$\dot{Q}$ ↔, SV ↓, HR ↑, BP ↔, a-vO2 diff ↔ (non-sign. ↓)	No	No
Klausen et al. 1968	CO inhalation (%COHb N/A)	$\dot{Q}$ in cycling	8 M, healthy	$\dot{Q}$ ↔	No	No
Koike et al. 1991	CO inhalation (11 and 20% COHb)	V_E, [La], $PeCO_2$ in incremental + constant load cycling	9 M, 1 F, untrained and trained	V_E at P < VT1 ↔, VE at P > VT1 ↑; V_E, [La], $P_{ET}CO_2$ ↑ heavy-int. load	No	Yes
Maehara et al. 1997	CO inhalation (15% COHb)	Deoxy-Hb, HR, VO_2, [La] in two constant cycling loads	7 M, healthy untrained	Deoxy-Hb ↑ during higher load in both CO and control	No	No
Sutehall et al. 2018	CO inhalation (4.7% (COHb)	Ventilatory threshold (VT)	11 M, moderately trained	VT ↓ 4.8%	No	Yes
Vogel & Gleser, 1972	CO inhalation (20% COHb)	$\dot{Q}$, SV, HR	8 M	$\dot{Q}$ ↑, SV ↔, HR ↑	No	No

Note: SV: Stroke volume; $\dot{Q}$: Cardiac output; V_E: Ventilation; TTE: Time to exhaustion; TT: Time trial; $P_{ET}CO_2$: End-tidal CO_2

Table 2.6. Key studies of submaximal exercise in response to other experimental manipulations of deliver oxygen.

Reference	Type of intervention	Outcome variables	Subjects (n, M/F, training status)	Main findings	Double-blind?	Cross-over?
Broxterman et al. 2015	Blood flow occlusion	CP in handgrip exercise	10 M, healthy	CP ↓ 100%	Yes	No
Heuberger et al. 2017	rHuEPO treatment	Cycling TT (45 min), Power at LT, VT1, VT2, race perf.	48 M, trained non-elite cyclists	$\dot{V}O_2$ and P at VT1 ↑, P at VT2 ↑, other outcomes ↔ (non-sign. ↑)	No	Yes
Pollock et al. 1991	Beta-blocker (propranolol) treatment, different doses	HR, BP, $\dot{V}O_2$, O_2 pulse, RPE	12 M, 1 F moderately trained	HR ↓, Sys BP ↓, $\dot{V}O_2$ ↔, O_2 pulse ↑, RPE ↔	Yes	Yes
Silva et al. 2019	Blood flow restriction	$\dot{V}O_2$, HR during walking (40% of VO2max)	22 M, active	VO_2 ↑, HR ↑	No	Yes
Sutehall et al. 2018	rHuEPO treatment	VT	14 M, trained	VT ↔ (non-sign. ↑of 2.2%)	No	No
Thomsen et al. 2007	rHuEPO treatment	TTE at 80% of baseline VO_2max + of new VO_2max	16 M, healthy	80% of baseline $\dot{V}O_2max$: TTE ↑ 54% 80% of new $\dot{V}O_2max$: TTE ↓ 27%	No	No
Willis et al. 2020	Blood flow restriction with or without hypoxia	[La], HR, V_E, SpO_2, Deoxy-Hb, HR, $\dot{V}O_2$, post blood flow, RPE for leg and arm cycling	5 M, 2 F	Most variables ↔, RPE ↑. When combined with hypoxia, V_E, RPE ↑	No	Yes

Note: SV: Stroke volume; $\dot{Q}$: Cardiac output; V_E: Ventilation; TTE: Time to exhaustion; TT: Time trial; VT: Ventilatory threshold; BP: Blood pressure

2.5 Neuromuscular fatigue and oxygen delivery

Physical performance depends on muscle force production, which is known to be affected by the intensity and duration of exercise (Allen et al., 2008; Amann, 2011). The classical measurement of exercise-induced fatigue involves assessing the decline in maximal voluntary (or evoked) force production during brief isometric contractions from baseline to immediately after the fatiguing task, i.e., neuromuscular fatigue (NMF) evaluation. This decline is evaluated through measurements of maximal voluntary contraction (MVC) force and evoked contraction force. NMF is broadly categorized into peripheral and central components. Peripheral fatigue involves processes occurring at or distal to the neuromuscular junction (Allen et al., 2008), assessed through supramaximal electrical or magnetic stimulation of motor nerves. Central fatigue, on the other hand, refers to a reduction in muscle activation during exercise and can be evaluated using techniques such as the interpolated twitch technique (ITT) (Gandevia, 2001). ITT enables the assessment of deficits in motor output vs. impairments in muscle contractile function. In short and intense exercise, peripheral fatigue mechanisms explain most of the loss in MVC force; however, with increasing exercise duration and decreasing exercise intensity, the contribution of central fatigue increases (Iannetta et al., 2022; Millet et al., 2018).

As sustained muscle contractions require an adequate supply of oxygen, disruptions in oxygen delivery accelerate impairments in contractile function, likely due to increase reliance on substrate-level phosphorylation and associated metabolic perturbations. The rate of NMF development has indeed been shown to be greater in the severe intensity domain in both small muscle mass exercise (Burnley et al., 2012) and whole-body exercise (Azevedo et al., 2021). Hence, measuring NMF in exercise tasks after altered oxygen delivery can be informative when assessing the exercise-induced metabolic perturbation in muscles as a consequence of such manipulations. While hypoxia has been used as a model for altered oxygen delivery (Fulco et al., 1996; Peyrard et al., 2019), it is unclear how reductions in oxygen carrying capacity in normoxia affects NMF development.

2.6 Regulation of Hb_{mass} and BV

Regulation of Hb_{mass} in response to changes in oxygen demand induced by changing FiO_2 is well documented (Bhoopalan et al., 2020). Erythropoiesis is the process of red blood cell (erythrocyte) maturation, which in adult humans, mainly occurs in the bone marrow. In steady state conditions, the production of new erythrocytes is matched by an equal rate of destruction of old erythrocytes in the spleen and the liver (de Back et al., 2014). In conditions of increased demand for oxygen transport, this balance must be transiently disrupted either by increasing erythropoiesis or decreasing the removal of old erythrocytes. Evidence from altitude studies suggest that the rate of cell destruction does not change when ascending to altitude but the increase in Hb_{mass} is due to stimulated erythropoiesis. On return to lower altitudes, both suppressed erythropoiesis and enhanced hemolysis are responsible for the subsequent fall in Hb_{mass} (Mairbäurl, 2018; Merino, 1950). However, despite the established importance of Hb_{mass} on aerobic capacity and known increase in Hb_{mass} in trained individuals, the mechanisms regulating the erythropoietic response to exercise are incompletely understood. Surprisingly, exercise-related changes in erythropoiesis have been studied less than altitude-related changes. The beginning of exercise training appears to transiently increase the proportion of reticulocytes (newly released red blood cells), but this effect diminishes with continued training (Díaz et al., 2011; Mairbäurl, 2013). Little is known about the specific adjustments in the RBC turnover needed to achieve a new level of BV when adapting to a higher training load.

Erythropoietin (EPO) is the main regulator of erythropoiesis and is required for the early steps of erythrocyte differentiation. For a comprehensive review of its action through binding to the EPO receptor, see Bhoopalan et al. (2020). While elevated serum EPO has been reported in some studies after demanding exercise (Schwandt et al., 1991), others have not found changes in EPO concentration after acute sea-level exercise (Bodary et al., 1999). The post-exercise expansion in PV is associated with a mild increase in erythropoietin (EPO), which could stimulate erythropoiesis (Bodary et al., 1999; Montero et al., 2017; Schmidt et al., 1991); however, the effect of regular training on EPO may be short-lived, as the hormone levels return to baseline: while the beginning of an exercise program resulted in elevated EPO the morning after exercise for two weeks of training, this effect coincided with the cortisol response and was diminished in the following weeks (Montero et al., 2017). In contrast to these results, a cross-sectional study on athletes vs. non-athletes differing ~4.5-fold in their activity levels found a significant difference in serum EPO and erythroferrone (markers of erythroid activity) in a morning sample after a rest day (Dziembowska et al., 2021).

EPO is produced in kidneys in response to lowered pO_2. Thus, a transiently lower [Hb] as a consequence of exercise-induced hypervolemia could be a direct source of stimulation of EPO release. An expansion in PV post-exercise induces changes in hormones regulating blood volume by enhancing fluid retention in kidneys: increase in aldosterone and antidiuretic hormone and decrease in atrial natriuretic peptide levels. The hypervolemic response to high-intensity or high-volume exercise is well documented, and the time-course of acute PV expansion has been described in detail (Convertino, 2007); however, its relationship with chronic improvements in cardiorespiratory fitness and increase in BV has not been extensively reported in the literature. The magnitude of acute post-exercise reduction in blood pressure (hypotension) has been reported to be intensity-dependent (Quinn 2000; Eicher et al. 2010) or work-dependent (Jones et al., 2007), with no differences between high-intensity interval exercise and steady-state exercise (Lacombe et al., 2011; Rossow et al., 2010).

Another possible erythropoiesis-stimulating mechanism is the direct effect of elevated stress hormones on erythroid progenitors. Unlike steady-state erythropoiesis, stress erythropoiesis is activated in response to tissue damage or inflammation and results in a rapid differentiation of erythroid progenitors (Ruan & Paulson, 2022). Evidence from both in vitro and in vivo experiments with mice suggest that glucocorticoids like cortisol are needed for stress-induced proliferation of immature erythroid cells. Unlike wild-type mice, mice lacking glucocorticoid receptors failed to respond to hemolytic or hypoxic stress by upregulating erythropoiesis (Bauer et al., 1999). It is likely that elevated levels of both EPO and cortisol in the early phase of intensified training are needed to stimulate erythroid progenitor maturation. Interestingly, stress erythropoiesis is restricted to a special erythroid compartment expressing specific surface antigens. In rodents, this response primarily occurs in the spleen, whereas bone marrow erythropoiesis is hardly affected (Ou et al., 1980). The location of stress erythropoiesis in humans has not been definitively identified, but in anemia patients many reports exist of stress erythropoiesis outside the bone marrow including the liver, spleen, and lymph nodes (Paulson et al., 2020). Whether stress-erythropoiesis is the main driver of early increases in Hb_{mass} with exercise training is not known.

While the stress-response described above might explain early enhancement of erythropoiesis with novel exercise stress or substantially increased training load, the diminishing EPO and cortisol responses suggest that another mechanism is responsible for the maintenance of higher Hb_{mass} in chronically trained individuals. Hyperplasia of the hematopoietic bone marrow has been reported in well-trained marathon runners and elite cyclists (Shellock et al., 1992; Vogt et al., 2008) and has been suggested as an adaptation to help sustain a high turnover of red blood cells. Not all studies have found significant bone marrow-related changes in endurance athletes and low iron reserves may contribute to hematopoietic hyperplasia more than high BV per se (Altehoefer et al., 2002; Caldemeyer et al., 1996).

Since chronic endurance training is characterized by an increase in BV due to an increase in both PV and RBCV (Schmidt & Prommer, 2008), derived from [Hb], Hct, and Hb_{mass} measured using the CO rebreathe method, a higher rate of Hb synthesis could be expected in athletes. Training is also associated with a higher turnover rate of RBC and a larger proportion of young RBCs with greater deformability (Smith et al., 1999; Weight et al., 1991), indicative of an elevated rate of Hb synthesis in endurance trained individuals. Deformability in these studies was measured using a technique called ektocytometry, which measures deformation of RBCs in response to shear stress, and the lifespan of RBCs by ^{51}Cr-labelling of RBCs. Further indirect evidence for a shorter survival time of RBCs in endurance athletes is lower serum haptoglobin and ferritin concentrations, as well as a higher fraction of reticulocytes (Schmidt et al., 1988).

Despite these reports of higher RBC turnover in athletes, published data are lacking on the protein synthesis rates of RBCs. A potential method to study the rate of Hb synthesis would be by oral administration of heavy water (^2H_2O), which has been used to establish fractional synthetic rates (FSR) of several other proteins in the body, including muscle contractile protein (MacDonald et al., 2013), mitochondrial protein (Kasumov et al., 2013), and plasma albumin (Previs et al., 2004). This technique would allow for direct comparisons of Hb synthesis between different populations, such as sedentary individuals and trained endurance athletes, which would directly quantify potential difference in RBC turnover. To apply the heavy water method to Hb requires some special considerations, as the site of sampling (blood) is not the same as the site of protein synthesis (bone marrow). This issue results in a delay between synthesis and when the labeled protein may be detected in a blood sample (**Figure 2.2**).

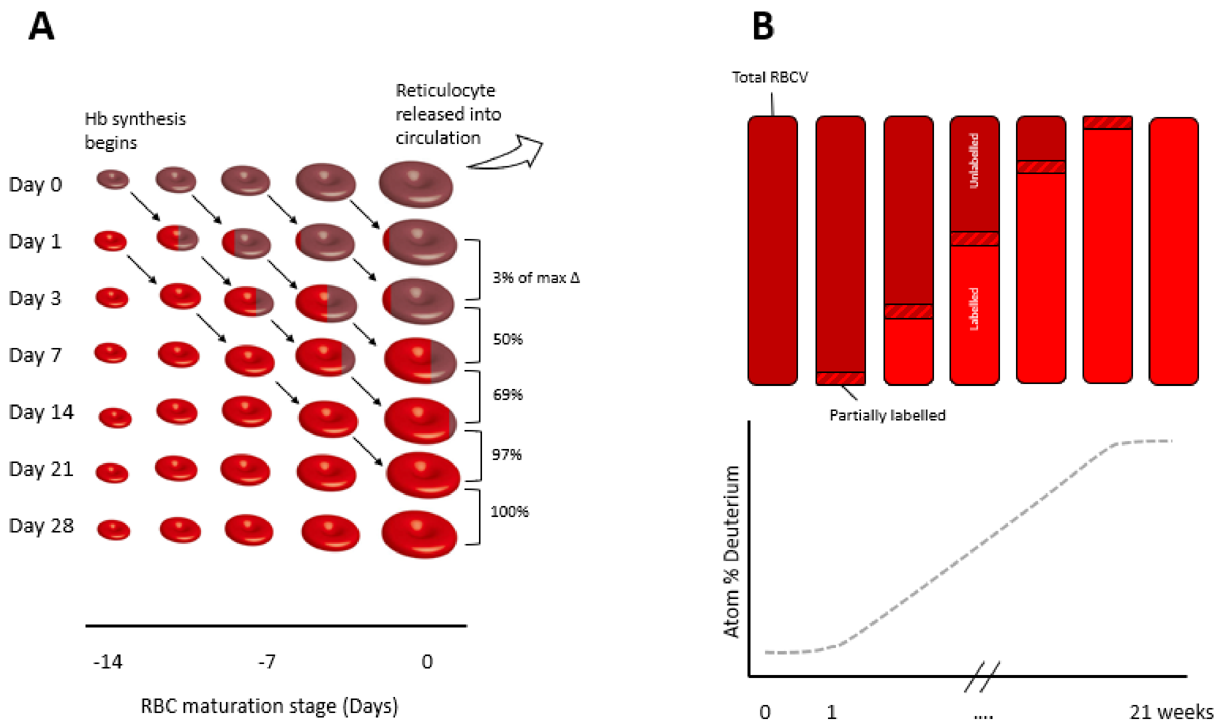

Figure 2.2. A schematic outlining the hypothetical measurement of hemoglobin (Hb) synthesis rates. A) During a 2-week phase of protein synthesis and accumulation, reticulocytes are released with varying levels of labeled Hb. B) The percentage of the label in total RBCV will reach a linear increase after ~2 weeks and only plateau when all circulating RBCs present at the beginning of labeling have been replaced.

2.7 Measurement of Hbmass and BV

The most widely used method for determination of Hb_{mass} is the CO-rebreathing technique utilizing the dilution principle (Burge & Skinner, 1995). The original 10-minute rebreathing procedure has been optimized to provide valid results in a 2-minute rebreathing protocol (Schmidt & Prommer, 2005; Siebenmann et al., 2017). This technique involves inhaling a known dose (typically ~0.8-1.4 mL/kg BM) of CO and collecting blood samples before and after to determine the percentage of carboxyhemoglobin (COHb). The inhaled CO transiently binds to Hb, which allows Hb_{mass} to be calculated from the change in COHb using the dilution principle (**Figure 2.3**). Assumptions of small CO losses from the intravascular bed, including remaining CO in the rebreathing system, exhaled CO after disconnection from the respirometer, and diffusion of CO into myoglobin are used to improve accuracy of the final value (Prommer &

Schmidt, 2007; Siebenmann et al., 2017). The equation to derive Hbmass from the measured test variables is as follows:

$$Hb_{mass} = K \cdot MCO \cdot (\Delta COHb \cdot 1.39\ ml \cdot g^{-1})^{-1} \qquad \text{(Eq. 2.3)}$$

Where K = current barometric pressure · 760^{-1} · 273 · current temperature $(K)^{-1}$; MCO = CO administered (corrected for losses); 1.39 = Hüfner's number (ml CO / g Hb); ΔCOHb = difference between fraction of COHb before and after CO inhalation.

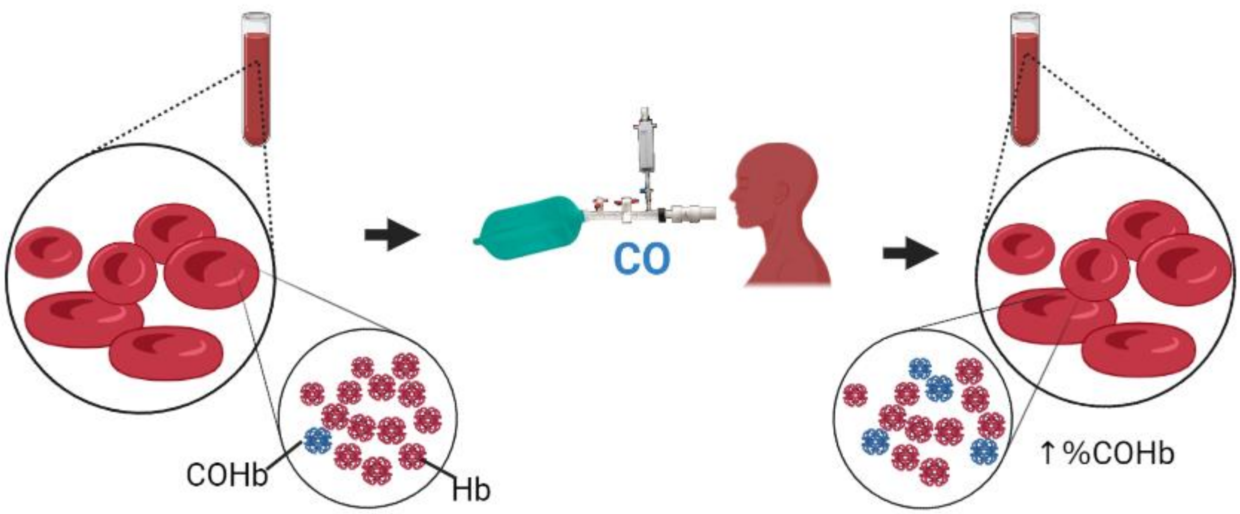

Figure 2.3. Measurement of hemoglobin mass (Hb_{mass}) by utilizing the dilution principle. Inhalation of carbon monoxide (CO) results in an increase in the fraction of carboxyhemoglobin (COHb). From the ΔCOHb measured in pre- and post-inhalation blood samples, Hb_{mass} can be calculated when the administered CO dose is known and CO losses from the intravascular space are estimated. Created with BioRender

The volume of any closed system can be determined if the concentration of a substance and the total amount of the substance is known. Since [Hb] and Hct are easily determined, BV, PV, and RBCV can be calculated from the measured Hb_{mass}. When Hct is known, RBCV can be derived by dividing Hb_{mass} by [Hb] and then multiplying by Hct (Equation 2.4). For the calculation of BV, a correction factor for venous Hct is used to obtain an estimate representing the overall intravascular average for Hct (Chaplin et al., 1953) (Equation 5). PV can then be calculated by subtracting RBCV from BV (Equation 6):

$$RBCV = Hct \cdot Hb_{mass} / [Hb] \quad \text{(Eq. 2.4)}$$

$$BV = RBCV \cdot 100 / (Hct \cdot 0.91) = 109.9 \cdot Hb_{mass} / [Hb] \quad \text{(Eq. 2.5)}$$

$$PV = BV - RBCV \quad \text{(Eq. 2.6)}$$

2.8 Relationship between aerobic fitness and Hb_{mass}: effect of sex

While hemoglobin mass is an important factor in $\dot{V}O_2max$, other variables also play a role. Regression slopes of Hb_{mass} and $\dot{V}O_2max$ published in the literature differ between athletes in different sports (Gore, Hahn, et al., 1997), trained and untrained subjects, as well as males and females (Mancera-Soto et al., 2022), demonstrating that this relationship is not strictly fixed but specific to the population studied. Cardiac output is only partly dependent on blood volume, meaning that stroke volume is still elevated independently of blood volume in the trained heart (Krip et al., 1997). Additionally, limb blood flow is affected by mode of exercise, including the amount of active muscle mass (Calbet & Joyner, 2010; Rowell, 1988), and the level of oxygen extraction is affected by the training status of the muscle (Kalliokoski et al., 2001; Skattebo, Capelli, et al., 2020). When equally fit males and females are compared, it is possible that females compensate for a lower [Hb] by increased extraction, perhaps due to differences in mitochondrial respiration (Cardinale et al., 2018) or due to estrogen-mediated differences in RBC rheology (Grau et al., 2018) or capillary hematocrit (Murphy, 2014).

To investigate whether the Hb_{mass}-$\dot{V}O_2max$ relationship is similar in both sexes, absolute data may need normalization. Males and females possess different average $\dot{V}O_2max$ mainly due differences in body mass (BM), body composition, [Hb], and possible differences in physical activity habits (Sparling, 1980). To draw sex-based conclusions from studies including both males and females, appropriate matching of aerobic fitness is essential to isolate the effect of sex rather than fitness or training status. If matching is achieved by BM-normalized $\dot{V}O_2max$, it may underestimate the true fitness of female participants by recruitment of better trained females than males, since fat mass does not contribute to aerobic capacity (Goran et al., 2000) but affects the calculated relative $\dot{V}O_2max$ value when included in the divisor.

Normalizing $\dot{V}O_2$max or other indicators of aerobic fitness (such as peak power output or $\dot{V}O_2$ or power associated with a given threshold) to fat-free mass (FFM), reduces the impacts of BM and body composition on measures of aerobic fitness (Cureton, 1981). The magnitude of a remaining sex difference after normalization to FFM is controversial. On average, studies have found a ~12% difference in $\dot{V}O_2$max even after accounting for body fat (Sparling, 1980).

When accepting Hb_{mass} as a surrogate for central oxygen delivery, important sex-differences must be considered. For a given BV, absolute Hb_{mass} is higher in males regardless of performance level (Schmidt & Prommer, 2010). Previous studies have demonstrated that Hb_{mass} remains greater in males when normalized to BM (Diaz-Canestro & Montero, 2022; Diaz-Canestro, Pentz, et al., 2021; Diaz-Canestro, Siebenmann, et al., 2021; Goodrich et al., 2020), and reports are also available for the comparison of FFM-normalized values (Goodrich et al., 2020; Ulrich et al., 2011). Overall, there appears to be a sex-difference in Hb_{mass} or RBCV even after normalization to FFM (Santisteban et al., 2022). These findings are expected due to the direct effect of testosterone on [Hb] and Hct, resulting in a different composition of blood in males and females (Coviello et al., 2008; Hero et al., 2005). Despite this sex-difference in Hb_{mass}, BV has been reported to be similar between sexes when corrected for body fat (Raes et al., 2006), meaning that PV tends to be higher in females per kg FFM. Indeed, lean body mass was found to be the best predictor of BV in a study including 50 males and 50 females (Falz et al., 2019). However, even if a sex difference in a given parameter seems likely, it's important to look at it with large sample sizes of individuals matched for aerobic fitness to reduce the confounding effect of training on hematological parameters.

2.9 Relationship between aerobic fitness and Hbmass: effect of training status

Debate exists on whether central oxygen delivery by the cardiovascular system, or the peripheral capacity to extract and use oxygen is limiting $\dot{V}O_2$max (Lundby et al., 2017). Several authors have previously demonstrated that training status may be a factor in that well-trained athletes are more likely to possess a limitation in oxygen supply rather than exhaustion of the peripheral oxidative capacity (Gifford et al., 2016; Lawler et al., 1988; Richardson et al., 1999; Roca et al., 1992; Skattebo et al., 2020). This is also evident in a study with 93 participants assessing the effect of beta-blockers, where reduced $\dot{Q}_{max}$ only lead to reduced $\dot{V}O_2$max in individuals with $\dot{V}O_2$max above 45-50 mL/kg/min while untrained subjects maintained their aerobic capacity

(Wilmore et al., 1985). In a meta-analysis combining results from studies using direct measurements of oxygen extraction, it was demonstrated that the contribution of enhanced oxygen delivery to increased $\dot{V}O_2max$ after endurance training is greater than the contribution of enhanced oxygen extraction (Skattebo et al., 2020). In other words, after a high extraction capacity of the trained muscles (>90% in trained individuals) has been achieved, there is little room for improvement on the extraction-side of the Fick equation, and thus oxygen delivery becomes limiting.

If the determinants of $\dot{V}O_2max$ differ between trained and untrained populations, it is plausible that the same applies to the MMSS. Traditionally, performance at submaximal exercise intensities, based on outcomes such as endurance time or fuel metabolism, has been suggested to be determined by peripheral and not central factors (Hinton et al., 2000; Lundby & Jacobs, 2016; Minotti et al., 1990). However, as exceeding the boundary between the heavy and severe exercise domains leads to an inevitable attainment of $\dot{V}O_2max$ and $\dot{Q}_{max}$ (Poole et al., 2016), it appears as though this threshold represents an intersection between stable and unstable oxygen delivery, too. To date, no studies have attempted to investigate whether the determinants of the MMSS differ in trained and untrained humans. Therefore, the extent to which this threshold relies on factors limiting central oxygen delivery in people with varying training status requires more research.

2.10 Objectives

The objectives of this thesis were:

1) To investigate the effect of an acute reduction of effective [Hb] on the maximal lactate steady state and associated physiological, metabolic, and neuromuscular responses.
2) To explore the association of training status and sex with exercise capacity and its reduction after oxygen carrying capacity manipulations.
3) To investigate the effect of acute BV reduction on physiological, metabolic, and neuromuscular responses in heavy prolonged submaximal exercise, and subsequent maximal performance.
4) To explore the relationship between hematological parameters and fitness, the effect of sex on hematological parameters, and how to best normalize hematological parameters considering body size and composition.

5) To develop and apply a method to measure the Hb synthetic rate in humans using heavy water.

2.11 Hypotheses

This thesis addresses the following hypotheses:

i) Impairment of oxygen carrying capacity by experimental manipulation of effective or actual Hb_{mass} would impair indicators of submaximal exercise capacity and steady state behaviour of said indicators in cycling.

ii) Greater fitness levels would be associated with greater stress responses and greater deviations of steady state behaviour when oxygen-carrying capacity is acutely reduced.

iii) The relationship between aerobic fitness and hematological parameters would strengthen after normalizing both variables to fat-free mass.

iv) Sex-based differences in hematological parameters and endurance performance would be reduced or diminished when participants are matched for fitness using $\dot{V}O_2max$ values expressed per kg fat-free mass.

v) The fractional and absolute synthetic rates of Hb can be determined using oral administration of deuterated water and Hb_{mass} measurements.

Chapter III: Impairment in maximal lactate steady state after carbon monoxide inhalation is related to training status

3.1 Introduction

Numerous studies have attempted to identify the extent to which "central" and "peripheral" factors limit maximal oxygen uptake ($\dot{V}O_2max$) or its surrogate, $\dot{V}O_2peak$, (Bassett & Howley, 2000; Johnson et al., 2019; Lundby et al., 2017), but the contribution of these factors to prolonged exercise is underexamined. $\dot{V}O_2max$ is primarily limited by convective O_2 delivery in most, and particularly in well-trained, individuals, as demonstrated by blood withdrawal studies (Kanstrup & Ekblom, 1982; Panebianco et al., 1995; Van Remoortel et al., 2017), but peripheral factors seem to limit $\dot{V}O_2max$ in untrained individuals to some extent (Bassett & Howley, 2000; Cardús et al., 1998; Gifford et al., 2016). Intensities of exercise that elicit $\dot{V}O_2max$ are unsustainable, as the attainment of $\dot{V}O_2max$ requires the continuous depletion of immediate energy substrates (Poole et al., 1988) and results in exercise intolerance in minutes (Hill & Rowell, 1997; Midgley et al., 2006). As prolonged endurance exercise is performed at intensities that do not elicit $\dot{V}O_2max$ (i.e., within the heavy and moderate intensity domains), the highest exercise intensity achievable via sustained aerobic ATP production may predict prolonged endurance performance better than $\dot{V}O_2max$ (Coyle et al., 1991; Faude et al., 2009).

Maximal lactate steady state (MLSS) and critical power (CP) are two commonly used methods to identify the threshold delimiting sustainable from non-sustainable exercise. These thresholds share the same conceptual basis: each represents the highest work rate that is fully supported by oxidative metabolism. Specifically, MLSS represents the highest intensity at which an elevated but stable blood lactate ([La]) is achieved (Billat et al., 2003), and CP represents the asymptote of the power-duration relationship for exhaustive exercise bouts lasting 2-20 min (Jones & Vanhatalo, 2017). The power output (PO) associated with MLSS during whole-body cycling is similar, but often slightly below CP (by ~7 %), and MLSS can be sustained for a longer duration than CP (~1 h vs. ~30 min in the majority of studies) (Jones et al., 2019). Reductions in MLSS can be assessed during a single exercise trial performed at the PO associated with MLSS by measuring the stability of [La], a surrogate for metabolic stability. Accordingly, MLSS is a useful model to study whether a reduced oxygen carrying capacity influences the highest metabolic steady state.

The MLSS occurs at ~75-90 % of $\dot{V}O_2max$ in most people (Iannetta et al., 2020), indicating that the maximal capacities of the body to deliver and utilize O_2 are not fully engaged at MLSS. Interventions that reduce the O_2 transport capacity, such as carbon monoxide (CO) inhalation or blood withdrawal, intensify physiological responses to submaximal exercise (Ekblom & Huot, 1972; Koike et al., 1991; Panebianco et al., 1995; Williams et al., 1981); however, whether similar perturbations influence the attainment of a metabolic steady state at a given PO (i.e., reduce the MLSS) has not been investigated. Estimates of CP for cycling are decreased by hypoxia (fraction of inspired O_2 (F_IO_2) =13%) (Simpson et al., 2015) and increased by hyperoxia (F_IO_2 = 50%) (Goulding et al., 2020), suggesting that O_2 delivery could influence MLSS; however, the hypoxia and hyperoxia conditions were relatively severe compared to normoxia. Whether less severe manipulations to the O_2 transport capacity during exercise influence the MLSS is unclear.

The aim of this study was to investigate maximal and submaximal physiological responses to exercise with a moderately elevated carboxyhemoglobin (COHb) concentration in healthy individuals with heterogeneous training status. An absolute increase of ~5 % COHb transiently decreases the Hb available to bind oxygen (i.e., it reduces the "effective [Hb]" by ~0.6-0.9 g/dL), which mildly reduces the O_2-carrying capacity and arterial oxygen content (CaO_2). We hypothesized that a reduced effective [Hb] would (i) decrease $\dot{V}O_2max$ and (ii) destabilize exercise performed at the MLSS. As training status seems to influence the extent to which central/peripheral factors limited $\dot{V}O_2max$ (Gifford et al., 2016; Skattebo, Calbet, et al., 2020), we also hypothesized that training status would affect the magnitude by which a reduced effective [Hb] would decrease (iii) $\dot{V}O_2max$ and (iv) MLSS, with trained individuals demonstrating a larger reduction in both variables compared to lesser trained individuals. A modest rather than substantial elevation in COHb was also chosen to assess the effect of a typical hemoglobin mass (Hb_{mass}) measurement on subsequent exercise testing, which is relevant when multiple tests are performed consecutively. We hypothesized that a recent Hb_{mass} test (i.e., while COHb remains elevated) would contraindicate submaximal and maximal exercise testing.

3.2 Methods

Ethical Approval

All participants provided written, informed consent prior to enrollment in the study. The study was approved by the University of Calgary Conjoint Health Research Ethics Board (REB19-1761) and conformed to the standards set by the Declaration of Helsinki, except for registration in a database.

Study design

This study had a double-blind cross-over design (**Figure 3.1**). In total, participants visited the laboratory 7-8 times over the course of 3-4 weeks. After being familiarized on the first visit, each participant performed two ramp incremental tests, on separate days, in a random order, following either a CO rebreathing test (CO) or a sham rebreathing test (SHAM) for Experiment I. During the next 2-3 visits, participants performed 30-min constant work rate trials to identify MLSS. Subsequently, participants performed two experimental trials at the PO corresponding to MLSS, on separate days, in a random order, following either a CO or SHAM rebreathing test for Experiment II.

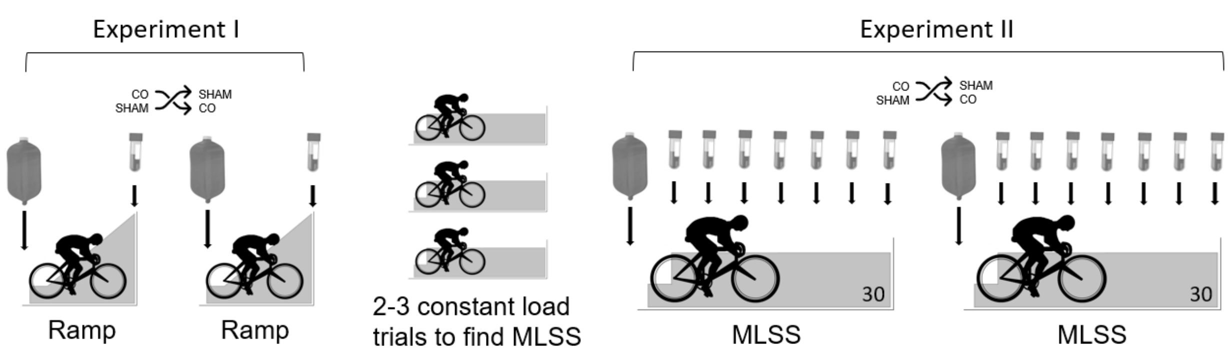

Figure 3.1. A schematic representation of the study design. Participants either inhaled carbon monoxide (CO) during the rebreathing test (CO trial) or no CO during the rebreathing test (SHAM trial) prior to ramp exercise (Experiment I) or exercise at the maximal lactate steady state (MLSS; Experiment II). The timing of rebreathing tests (bag icon) and blood samples (tube icon) are indicated on the figure. Cardiorespiratory data were recorded continuously for the four trials in Experiments I and II, and neuromuscular fatigue was assessed before and after each MLSS visit in Experiment II (not indicated on figure). Each exercise test was conducted on separate days.

Participants

Twenty participants (10 males, 10 females) of heterogeneous training status (competitive endurance athletes to sedentary) were recruited and completed the first part of the study (Experiment I). Of these participants, 16 (8 males, 8 females) completed the second part consisting of MLSS testing (Experiment II). The four participant “drop-outs” from Experiment II were lost due to laboratory shutdown during the COVID-19 pandemic. Note that MLSS data were obtained for three of these participants, as they had completed their visits to identify MLSS, whereas the other participant had not (i.e., MLSS data were available for 19 participants: 10 males, 9 females). All participants were habitually residing at mild altitude in Calgary, Alberta (approximately 1100 m above sea level, with a barometric pressure of ~660 mmHg).

Randomization and blinding

Using a concealed allotment procedure, participants were randomized into receiving the order of conditions as either SHAM-CO or CO-SHAM independently for Experiments I and II. The participants and the primary researcher administering the exercise tests were blinded to all conditions. A second, unblinded researcher led the CO rebreathing tests, filling the syringe with either CO or room air, analyzing all blood samples for COHb, and measuring the residual CO content of the rebreathing bag in a separate laboratory space. The second researcher did not directly assist with exercise tests. After finishing each experiment, the participants were asked to indicate the condition to determine the effectiveness of blinding. Participants were not unblinded until they completed the entire study, and all data were analyzed by the blinded researcher.

Body composition

Body composition was measured using whole-body dual energy X-ray absorptiometry (DXA) scans (Horizon DXA system, Hologic, Marlborough, MA) to determine fat-free mass (FFM). To facilitate comparisons between males and females (Tarnopolsky, 2008), all measures of whole-body fitness (i.e., $\dot{V}O_2$peak, peak power output (PPO), gas exchange threshold (GET), respiratory compensation point (RCP), and $\dot{V}O_2$ at MLSS) were normalized to FFM. Throughout this report, we use FFM-normalized $\dot{V}O_2$peak as a surrogate for aerobic fitness.

Hb_{mass} and vascular volumes

Hb_{mass} was measured using a modified CO rebreathing procedure, as described by (Schmidt & Prommer, 2005). Briefly, 1.0 to 1.5 mL/kg body mass (BM) of CO (determined based on sex and habitual physical activity) was injected into a closed respirometer system (Blood tec GmbH, Bayreuth, Germany) with a 3-L anesthetic bag filled with 100 % O_2, and this gas mixture was rebreathed for 2 min. Before the rebreathing, and 7 minutes post-rebreathing, blood samples were drawn via an intravenous catheter that was inserted into a forearm vein. This catheter remained in place for the duration of the cycling exercise test to collect blood samples during exercise for the determination of COHb immediately before and after the ramp incremental exercise test in Experiment I and every 5 min during exercise at MLSS in Experiment II.

COHb and [Hb] were measured using a blood gas analyzer (ABL80 FLEX; Radiometer, Brea, CA). Hematocrit (Hct) was determined using a capillary centrifuge (StatSpin MP, Beckman-Coulter, Brea, CA). CO from exhaled air was measured using a hand-held CO detector (Dräeger Pac 7000, Dräeger AG, Lübeck, Germany) connected to a mouthpiece. To prevent inadvertent unblinding, the screen of the CO detector was not visible to the participants or the blinded researcher, and the CO detector was reprogrammed such that the alarms did not sound. In the SHAM conditions, all procedures, including blood sampling, remained identical to the real Hb_{mass} measurement, and COHb was analyzed in these samples, despite expecting no change from baseline. Blood volume (BV), plasma volume (PV), and red blood cell volume (RBCV) were calculated using 0.91 as the correction factor for venous Hct (Keiser et al., 2017).

Ramp incremental tests (Experiment I)

All exercise tests were conducted on an electromagnetically braked cycle ergometer (Velotron; Dynafit Pro, Racer Mate, Seattle, WA, USA) in which the work rate is externally controlled and independent of cadence. The two ramp incremental tests were identical. After completing a 4-min warm-up at 50 W, a 30 W/min ramp (i.e., 1W/2s) began. Participants cycled until they reached volitional exhaustion or were unable to maintain a cadence within 20 rpm of their self-selected cadence. Perceptual responses were recorded during warm-up and after task failure. Capillary [La] was measured during warm-up and 1-2 min after task failure. The PO at task failure was defined as the PPO.

Trials to identify MLSS

Participants completed 2-3 constant work rate trials to identify the highest PO at which [La] was stable, defined as an increase in [La] of ≤1 mM between 10 and 30 minutes. The initial PO was chosen using a previously published prediction equation based on the RCP corrected for mean response time and body weight (Iannetta, Fontana, et al., 2018). If [La] was stable, the work rate was increased by 10 W for the next trial, and if [La] was unstable (i.e., increased by >1mM), the work rate reduced by 10 W, until the highest PO with a stable [La] was identified. During these trials, cardiopulmonary data were recorded continuously, and [La] and perceptual responses were recorded every 5 min.

Experimental MLSS trials (Experiment II)

Participants completed two constant work rate tests at their individual MLSS, with either CO or SHAM rebreathing tests performed before the exercise bout in a random order. In these trials, venous blood and capillary blood were collected every 5 min during exercise at MLSS to measure COHb and [La], respectively. Peripheral O_2 saturation (SpO_2) data were collected continuously from a finger. Neuromuscular fatigue (NMF) measurements were performed at baseline and immediately after the 30-min bout (or at task failure if participants could not cycle for 30 min). The test started with a 4-min warm-up at 50 W and then the PO increased to the participant's individual MLSS. Participants were not informed of any physiological variables during exercise at MLSS, but they received feedback on their cadence and were instructed to keep cadence constant at a self-selected value.

Pulmonary gas exchange variables

Ventilatory and gas exchange variables were measured with a metabolic cart (Quark CPET, Cosmed, Rome, Italy) using a physical mixing chamber and 10-s data averaging. The flowmeter was calibrated using a 3-L syringe as per manufacturer's instructions. Gas analyzers were calibrated using a precision-analyzed gas mixture of known concentration (15.90% O_2, 4.95% CO_2, and balanced N_2). Heart rate (HR) was monitored and recorded continuously using a chest strap HR monitor (Polar Electro, Kempele, Finland). The gas exchange threshold (GET) and the respiratory compensation point (RCP) were identified from blinded data to the nearest 0.1 L/min independently by two researchers, using previously published methods (Beaver et al., 1986; Beever et al., 2020). Any discrepancies were discussed until agreement was achieved.

Blood lactate concentration

The [La] was measured from capillary blood (finger) using a lactate analyzer Biosen C-line (EKF Diagnostic, Barleben, Germany). In Experiment I, samples were collected during warm-up and 1-2 min post-task failure. In Experiment II, duplicate capillary blood samples were analyzed every 5 min, except for timepoints 10 and 30 minutes, where measures were made in triplicate. Duplicates and triplicates were averaged to yield final values for analysis.

Peripheral O_2 saturation

As a surrogate for arterial O_2 saturation, pulse oxygen saturation (SpO_2) was measured continuously during the experimental exercise tests using a finger pulse oximeter (ADInstruments, Sydney, Australia). Since the wavelength used in pulse oximeters is unable to differentiate O_2Hb from COHb (Hampson, 1998), the values where corrected for the measured COHb to allow for comparison between the SHAM and CO conditions. As SpO_2 was measured continuously, COHb values between measurement points (warm-up and post-ramp in Experiment I; 5-min intervals in Experiment II) were linearly interpolated to obtain second-by-second data for SpO_2 correction. At each measurement point, the effective [Hb] was calculated using the following equation:

$$\text{Effective [Hb]} = \text{[Hb]} - (\text{COHb (\%)} * \text{[Hb]}/100). \qquad \text{(Eq. 3.1)}$$

Perceptual variables

Perceptual responses were recorded in the exercise tests before and after (Experiment I) or every 5 minutes (Experiment II). At these time points, participants were asked to indicate their rating of perceived exertion (6-20; RPE) (Borg, 1982), rating of fatigue (1-10; ROF) (Micklewright et al., 2017), and feeling scale (-5 to +5; FS) (Hardy & Rejeski, 1989). The questions for RPE, ROF, and FS were presented as “How hard are you working?”, “How fatigued do you feel?”, and “How are you feeling overall?”, respectively.

Dietary standardization

The testing was performed at the same time of day for each individual (±1 hour). The participants completed a 24-h food and physical activity log before the experimental days and were instructed to repeat them for subsequent tests. Participants refrained from consuming caffeinated drinks (on day of testing) and alcohol (24 hours prior to testing). To ensure similar carbohydrate availability between the visits, participants were provided with two individualized meals to consume at standardized times before their tests.

Neuromuscular fatigue

NMF was assessed pre- and post-exercise in Experiment II using a custom-made chair to which the participants could move directly from the cycle ergometer at the end of the MLSS trial (30 min or task failure). For all NMF testing, participants were strapped to the chair at their hips and shoulders, with their hips and knees at 90°. The right leg of each participant was secured to an S-beam load cell (OMEGA Engineering, Norwalk CT). Prior to any testing, participants were instrumented for NMF assessment. The stimulating cathode electrode (Ag-AgCl discs, 20 mm diameter, Kendall MediTrace foam electrodes, MA) was placed in proximity to the femoral nerve in the inguinal triangle. The anode electrode (Durastick Plus, DJO Global, Vista, CA) was placed between the greater trochanter and gluteal fold. The amperage was increased gradually until a plateau in the force response was observed.

Before the two MLSS rides in Experiment II, the participants performed a baseline NMF assessment beginning with a warm-up of 2 x 10%, 2 x 30%, 2 x 50%, and 1 x 70% MVC contraction for 5 s, then proceeded to the stimulation protocol after ~1 min of rest. The NMF stimulation protocol was similar to procedures reported previously from our faculty (Azevedo et al., 2021). Briefly, participants were instructed to perform an isometric maximal voluntary contraction (MVC), and a high-frequency doublet (100 Hz) was delivered at the force plateau. The peak force elicited by this stimulation was the superimposed twitch (SIT) force. After a 5-s delay, a 100 Hz doublet, a 10 Hz doublet, and a single stimulus followed, at 2-s intervals to measure potentiated high- and low-frequency and single twitch forces. The amperage for all pulses was equal to 130% of the amperage that elicited maximal twitch force during instrumentation on the same day. The time delay from the end of exercise until the start of the MVC was 25 [7] s.

Data analysis

Ventilatory data

All mixing chamber measurements were filtered using the software Origin 2020 (OriginLab Corporation, Northhampton, MA) to remove any data points laying outside 3 SD of the local mean, time aligned, and interpolated from 10-sec averages to 1-sec data. The highest 30-sec rolling averages from the ramp incremental tests were considered $\dot{V}O_2$peak. Other ventilatory peak values (i.e., $\dot{V}_E$, f_B, and V_T) were determined in the same manner. For the constant-load exercise time points in Experiment II, 2-min averages of data around the timepoint (e.g., minutes 9 to 11 for 10 min) or the last 2 min for the endpoint were calculated. The O_2 pulse was calculated by dividing the $\dot{V}O_2$ at these timepoints by the corresponding HR. For all ventilatory data, the 10-min, 20-min and end-point ("END") were used for analysis.

NMF data

All NMF data were analyzed in Labchart 8 (ADInstruments). Global fatigue was determined by the magnitude of decline in peak force during the isometric MVC. Peripheral fatigue was determined by assessing the declines in the potentiated 100 Hz peak force, the ratio of peak forces at 10 Hz and 100 Hz (i.e., low-frequency fatigue), and the potentiated single twitch peak force (Pt). As a measure of central fatigue, voluntary activation (%) with femoral nerve electrical stimulation was calculated with the following equation:

$$VA = 1 - \frac{(SIT - Force\ before\ SIT) \cdot Force\ before\ SIT}{MVC \cdot Db100Hz} \cdot 100\% \qquad \text{(Eq 3.2)}$$

Sample size justification

For our main outcome variable, the [La] response (stable/unstable), estimation of the sample size needed was based on the McNemar's test. Since no previous data on the stability of exercise at MLSS after CO exposure existed, we used published physiological responses to CO in heavy exercise (Ekblom & Huot, 1972; Koike et al., 1991) to estimate possible outcomes. The primary outcome was the attainment of a steady [La] response (increase $\leq$ 1.0 mmol/L between minutes 10 and 30) in submaximal exercise (i.e., MLSS). If [La] increased > 1.0 mmol/L after inhaling CO, then the participant is unstable and MLSS is considered to have decreased. Assuming an odds ratio of 0.11 (45% stable in both SHAM and CO, 45% stable in SHAM and not CO, 5%

stable in CO but not SHAM, and 5% stable in neither), a proportion of discordant pairs of 0.5, 80% statistical power, and an alpha of 5%, the anticipated sample size needed to detect an effect was 16 with a one-tailed McNemar test, which was the sample size achieved for Experiment II.

Statistics

Blinding success was evaluated using binomial tests to compare the percentage of correctly identified conditions against the random chance of guessing correctly (i.e., 50%). The main outcome, attainment of a stable [La] response, was dichotomous (i.e., stable/unstable) and was analyzed with McNemar's test between the CO and SHAM conditions.

For continuous variables in Experiment I, a paired sample t test was used to compare the outcomes between CO and SHAM for all participants. The Kolmogorov-Smirnov test was used to assess normality. For non-normal data (only $[La]_{peak}$), the Wilcoxon signed-rank test was used. Sex differences in the effect of CO on threshold (GET, RCP) and maximal performance (PPO, $\dot{V}O_2$peak) were assessed using independent sample t tests. Pearson's correlation coefficient (r) was calculated to assess the relationship between fitness status ($\dot{V}O_2$peak in units of $mL \cdot kg\ FFM^{-1} \cdot min^{-1}$) and change in performance variables between SHAM and CO. For most statistical tests in Experiment I, the sample size was n = 20; however, due to missing data (i.e., technical errors and quality control), sample sizes range from 17-20, depending on the measure (see figures/tables).

Time course data in Experiment II were analyzed with two-way repeated measures ANOVA with condition (SHAM or CO) as one factor and time (10, 20, and END; or Pre and Post for NMF variables) as the other factor. If a violation of sphericity occurred, the Greenhouse-Geisser correction was applied. When appropriate, post hoc testing was performed with Šidák correction. Additional analysis was performed to compare males and females and stable/unstable individuals using independent sample t tests. For Experiment II, the sample size was limited to 16 participants; however, due to missing data (i.e., technical errors and quality control) and one participant not lasting 20 min at MLSS in CO, sample sizes range from 13-16, depending on the measure (see figures/tables). The reliability estimates for the NMF parameters are derived from the baseline measurements which were repeated twice (typical error = $SD_{Difference} / \sqrt{2}$).

The effect of aerobic fitness on [La] stability at MLSS in CO was assessed by comparing the $\dot{V}O_2$peak and $\dot{V}O_2$ at MLSS of stable individuals to the unstable individuals using independent sample t tests. Similar comparisons were made for COHb and SpO_2 at the END time point in the CO and SHAM trials as well as hematological values (i.e., Hb_{mass} and BV). In addition, the CO-SHAM difference of drift in the physiological and perceptual variables from minute 10 to END were compared between these groups to ascertain whether the unstable [La] response was associated with other unstable responses.

For hematological variables, measured both in Experiment I and II, the typical error from 15 pairs was calculated. Although 16 participants partook in both experiments, one outlier with a difference between tests >3 SD of the group was removed. Statistical analysis of hematological variables was based on a combination of the average values from Experiments I and II (n = 16, with 8 participants per sex) or a single value from Experiment I (n = 4, with 2 participants per sex). For correlations with hematological variables, sample sizes were n=20 when data were derived from ramp incremental tests (Experiment I) and n=19 when data were derived from MLSS tests (n = 16 from Experiment II and n = 3 from MLSS testing). Fisher's exact test was used to compare proportions of males and females who were stable in the CO condition.

Effect sizes were measured as Cohen's d or d_z for independent and paired-sample t tests, respectively.

All statistical analysis was performed in Prism 9 (GraphPad V9.2.0). Significance was set to α = 0.05 for all outcomes. Data are presented as mean [standard deviation].

3.3 Results

Participant characteristics

The characteristics of participants are presented in **Table 3.1**. For Experiment I (n=20) and Experiment II (n=16), FFM and absolute $\dot{V}O_2$peak, PPO, and MLSS $\dot{V}O_2$ and PO were significantly different between sexes. Age, $\dot{V}O_2$peak, PPO, and MLSS $\dot{V}O_2$ normalized to BM and FFM, MLSS PO normalized to FFM, and the $\dot{V}O_2$ at RCP and MLSS as percentages of $\dot{V}O_2$peak were not significantly different between sexes for either experiment. The only discrepancies between participant characteristics across the two experiments were that the BM of males was greater than females in Experiment I only and the MLSS PO normalized to BM was greater in males compared to females in Experiment II only.

Experiment I – Blinding success

11 out of 20 participants correctly identified the condition when asked after completing the two ramp tests. This result (55% correct) was not significantly different ($P = 0.82$) from a distribution obtained by random guessing (50%); hence the blinding was considered successful.

Experiment I – Manipulation of effective [Hb]

The effects of CO on blood parameters in Experiment I are reported in **Table 3.2.** [Hb] was not different between conditions at both time points and increased from pre- to post-ramp in both conditions. COHb was significantly elevated pre-ramp and post-ramp in the CO condition compared to SHAM and decreased significantly in both conditions, but to a greater extent in CO. As a result, effective [Hb] increased in both conditions (to a greater extent in CO than SHAM) and was lower pre- and post-ramp in CO compared to SHAM. Finally, SpO_2 was also significantly lower in CO compared to SHAM and the decline was not different in the conditions.

Experiment I – Physiological responses

As shown in **Figure 3.2**, $\dot{V}O_2$peak (-0.14 [0.12] $L \cdot min^{-1}$, -4.2 [3.7] %, $P < 0.001$, d. = -1.2) and $\dot{V}O_2$ at the RCP (-0.18 [0.13] $L \cdot min^{-1}$, -6.3 [4.5] %, $P < 0.001$, d=-1.4) were lower in CO relative to SHAM, but CO did not have a statistically significant effect on the $\dot{V}O_2$ at the GET (-0.07 [0.16] $L \cdot min^{-1}$, -3.3 [7.1] %, $P = 0.077$, d = -0.4). PPO was reduced in CO (-7 [11] W, -3.3 [2.2] %, $P < 0.001$ d = 1.1). As reported in **Table 3.3**, O_2 pulse was also significantly lower in CO compared to SHAM, whereas $\dot{V}_E/\dot{V}O_2$ was significantly greater in CO compared to SHAM. Peak HR, [La], $\dot{V}_E$, f_B, and V_T were not significantly different between the two conditions (**Table 3.3**).

The reductions in PPO ($r = -0.49$) and $\dot{V}O_2$peak ($r = -0.45$) due to CO inhalation were significantly correlated to aerobic fitness, but the reductions in GET ($r = -0.14$) and RCP ($r = -0.40$) were not (**Figure 3.3**). In addition, COHb post-ramp ($r = -0.74$) was also correlated with aerobic fitness, but pre-ramp COHb ($r = -0.10$) was not (**Figure 3.3**).

Table 3.1. Participant characteristics in Experiments I and II.

	Experiment I (n=20)					Experiment II (n=16)				
	All	F (n=10)[a]	M (n=10)	P	d	All	F (n=8)	M (n=8)	P	d
Age (y)	29 [6]	28 [6]	30 [7]	0.432	0.3	29 [6]	29 [6]	29 [7]	0.938	0.0
BM (kg)	70.6 [13.9]	64.1 [8.8]	77.2 [14.2] §	**0.030**	1.1	69.4 [13.0]	64.0 [8.4]	74.7 [15.1]	0.100	0.9
FFM (kg)	57.1 [10.8]	48.9 [3.6]	65.4 [8.5] §	**<0.001**	2.7	56.6 [10.7]	49.6 [3.7]	64.9 [9.8] §	**0.001**	2.2
$\dot{V}O_2$peak ($L \cdot min^{-1}$)	3.45 [0.78]	2.93 [0.39]	3.97 [0.67] §	**0.001**	1.9	3.49 [0.77]	2.91 [0.46]	4.08 [0.53] §	**<0.001**	2.5
$\dot{V}O_2$peak ($mL \cdot kg\ BM^{-1} \cdot min^{-1}$)	49.9 [11.7]	46.7 [9.3]	53.0 [12.3]	0.233	0.6	51.1 [11.1]	46.4 [10.7]	55.8 [11.3]	0.089	0.9
$\dot{V}O_2$peak ($mL \cdot kg\ FFM^{-1} \cdot min^{-1}$)	60.8 [10.6]	60.1 [8.5]	61.5[12.9]	0.834	0.1	61.9 [9.8]	59.5 [9.4]	64.3 [10.1]	0.372	0.5
PPO (W)	325 [69]	283 [40]	368 [68] §	**0.003**	1.5	328 [66]	282 [45]	374 [49] §	**0.001**	2.0
PPO ($W \cdot kg\ BM^{-1}$)	4.7 [1.1]	4.5 [1.0]	4.9 [1.1]	0.423	0.4	4.8 [1.0]	4.5 [1.1]	5.1 [0.8]	0.220	0.6
PPO ($W \cdot kg\ FFM^{-1}$)	5.7 [1.0]	5.8 [0.9]	5.7 [1.1]	0.792	0.1	5.8 [0.9]	5.8 [1.0]	5.9 [0.8]	0.815	0.1
PO at MLSS (W)	203 [60]	164 [58]	239 [59] §	**0.003**	1.3	204 [58]	162 [28]	246 [49] §	**0.001**	2.1
PO at MLSS ($W \cdot kg\ BM^{-1}$)	2.9 [0.8]	2.7 [0.6]	3.2 [0.9]	0.173	0.7	3.0 [0.7]	2.6 [0.6]	3.3 [0.7] §	**0.034**	1.1
PO at MLSS ($W \cdot kg\ FFM^{-1}$)	3.6 [0.8]	3.4 [0.6]	3.7 [1.0]	0.436	0.4	3.6 [0.7]	3.3 [0.6]	3.9 [0.8]	0.132	0.8
$\dot{V}O_2$ at MLSS ($L \cdot min^{-1}$)	2.94 [0.80]	2.38 [0.28]	3.27 [0.75] §	**0.004**	1.5	2.87 [0.70]	2.39 [0.30]	3.35 [0.67] §	**0.002**	1.6
$\dot{V}O_2$ at MLSS ($mL \cdot kg\ BM^{-1} \cdot min^{-1}$)	41.3 [10.1]	38.7 [7.5]	43.6 [11.9]	0.301	0.5	41.9 [9.5]	38.1 [7.9]	45.6 [9.9]	0.115	0.8
$\dot{V}O_2$ at MLSS ($mL \cdot kg\ FFM^{-1} \cdot min^{-1}$)	50.8 [8.9]	49.0 [6.7]	52.6 [10.9]	0.432	0.4	50.0 [9.8]	49.3 [6.3]	50.7 12.5]	0.765	0.1
RCP (% of $\dot{V}O_2$peak)	87 [4]	86 [6]	85 [4]	0.668	0.2	86 [5]	86 [7]	85 [3]	0.786	0.2
MLSS (% of $\dot{V}O_2$peak)	82 [6]	83 [6]	82 [7]	0.388	0.2	82 [7]	83 [6]	82 [8]	0.728	0.2

Note: All individuals in Experiment II also completed Experiment I.
BM, body mass; Fat-free mass, FFM; $\dot{V}O_2$peak, maximal oxygen uptake; RCP, respiratory compensation point; MLSS, maximal lactate steady state.

All data are presented as the mean [SD]. The § symbol indicates a statistically significant difference between males and females (P<0.05). Sample size is indicated in each column header. Cohen's d indicates the effect size for sex comparisons.
[a] For MLSS data in Experiment I, n = 19.

Males and females responded similarly to CO inhalation during ramp incremental exercise with respect to GET (males -3.8 [8.3]%, females -2.7 [6.1], P = 0.74, d = 0.2), RCP (males -5.9 [4.0]%, females -6.7 [5.1], P = 0.71, d = -0.2), $\dot{V}O_2$peak (males -3.7 [3.1]%, females -4.8 [4.3], P = 0.53, d = -0.3), and PPO (males -2.4 [1.8]%, females -4.1 [2.3], P = 0.09, d = -0.7).

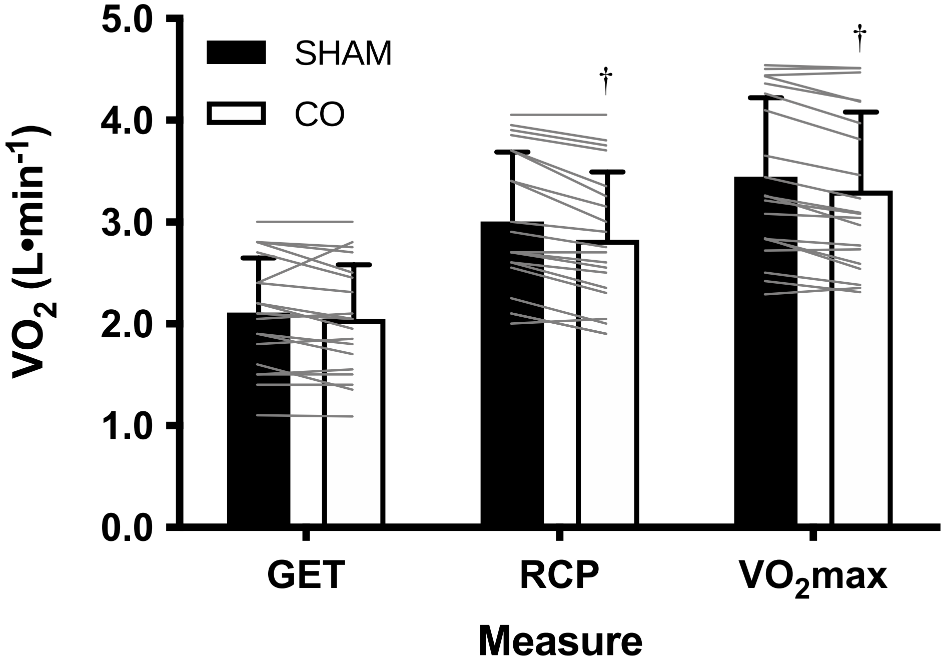

Figure 3.2. The effect of carbon monoxide (CO) inhalation on the mean gas exchange threshold (GET), respiratory compensation point (RCP), peak oxygen uptake ($\dot{V}O_2$peak). The mean SHAM (no CO inhalation) and CO (CO inhalation) data are shown in black and white bars, respectively. Error bars indicate one standard deviation of the mean. Individual data are shown as grey lines. The † symbol indicates a statistically significant difference between CO and SHAM (p<0.05). n = 20 for all variables.

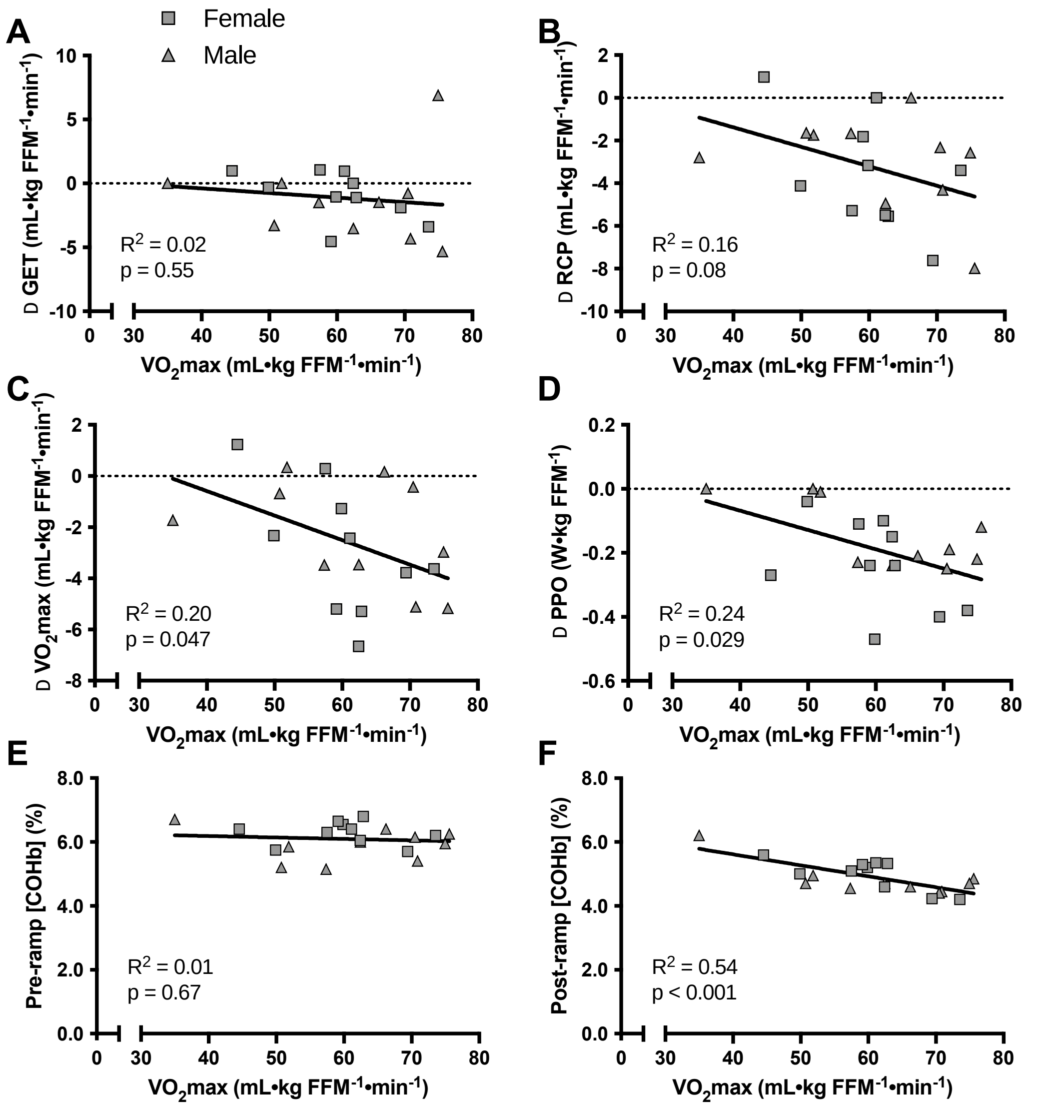

Figure 3.3. The relationship between aerobic fitness and the effects of carbon monoxide (CO) inhalation on indices of aerobic fitness. Individual data and regression lines are plotted for the decrease in gas exchange threshold (GET; Panel A), respiratory compensation point (RCP; Panel B), maximal oxygen uptake ($\dot{V}O_2$peak; Panel C), peak power output (PPO; Panel D) and the carboxyhemoglobin concentration (COHb) pre- (Panel E) and post-ramp incremental test (Panel F). For all panels, the x-axis variable is the $\dot{V}O_2$peak measured in SHAM, normalized to fat-free mass (FFM). The GET, RCP, $\dot{V}O_2$peak, and PPO data were also normalized to FFM. Squares represent females and triangles represent males. n = 20 for all panels.

Experiment II – Blinding success

14 out of 16 participants guessed the condition correctly when asked after completing the two MLSS trials, which was significantly different from the random probability of correctly guessing

the condition (P = 0.004). Anecdotally, participants indicated that their selections were based on the different perceptual responses during the trial, and no participants reported accidental unblinding during the CO/SHAM rebreathing or before beginning the exercise protocol. All participants finished the 30-min task in SHAM, but two individuals were unable to finish the exercise bout in CO (time to task failure of 19.8 and 23.3 minutes), resulting in an average task duration of 28.9 [3.0] min for CO.

Experiment II – Manipulation of effective [Hb]

The effects of CO on blood parameters in Experiment II are reported in **Table 3.2**. The [Hb] was not different across conditions at both time points and increased from pre-MLSS to END in both conditions. COHb was significantly elevated at both time points in the CO condition compared to SHAM and decreased significantly in CO but was stable in SHAM. As a result, effective [Hb] was lower in CO compared to SHAM pre-MLSS and at END and increased in both conditions but to a greater extent in CO than SHAM. SpO_2 was also significantly lower in CO compared to SHAM and declined in both conditions, but to a greater extent in SHAM.

Experiment II – Physiological and perceptual responses to MLSS

Compared to SHAM (14/16 stable; 88%), the [La] response was less likely to be stable in CO (7/16 stable; 44%) during the 30-min bout at MLSS (P=0.023, two-sided, test statistic 5.14). The [La] responses to CO and SHAM for all participants (n=16), including ANOVA results, are shown in **Figure 3.4** (Panel A), with those who were stable (n=7) or unstable (n=9) in the CO condition plotted separately (Panels B and C). There was a significant interaction effect for the overall group, with the SHAM [La] stable and lower at all time points than the [La] in CO, which increased over time. There was a main effect of condition but no effect of time in the stable group, with CO being greater than SHAM at all time points, but both conditions being stable. Finally, there was an interaction effect for [La] in the unstable group, with [La] stable in SHAM and lower at all time points compared to CO, which increased over time.

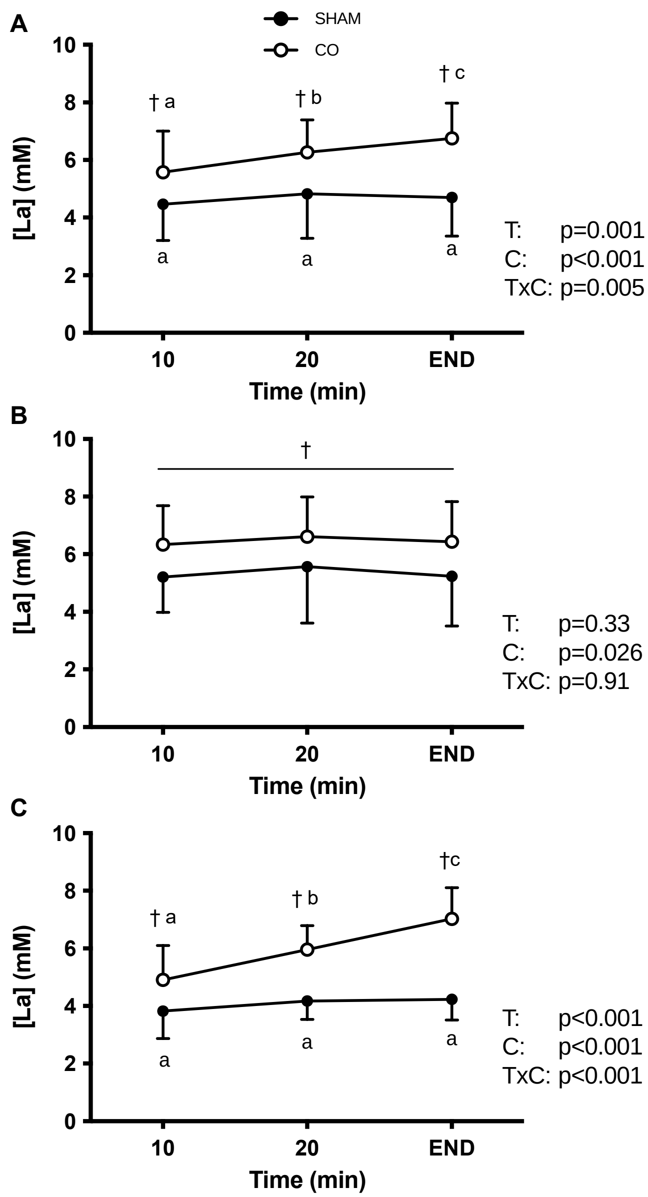

Figure 3.4. The effect of carbon monoxide (CO) inhalation on capillary lactate concentration ([La]) during cycling at the maximal lactate steady state (MLSS). The SHAM (no CO inhalation) and CO (CO inhalation) mean data are shown in black and white circles, respectively. Panels show the overall response (n=16) (Panel A) and the responses of participants who were stable (n=7; Panel B) or unstable (n=9; Panel C) in the CO condition. Error bars indicate the standard deviation. P values for main effects (time and condition) and interaction effects (time x condition) are included in each panel. The † symbol indicates a statistically significant difference ($p<0.05$) between means in opposite conditions (SHAM vs. CO) at the specified time point(s). When letters are present, pairs of means within the same condition are statistically significant if the corresponding time points do not share a common letter.

Table 3.2. The effect of CO inhalation on parameters related to the oxygen carrying capacity of blood for Experiments I and II.

		SHAM			CO			P value (T, C, TxC)
	n	Pre	End	Δ	Pre	End	Δ	
<u>Ramp</u>								
[Hb] ($g \cdot dL^{-1}$)	19	15.5 [1.4]	16.5 [1.5] *	1.0 [0.3]	15.4 [1.3]	16.5 [1.4] *	1.1 [0.4]	**<0.001**; 0.52; 0.13
COHb (%)	20	0.95 [0.12]	0.83 [0.13] *	-0.11 [0.09]	6.09 [0.47] †	4.90 [0.50] †*	-1.19 [0.43]	**<0.001; <0.001; <0.001**
Effective [Hb] ($g \cdot dL^{-1}$)	19	15.4 [1.3]	16.5 [1.5] *	1.0 [0.3]	14.5 [1.2] †	15.7 [1.4] †*	1.3 [0.4]	**<0.001; <0.001; 0.007**
SpO_2 (%)	18	96 [1]	90 [5] *	-5 [4]	90 [2] †	87 [4] †*	-3 [5]	**<0.001; <0.001**; 0.076
<u>MLSS</u>								
[Hb] ($g \cdot dL^{-1}$)	16	15.3 [1.2]	15.9 [1.3] *	0.7 [0.4]	15.3 [1.2]	16.0 [1.4] *	0.7 [0.4]	**<0.001**; 0.45; 0.72
COHb (%)	16	0.89 [0.12]	0.79 [0.13]	-0.11 [0.08]	5.94 [0.54] †	3.72 [0.51] †*	-2.22 [0.47]	**<0.001; <0.001; <0.001**
Effective [Hb] ($g \cdot dL^{-1}$)	16	15.1 [1.2]	15.8 [1.3] *	0.7 [0.4]]	14.4 [1.1] †	15.4 [1.4] †*	1.0 [0.4]	**<0.001; <0.001; 0.003**
SpO_2 (%)	13	95 [1]	92 [2] *	-3 [2]	90 [2] †	88 [3] †*	-2 [3]	**<0.001; <0.001; 0.029**

Note: SHAM, condition with sham CO rebreathe procedure; CO, condition with genuine CO rebreathe procedure. [Hb], hemoglobin concentration; COHb, carboxyhemoglobin concentration; SpO_2, pulse oxygen saturation.

All data are presented as the mean [SD]. The * symbol indicates a statistically significant difference ($p<0.05$) between means at different time points (pre vs. end) in the same condition. The † symbol indicates a statistically significant difference ($p<0.05$) between means in opposite conditions (SHAM vs. CO) at the same time point. Sample size is indicated in each row.

Table 3.3. The effect of CO inhalation on peak physiological parameters derived from ramp incremental tests.

	n	SHAM	CO	Δ (units)	Δ (%)	P-value	d_z
HR_{peak} (beat·min^{-1})	20	175 [10]	176 [8]	1 [4]	0.8 [2.5]	0.16	0.3
O_2 pulse (mL·beat^{-1})	20	20.2 [4.9]	19.0 [4.5] †	-1.2 [1.0]	-6.0 [5.1]	**<0.001**	-1.2
[La] (mM)	20	9.9 [2.4]	10.4 [2.1]	0.5 [1.7]	2.7 [11.5]	0.17	0.3
$\dot{V}_E$ (L·min^{-1})	20	146 [44]	146 [43]	-0.6 [11.1]	-0.2 [9.2]	0.80	0.0
V_T (L)	20	3.11 [0.77]	3.10 [0.75]	-0.01 [0.22]	-0.3 [6.7]	0.80	0.0
f_B (breath·min^{-1})	20	55 [10]	54 [10]	-1 [4]	-1.2 [7.4]	0.50	0.1
($\dot{V}_E/\dot{V}O_2$)	20	42.1 [6.5]	43.8 [6.7] †	1.7 [2.9]	4.0 [7.3]	**0.017**	0.6

Note: SHAM, condition with sham CO rebreathe procedure; CO, condition with genuine CO rebreathe procedure; HR, heart rate; [La], blood lactate concentration; $\dot{V}_E$, minute ventilation; V_T, tidal volume; f_B, breathing frequency.
All data are presented as the mean [SD]. The † symbol indicates a statistically significant difference between CO and SHAM ($p<0.05$). Sample sizes are indicated for each row. Cohen's d_z indicates the effect size for CO vs. SHAM.

The ANOVA results for physiological responses are included in **Figure 3.5**. As shown in Panels 5A-C, the $\dot{V}O_2$ was similar across conditions and increased significantly over time, whereas $\dot{V}CO_2$ was stable but significantly higher in CO compared to SHAM at all time points. Subsequently, RER decreased over time but was not different across conditions at any time point. Panels 5D-G show ventilatory responses to CO. There were statistically significant interaction effects and main effects (time and condition) for $\dot{V}_E$, f_B, and $\dot{V}_E/\dot{V}O_2$, with $\dot{V}_E$,f_B, and $\dot{V}_E/\dot{V}O_2$ being greater in CO at all time points and increasing throughout the 30 min in CO but increasing from 10-20 min and then remaining stable from 20 min to END in SHAM. In contrast, there was only a main effect of time for V_T, with significantly lower means at 20 min and END compared to 10 min. Panels 5H and 5I show HR and O_2 pulse responses to CO. There were significant interaction and main effects (time and condition) for HR, with CO being greater than SHAM and the mean increasing in both groups over time. There was also a significant interaction for O_2 pulse as well as a main effect of time, with O_2 pulse declining throughout exercise in SHAM but declining only from 10 to 20 min and then remaining stable to END in CO.

In terms of perceptual responses (**Figure 3.5, Panels J-K**), there were significant interaction and time effects for ROF and FS and significant main effects of condition for RPE, ROF, and FS. The RPE and ROF were both greater in CO at all time points compared to SHAM, and both increased over time in each condition. FS was lower in CO than SHAM at all time points, and continually declined in CO but remained unchanged from 20 min until END in SHAM after declining from 10-20 min.

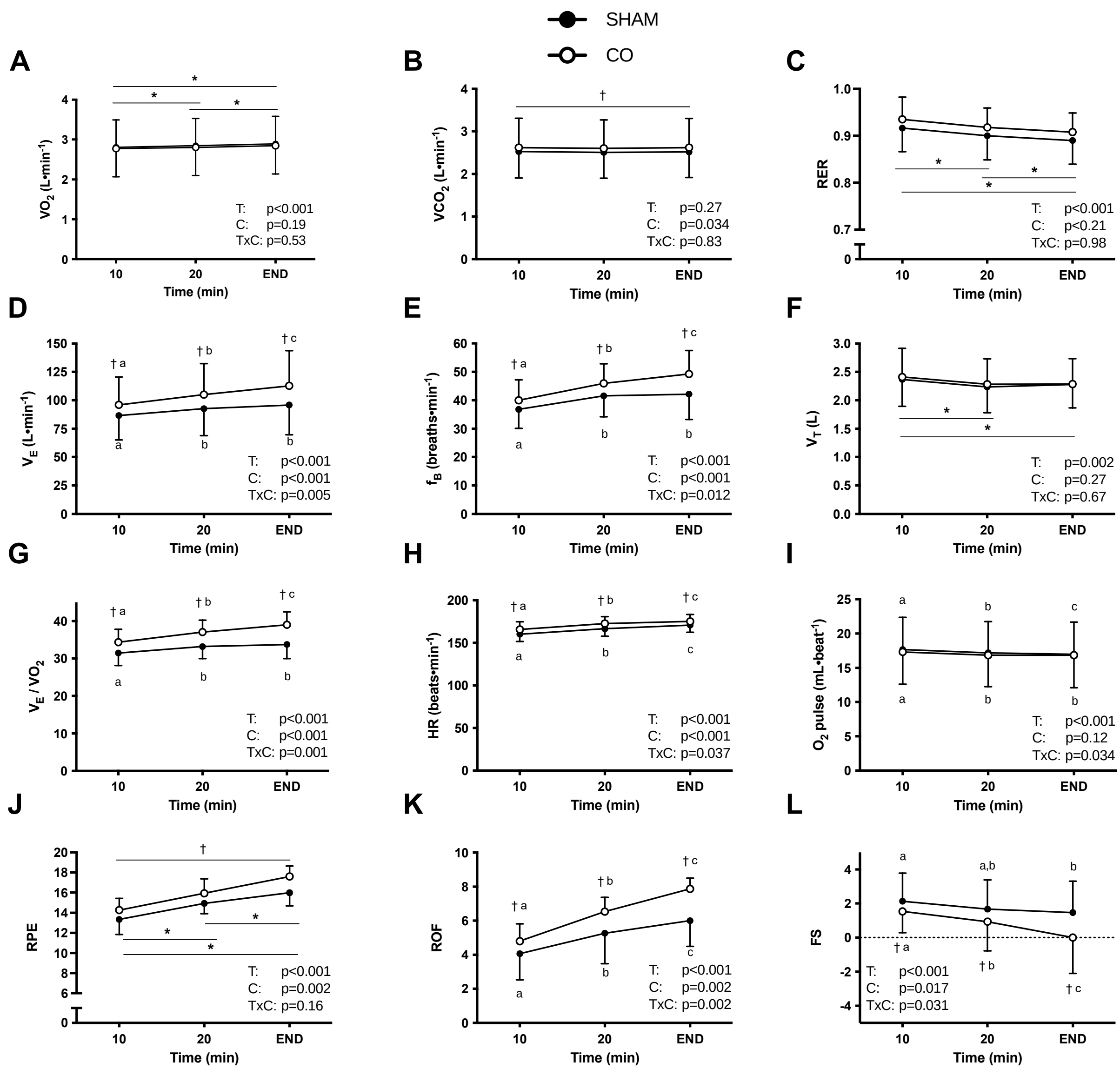

Figure 3.5. The effect of carbon monoxide (CO) inhalation on physiological responses to cycling at the maximal lactate steady state (MLSS). The mean SHAM (no CO inhalation) and CO (CO inhalation) data are shown in black and white circles, respectively. Separate plots are included for oxygen uptake ($\dot{V}O_2$; Panel A), carbon dioxide production ($\dot{V}CO_2$; Panel B), respiratory exchange ratio (RER; Panel C), ventilation ($\dot{V}_E$; Panel D), breathing frequency (f_B; Panel E), tidal volume (V_T; Panel F), the $\dot{V}_E/\dot{V}O_2$ ratio (Panel G), heart rate (HR; Panel H), O_2 pulse (Panel I), rating of perceived exertion (RPE; Panel J), rating of fatigue (ROF; Panel K), and feeling scale (Panel L). Error bars represent one standard deviation. P-values for main effects (time [T] and condition [C]) and interaction effects (time x condition [TxC]) are included in each panel. The * symbol indicates a statistically significant difference ($p<0.05$) between means at different time points for both conditions. The † symbol indicates a statistically significant difference ($p<0.05$) between means in opposite conditions (SHAM vs. CO) at the specified time point(s). When letters are present, pairs of means within the same condition are statistically

significant if the corresponding time points do not share a common letter. Sample sizes are as follows (see Methods): n = 15 (Panels J-L), n = 14 (Panels A-H), n = 13 (Panels I).

NMF responses to CO, including ANOVA results, are reported in **Table 3.4**. Cycling at MLSS resulted in similar overall fatigue (i.e., decrease in the isometric MVC force of the quadriceps muscles), as indicated by a main effect of time (without a significant main effect of condition or interaction effect). No central fatigue was evident, as voluntary activation post-exercise was unchanged from baseline (no significant main effects). There was a significant condition x time interaction and main effect of time for low-frequency fatigue. Post hoc testing revealed that low-frequency fatigue decreased in both conditions but without significant differences across conditions at either time point, indicating that the decrease in CO was greater than the decrease in SHAM (**Table 3.4**). In contrast, Pt force decreased similarly in the two conditions (main effect of time only).

Experiment II – [La] stability in CO

In an attempt to explain why some participants were unstable in CO, we compared characteristics of the stable and unstable groups. As shown in **Figure 3.6**, COHb at baseline (6.07 [0.34] vs. 5.83 [0.74] %; p = 0.41, d = 0.4) and End (3.76 [0.36] vs. 3.84 [0.56] %; p = 0.72, d = 0.2) were similar in participants who were unstable and stable. Those who were unstable in CO had higher $\dot{V}O_2$peak (66.2 [8.5] vs. 56.4 [8.8] mL· kg FFM^{-1}·min^{-1}, P = 0.042, d = 1.1) and $\dot{V}O_2$ at MLSS (55.8 vs. 44.3 mL· kg FFM^{-1}·min^{-1}, P = 0.006, d = 1.7) values in SHAM compared to those who were stable. Hb_{mass} (14.2 [1.2] vs. 14.2 [1.8] g· kg FFM^{-1}; p = 0.97, d = 0) and BV (106.3 [7.1] vs. 103.0 [7.6] mL· kg FFM^{-1}; P = 0.39, d = 0.5) were not different between the unstable and stable groups. An unstable [La] response was not associated with change in other responses measured (i.e., from 10 min to END for $\dot{V}O_2$, V_E, RPE, RoF, or FS; data not shown). Also, no differences in NMF parameters were observed between those with stable vs. unstable [La] in the CO condition (data not shown). No sex difference in [La] stability was found, as 4/8 males and 3/8 females were stable (P = 1.00).

Table 3.4. Changes in neuromuscular fatigue variables in the CO and SHAM conditions.

		SHAM			CO			P value (T, C, TxC)	Typical error (%)
	n	**Pre**	**Post**	**Δ**	**Pre**	**Post**	**Δ**		
MVC (N)	16	686 [132]	553 [123]	-133 [42]	673 [34]	531 [22]	-142 [82]	**<0.001**, 0.052, 0.56	5.3
VA (%)	14	91.7 [4.1]	91.8 [4.7]	0.1 [6.0]	91.4 [4.5]	89.4 [6.4]	-2.1 [6.5]	0.50, 0.19, 0.30	3.4
LFF (%)	14	99.4 [12.6]	76.4 [11.7]	-23 [14]	102.9 [12.4]	71.4 [12.6]	-32 [19]	**<0.001**, 0.76, **0.023**	4.5
Pt (N)	14	171 [35]	129 [33]	-42 [29]	172 [42]	116 [27]	-56 [37]	**<0.001**, 0.38, 0.082	5.3

Note: SHAM, condition with sham CO rebreathe procedure; CO, condition with genuine CO rebreathe procedure; T, time main effect; C, condition main effect; TxC, time x condition interaction effect; MVC, maximal voluntary contraction force; VA, voluntary activation; LFF, low-frequency fatigue; Pt, single potentiated twitch force.

Delta (Δ) indicates the difference between pre- and post-trial values, in the same units.

Typical error was determined from duplicate pre-trial values.

All data are presented as the mean [SD].

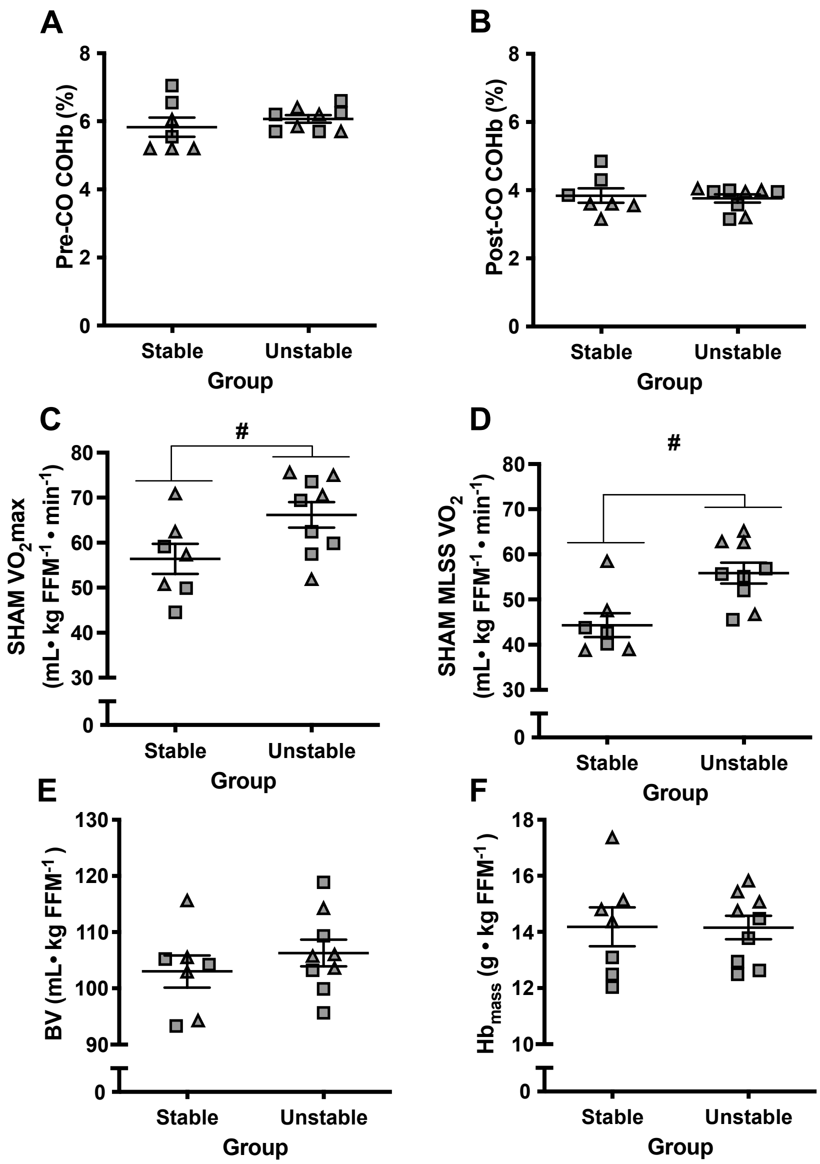

Figure 3.6. Physiological differences between participants who were stable and unstable while cycling at the maximal lactate steady state (MLSS) after carbon monoxide inhalation (i.e., the CO trial). Data are reported for pre-ramp (Panel A) and post-ramp (Panel B) carboxyhemoglobin content (COHb) for the CO trial, maximal oxygen uptake ($\dot{V}O_2$peak; Panel C) and the $\dot{V}O_2$ at MLSS (Panel D) measured for exercise without CO inhalation (SHAM), and baseline blood volume (BV; Panel E) and hemoglobin mass (Hb_{mass}; Panel F). For each panel, the mean (middle line) and standard deviation (error bars) are reported. Significant differences between groups are indicated by the # symbol. Sample sizes are n = 7 for the stable group and n = 9 for the unstable group.

Experiments I and II – Hematological variables

Hb_{mass} and vascular volumes for male and female participants matched for $\dot{V}O_2$peak normalized to FFM (see **Table 3.1**) are reported in **Table 3.5**. The absolute Hb_{mass}, BV, RBCV, and PV were greater for males compared to females; however, when normalized to BM or to FFM, only RBCV and Hb_{mass} were greater in males. The typical errors from duplicate measures in 15 participants were 1.8% (Hb_{mass}), 3.4% (RBCV), 4.2% (BV), and 5.1% (PV).

Table 3.5. Comparison of hematological variables between males and females.

Variable	Units	All (n=20)	Female (n=10)	Male (n=10)	P value (Sex)	Effect size (d)
Hb_{mass}	g	801 [205]	639 [82]	963 [154] §	**<0.001**	2.7
	$g \cdot kg\ BM^{-1}$	11.4 [2.1]	10.0 [1.2]	12.7 [2.0] §	**0.002**	1.7
	$g \cdot kg\ FFM^{-1}$	13.8 [1.7]	12.9 [1.1]	14.5 [1.5] §	**0.010**	1.2
BV	mL	5810 [1151]	5025 [584]	6595 [1044] §	**0.001**	1.9
	$mL \cdot kg\ BM^{-1}$	83.1 [11.9]	79.1 [9.1]	87.0 [13.6]	0.145	0.7
	$mL \cdot kg\ FFM^{-1}$	102.1 [10.0]	101.5 [8.2]	103.9 [6.9]	0.990	0.3
RBCV	mL	2380 [587]	1926 [284]	2835 [434] §	**<0.001**	2.5
	$mL \cdot kg\ BM^{-1}$	33.9 [6.3]	30.3 [4.2]	37.4 [6.1] §	**0.007**	1.4
	$mL \cdot kg\ FFM^{-1}$	41.5 [5.1]	38.8 [4.1]	43.8 [4.6] §	**0.039**	1.2
PV	mL	3430 [612]	3100 [347]	3760 [655] §	**0.011**	1.3
	$mL \cdot kg\ BM^{-1}$	49.2 [6.8]	48.8 [5.7]	49.6 [8.2]	0.821	0.1
	$mL \cdot kg\ FFM^{-1}$	60.6 [7.2]	62.6 [5.4]	60.1 [4.0]	0.130	-0.5
[Hb]	g/dL	14.7 [1.7]	13.6 [1.3]	15.9 [1.2] §	**0.001**	1.9
Hct	%	44.2 [4.4]	41.4 [2.7]	47.0 [4.0] §	**0.002**	1.7

Note: Hemoglobin mass (Hb_{mass}), blood volume (BV), red blood cell volume (RBCV), and plasma volume (PV) data are presented in absolute units, in units normalized to body mass (BM), and in units normalized to fat-free mass (FFM). Hemoglobin concentration ([Hb]) and hematocrit (Hct) are presented in their typical values. All data are presented as the mean [SD]. The § symbol indicates a statistically significant difference between males and females ($p<0.05$). Sample size is indicated in each column. Cohen's d indicates the effect size for sex comparisons.

3.4 Discussion

The present experiments demonstrated that maximal and submaximal exercise capacity are dependent on O_2-carrying capacity, as both $\dot{V}O_2$peak and MLSS were impaired by an acute reduction in the effective [Hb], induced by an elevated COHb (~5%). The novel aspect of this study was the focus on the MLSS. Compared to SHAM, an elevated COHb exacerbated physiological and perceptual indicators of exercise-related stress (e.g., [La], $\dot{V}_E$, HR, and RPE) while cycling at the predetermined MLSS and, based on unstable [La], MLSS was reduced in nine out of 16 participants. Whether [La] was stable after CO inhalation at this work rate depended on training status, with indicators of aerobic fitness being significantly lower in the stable group relative to the unstable group. Overall, our results suggest that factors related to oxygen delivery limit MLSS to some extent but that this limitation depends on training status and confirm that the effect of reduced O_2-carrying capacity on $\dot{V}O_2$peak is dependent on aerobic fitness.

In experiment I, ramp incremental test results were negatively impacted by CO inhalation, suggesting a dependency on O_2-carrying capacity. In agreement with previous studies (Ekblom & Huot, 1972; Ekblom et al., 1975; Schmidt & Prommer, 2005), CO inhalation significantly decreased $\dot{V}O_2$peak and PPO, further indicating that both variables are generally limited by O_2 carrying capacity (Skattebo, Calbet, et al., 2020). Previous research has mostly produced comparable results regarding the general effect of CO on $\dot{V}O_2$max: changes of -9.0% with 7% COHb (Ekblom & Huot, 1972), -6.3% with 4.3% COHb (Klausen et al., 1983), -4.8 % with 4.7% COHb (Sutehall et al., 2018), and -3.0% with 4.9 % COHb (Schmidt & Prommer, 2005) were reported in groups of individuals with similar average $\dot{V}O_2$peak (45-55 mL·kg $BM^{-1}·min^{-1}$). The reduced O_2-carrying capacity did not impair other peak physiological responses to exercise, except for O_2 pulse and $\dot{V}_E/\dot{V}O_2$, which were lower and higher in CO, respectively. We also found that CO inhalation reduced the $\dot{V}O_2$ at the RCP but not at the GET. While we are unaware of a previous study that examined the effect of an elevated COHb on metabolic rate at the RCP, this threshold is decreased in hypoxia (Azevedo et al., 2020), and increasing COHb to 11% and 20% affected $\dot{V}_E$ and $P_{ET}CO_2$ in a manner consistent with large reductions in RCP, although no statistical tests were performed on the change in $\dot{V}O_2$ at RCP (Koike et al., 1991). Similarly, the lack of effect on the $\dot{V}O_2$ at the GET is consistent with previous studies that reported only small effects of elevated COHb on HR, [La], and $\dot{V}_E$ at relatively low intensities of exercise (Ekblom & Huot, 1972; Vogel & Gleser, 1972). High intensities of exercise seem to be more negatively impacted by COHb than low intensities.

In ramp incremental tests, the effect of CO inhalation was dependent on aerobic fitness, as baseline $\dot{V}O_2$peak correlated with impairments in $\dot{V}O_2$peak and PPO. The inability to standardize COHb across fitness levels—particularly at the later portions of ramp incremental tests—and maintain a constant COHb during exercise likely attenuated some of the relationships and effects reported herein. The rate of CO loss is dependent on exercise duration and is accelerated at higher ventilation rates (Schmidt & Prommer, 2005; Zavorsky et al., 2012). Accordingly, that fitter individuals reach a greater PPO (i.e., longer duration test) and a greater peak $\dot{V}_E$ likely explains the negative correlation between post-ramp COHb and aerobic fitness (**Figure 3.3F**) despite pre-ramp COHb being unrelated to aerobic fitness (**Figure 3.3E**). Nevertheless, results from Experiment I agree with previous studies reporting an O_2 delivery limitation to $\dot{V}O_2$peak in trained individuals (Gifford et al., 2016; Lawler et al., 1988; Richardson et al., 1999; Roca et al., 1992),

Our data indicate that MLSS is also dependent on the O_2-carrying capacity. In experiment II, we observed that CO inhalation resulted in a failure to maintain stable [La] at the PO associated with MLSS, indicative of a decrease in MLSS. To our knowledge, few studies have investigated whether decreasing the O_2-carrying capacity affects the boundary between the heavy and severe intensity domains; however, our MLSS results are supported by the significant reduction in the $\dot{V}O_2$ at the RCP in experiment I, as the $\dot{V}O_2$ of RCP is considered to be a proxy for the $\dot{V}O_2$ associated with MLSS (Keir et al., 2018). Furthermore, CP is sensitive to hypoxia (Simpson et al., 2015), further supporting our findings, despite differences between CP and MLSS (Jones et al., 2019). Reducing O_2-carrying capacity by elevating COHb also exacerbated the physiological stress of exercising at the predetermined MLSS. This finding agrees with previous studies that administered CO prior to submaximal exercise, albeit at intensities not anchored to individual thresholds (Ekblom & Huot, 1972; Pirnay et al., 1971). In agreement with our NMF data, which indicated a greater reduction in the low-frequency fatigue ratio in CO relative to SHAM, reducing the F_IO_2 to manipulate O_2 delivery has previously been shown to affect quadriceps fatigue during cycling exercise, albeit in the severe intensity domain (Romer et al., 2006).

Similar to Experiment I, the reduction in MLSS induced by CO inhalation was related to training status. Although the reduction in O_2-carrying capacity was elevated in general, in the CO condition, [La] reached a steady state in seven participants, but was unstable in nine others, increasing throughout the exercise bout until the end (30-min or task failure; **Figure 3.4**). Unlike Experiment I, and likely due to the lower $\dot{V}_E$ and nearly consistent exercise duration, the reduction in COHb during exercise at MLSS was unrelated to training status, and post-exercise COHb was not different between stable and unstable groups, ruling out the possibility that differences in COHb during exercise influenced these results. The group with an unstable [La] response had greater aerobic fitness than the group with stable [La], as indicated by several variables (**Figure 3.6**), albeit not Hb_{mass} or BV. As was shown for $\dot{V}O_2$max (Lawler et al., 1988; Richardson et al., 1999), we hypothesize that the MLSS of the more trained group was limited by convective O_2 delivery whereas the MLSS of the stable group was not. Both groups experienced similar ventilatory, cardiovascular, neuromuscular, and perceptual changes in response to exercise after CO inhalation relative to SHAM. If cardiac output distribution was already optimized to ensure adequate O_2 supply to other tissues (e.g. respiratory muscles, brain, skin) in trained individuals, then hyperventilation (and greater respiratory muscle blood flow requirement) may have prevented the allocation of more cardiac output towards the exercising muscles (Calbet et al., 2004; Harms et al., 1998; Sheel et al., 2018). In fact, the two participants who were unable to finish the exercise bout in the CO condition possessed the highest values for $\dot{V}O_2$max (76.1 and 74.4 mL·kg FFM^{-1}·min^{-1}) and $\dot{V}O_2$ at MLSS (65.6 and 62.3 mL·kg FFM^{-1}·min^{-1}). That a submaximal intensity eliciting ~85 % of $\dot{V}O_2$max is unsustainable in the most trained individuals after a modest 4 % reduction in O_2-carrying capacity further demonstrates the importance of this variable to MLSS in trained individuals.

It has been suggested that the assumption of the MLSS as an indicator of a steady state whole-body metabolism may be incorrect since steady state $\dot{V}O_2$ behavior can be observed at intensities slightly above MLSS (Hill et al., 2021; Iannetta et al., 2021; Nixon et al., 2021). The assumption ignores the complex dynamics of blood [La], which may differ in trained and untrained individuals and is not captured without measuring lactate appearance and disappearance rates (Bang, 1936; Bergman et al., 1999). The capacity to oxidize lactate does not limit MLSS, since infused exogenous lactate can be utilized during exercise at an intensity that corresponds approximately to MLSS without destabilizing the [La] (Messonnier et al., 2013). Therefore, a ceiling in neither lactate uptake nor muscle oxidative capacity can explain the existence of MLSS in moderately trained individuals during whole-body exercise; however, the capacity to deliver sufficient O_2 to tissues to increase lactate oxidation could set a barrier for increasing the lactate disappearance above this threshold. Since skeletal muscle is both the main source and main disposal site of lactate, at least during the first 30 minutes of exercise (Bergman et al., 1999), a small reduction in O_2-carrying capacity induced by elevated COHb may disturb this fine-tuned balance: intracellular PO_2 has been shown to influence lactate production mediated by changes in the [ADP]/[ATP] ratio (Ferguson et al., 2018). Future research should measure changes to blood flow and lactate kinetics at and above the MLSS to assess whether insufficient convective O_2 delivery plays a role in exceeding this threshold.

Training-status dependent responses to exercise also highlight the importance of appropriate matching of aerobic fitness when sex-related differences are investigated. Matching of males and females, using the gold-standard approach of normalization to FFM, reduces the impacts of BM and body composition on measures of aerobic fitness (Cureton, 1981). If matching is achieved by BM-normalized $\dot{V}O_2max$, it may underestimate the true fitness of female participants (i.e., result in recruitment of better trained females than males). In the present study, no sex differences were observed for responses to exercise after CO inhalation. Based on the present data, it is likely that trained males and females both possess a greater dependency on O_2-carrying capacity for high-intensity exercise than untrained males and females. Overall, a similar impairment in $\dot{V}O_2max$ following CO inhalation was observed in both sexes, and [La] stability in CO also did not reveal any association with sex, as female participants were as likely as males to be unstable in CO. Few previous investigations have included females, and therefore limited data exists for comparison. Even when studies have included both sexes (Schmidt & Prommer, 2005), possible sex differences have not been discussed; however, a recent study in older men and women (average age 63 years) also did not indicate any sex difference in performance decrements after CO inhalation (Diaz-Canestro, Siebenmann, et al., 2021), aligning well with our results.

The negative effects of elevated COHb on exercise responses reported herein have implications for research, health, and sport. In the present study, the CO rebreathing method, which is commonly used to measure Hb_{mass}, was used to increase COHb prior to ramp incremental and MLSS testing. As both types of exercise were negatively affected by a moderately increased COHb, the CO rebreathing method should not be performed prior to exercise testing. If the CO rebreathe must be performed before exercise on the same day, to be conservative, exercise testing should not be performed until COHb has returned to approximately baseline. While exercise can accelerate the decrease in COHb (Schmidt & Prommer, 2005; Zavorsky et al., 2012), the present study suggests that this is not a practical strategy to accelerate the decrease in COHb, as even 30 min of relatively high-intensity exercise was insufficient to return COHb to baseline. Alternatively, the CO rebreathe method could be performed after exercise testing, at least after light intensity exercise (Naef et al., 2015; Plumb et al., 2018). Some evidence exists to suggest an acute increase in measured Hb_{mass} after ultra-endurance exercise (Gough et al., 2012), but it is not clear whether prior maximal exercise affects the measurement of Hb_{mass}. Maximal exercise does, however, lead to acute changes in BV and PV due to hemoconcentration (Schierbauer et al., 2021). The COHb achieved in this study was also in the range of COHb observed in cigarette smokers (~3-7%) (Hee et al., 1995) and hookah lounge visitors (~7%) (Martinasek et al., 2021), indicating that the CO inhaled during these activities is sufficient to reduce exercise capacity, albeit such an effect would depend on individual's aerobic fitness. Finally, runners exercising for 30 min near heavy traffic also have elevated COHb (~4-5%) (Nicholson & Case, 1983), suggesting their performance could be acutely impaired. That similar elevations in COHb occur outside of a laboratory setting demonstrates the ecological validity of the chosen CO dose.

Our use of CO inhalation to reduce O_2-carrying capacity has some limitations for investigating limitations to exercise performance. Firstly, it is difficult to know the full physiological effect of CO inhalation on exercise. Unlike hypoxia, which decreases the CaO_2 via a lower PaO_2 (and SaO_2), CO exposure reduces the CaO_2 by lowering the effective [Hb] but maintaining the PaO_2 (Calbet et al., 2009); thus, CO inhalation may be more analogous to decreasing RBCV while maintaining BV than it is to altitude exposure. Yet, CO inhalation is not synonymous with hemodilution either. CO shifts the Hb dissociation curve leftward, potentially causing a decrease in O_2 offloading in tissues, although 6-7% COHb did not impair extraction—measured directly from femoral arterial and venous blood gases—during sprint exercise (Calbet et al., 2015). As a vasodilator, CO increases blood flow in active muscle (Gonzalez-Alonso et al., 2001; Richardson et al., 2002), but the detrimental effects of increased perfusion on extraction (Gonzalez-Alonso et al., 2001; Lundby et al., 2008; Roca et al., 1992), could be disadvantageous in whole body exercise, where distributing a limited cardiac output becomes critical (Sheel et al., 2018). Secondly, the loss of CO from the vascular bed during heavy and especially severe exercise (exacerbated in the fittest individuals) likely reduced the impact of CO on the variables measured herein, potentially in a fitness-dependent manner for Experiment I. An effect of CO on both $\dot{V}O_2$peak and MLSS was still evident, but this effect may be greater than we have reported. Thirdly, the double-blind crossover design likely helped eliminate nocebo effects, as the known toxicity of CO may have impacted perceptual responses and motivation to reach maximal levels of effort during ramp incremental testing if a sham treatment were not performed (Raglin et al., 2020). While the sham was effective in the ramp tests, most participants correctly identified the sham condition for MLSS tests due to higher perceived effort. Regardless, the measured perceptual responses to MLSS align with the physiological responses, suggesting little to no impact on the overall study results, particularly because 14/16 participants completed the task. Knowing that they had already performed maximal exercise after CO inhalation without any harmful effect may have also reduced a potential nocebo effect during Experiment II. While some sex comparisons were made, our study was not specifically powered to compare data between males and females; therefore, these comparisons should be viewed as exploratory analyses. The present study investigated limitations to MLSS by manipulating O_2 transport; however, peripheral factors also seem to play an important role in exercise thresholds (Beever et al., 2020; Coyle et al., 1988; Iannetta et al., 2019; Ivy et al., 1980; Mitchell et al., 2018), and future studies should consider these factors when investigating limitations to MLSS. Finally, our experiment was conducted at mild altitude (~1100 m), which could influence the generalizability to sea-level conditions. As an example, the decrease in SpO_2 during the SHAM ramp incremental test indicated "moderate" exercise induced arterial hypoxemia (Dempsey & Wagner, 1999) at the group level (post-ramp mean of 90%), which may have been driven in part by the reduced inspired PO_2 of mild altitude (Gore, Little, et al., 1997). While speculative, we suggest that the participants' long-term residency at mild altitude likely mitigated the extent to which hypoxia may have exacerbated the effect of CO.

To conclude, maximal and heavy exercise is negatively impacted by CO inhalation in a fitness-dependent manner. Exercise at the MLSS was negatively impacted by a mild elevation of COHb, indicating that this threshold is dependent on O_2-carrying capacity. Specifically, CO inhalation increased indicators of physiological stress during exercise at the MLSS and the ability to maintain a stable [La] was related to indicators of aerobic fitness, with higher fitness associated with an unstable [La] response after CO inhalation. These novel results agree with previous studies of maximal exercise. Future research should examine the role of competition in blood flow distribution in setting the boundary between heavy and severe intensity exercise and whether the use of blood [La] accurately captures the highest metabolic steady state. From a practical perspective, the CO rebreathing method should be avoided prior to exercise testing or a return to baseline COHb should at least be ensured prior to testing.

Chapter IV: The effect of phlebotomy on prolonged exercise

4.1 Introduction

An increase in blood volume is one of the key adaptations to endurance training (Convertino, 1991), and increases in blood volume, whether from exercise training or blood transfusion, are directly linked with increases in maximal aerobic capacity ($\dot{V}O_2max$) (Bejder et al., 2019; Ekblom et al., 1976; Lundby et al., 2017). Conversely, the loss of one unit (~5-10%) of blood immediately reduces $\dot{V}O_2max$ (Balke et al., 1954; Ekblom et al., 1972; Krip et al., 1997) and impairs endurance at constant work rates in the severe intensity domain (Burnley et al., 2006; Ekblom et al., 1972), with effects lasting up to four weeks (Christensen & Christensen, 1978; Panebianco et al., 1995; Stangerup et al., 2017). While there is a rapid restoration of plasma volume after phlebotomy (Mora-Rodriguez et al., 2012; Saito et al., 2013), several weeks are required to replenish the lost red blood cells and total blood volume (Pottgiesser et al., 2008; Ziegler et al., 2015), leading to a decline in oxygen-carrying capacity. Accordingly, the acute impairment in $\dot{V}O_2max$ is due to reduced oxygen delivery as a consequence of lower stroke volume and/or lower [Hb], which are reduced in proportion to the amount of blood withdrawn (Calbet et al., 2006). While $\dot{V}O_2max$ is a key parameter of performance, the effect of blood withdrawal on submaximal intensities, which are also important for performance (Bassett & Howley, 2000), has not been studied in depth.

Few studies have addressed the effect of blood withdrawal on submaximal exercise capacity, particularly for exercise tasks ≥30 minutes involving heavy, steady-state exercise (Johnson et al., 2019). While studies investigating short, steady-state exercise (3-6 min) show unclear (Janetzko et al., 1998) but potentially intensity-dependent effects of phlebotomy (Celsing & Ekblom, 1986; Kanstrup & Ekblom, 1984), these findings cannot necessarily be extended to prolonged exercise. We recently demonstrated that a reduction in the effective [Hb] (via low-dose carbon monoxide inhalation) increased ventilation ($\dot{V}_E$), heart rate (HR), perception of exertion (RPE), and low-frequency fatigue (LFF) and destabilized the maximal lactate steady state (MLSS) during 30 min of exercise (Kontro et al., 2022). Similar effects would be expected in response to blood withdrawal; however, a potential reduction in blood volume may also have independent effects on these variables, as demonstrated in severe-intensity exercise (Kanstrup & Ekblom, 1984). To our knowledge, the only studies investigating acute blood loss on prolonged submaximal exercise combined phlebotomy with heat stress, obscuring the independent effect of phlebotomy (Fortney et al., 1981; Mora-Rodriguez et al., 2012). Evidence from studies augmenting red blood cell volume before longer (>30min) performance trials demonstrated that transfusions can improve prolonged exercise capacity (Bejder et al., 2019; Brien & Simon, 1987; Williams et al., 1981), suggesting a role for blood volume in prolonged exercise; however, the effects of blood withdrawal on prolonged exercise need to be addressed directly.

The purpose of this study was to investigate the extent to which reduced blood volume impairs submaximal exercise performance and affects the development of neuromuscular fatigue and the cardiovascular, respiratory, and metabolic indicators of exercise-induced stress during prolonged submaximal exercise We hypothesized that 7% blood loss would exacerbate physiological responses to heavy-intensity exercise and reduce time to task failure (TTF) at a constant power output following the prolonged bout of submaximal exercise.

4.2 Methods

Participant characteristics and ethical approval

17 healthy subjects (12 male, 5 female) who habitually participated in endurance training 3 or more times per week (and ≥3h/week) were recruited (Table 5.1). All participants provided written, informed consent prior to enrollment in the study. The study was approved by the University of Calgary Conjoint Health Research Ethics Board (REB20-2005) and conformed to the standards set by the Declaration of Helsinki, except for pre-trial registration. One participant in the PHLE group was excluded due to an adverse reaction to the phlebotomy (i.e., presyncope) and subsequent inability to exercise in the experimental trial. The participants were required to be free from any conditions and medication use affecting hematological parameters or cardiorespiratory responses to exercise and to have abstained from donating blood for a minimum of 3 months prior. Participants were required to pass the PARQ+ questionnaire. All participants were accustomed to indoor cycling and maximal endurance efforts.

Experimental protocol

Study design

This was a randomized, double-blinded, sham-controlled study. The intervention group underwent phlebotomy while the control group underwent catheterization without phlebotomy.

Participants were block randomized to either the SHAM or PHLE, stratified by sex and fitness level (defined as $\dot{V}O_2max \cdot$ kg fat free mass $(FFM)^{-1}$). Randomization was performed by an assisting researcher not involved in testing. Furthermore, only the individual performing the phlebotomy procedure and one assistant were aware of the condition, while the participants and other study personnel remained blinded until the participant completed the visit. All data were analyzed blinded.

Participants visited the lab five times during the baseline testing phase and one time for the experimental trial, which involved the phlebotomy or sham procedure and subsequent exercise testing (**Figure 4.1**). During visit 1, after measuring body composition and anthropometrics, $\dot{V}O_2max$ and exercise thresholds were assessed and a 20-min familiarization to submaximal cycling protocol, including the associated data collection, was performed. Subsequently, participants performed the full 60-min submaximal cycling protocol for familiarization (visit 2), as a control trial (visit 3), and during the experimental trial (visit 6). The amount of blood that was withdrawn for the phlebotomy group during visit 6 was determined from duplicate hemoglobin mass (Hbmass) tests performed during visits 4 and 5. The visits were scheduled over 2-3 weeks. All exercise tests were conducted on an electromagnetically braked cycle ergometer (Velotron; Dynafit Pro, Racer Mate, Seattle, WA, USA) using an externally controlled work rate that was independent of cadence. All visits were scheduled for the same time of day (±1h) for each participant with a minimum of 1 day between each test and maximum of 2 visits per week. Testing was conducted in a ventilated laboratory with a temperature of 22-23 °C. A floor fan was used for body cooling.

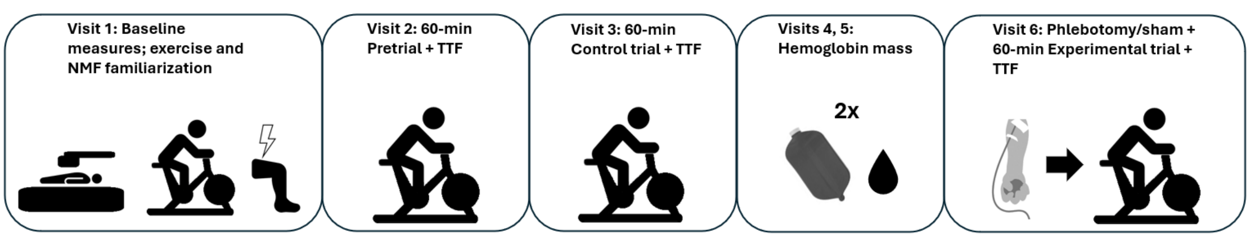

Figure 4.1. schematic of the study protocol outline the six visits that participants completed. NMF = Neuromuscular fatigue assessment; TTF = Time-to-task failure.

Exercise sessions

Step-ramp-step test

Participants completed a step-ramp-step test to measure $\dot{V}O_2$max and exercise thresholds (Iannetta et al., 2020). Briefly, a 6-min constant-load step in the moderate domain (80 or 100W) was included in the warm-up (50W) before a ramp at the rate of 30W/min began. Participants cycled until they reached volitional exhaustion or were unable to maintain their self-selected cadence within 20 rpm. After ~15 min of recovery, a 20-min heavy domain step min was performed to quantify the magnitude of the $\dot{V}O_2$ slow component. Power outputs corresponding to constant work rates at the gas exchange threshold (GET) and the respiratory compensation point (RCP) were calculated using the mean response time and the slow component corrections. The GET and RCP were independently identified to the nearest 0.1 L/min by two researchers, using previously published methods (Beaver et al., 1986; Beever et al., 2020).

Submaximal and maximal cycling test

To assess responses to submaximal and maximal exercise, participants completed 60-min constant load bouts of cycling in the heavy domain (Δ50% RCP-GET) followed by a time-to-task failure (TTF) trial at 80% of the peak power output (PPO) achieved in the ramp test. Pulmonary gas exchange variables and pulse oximetry were assessed continuously, and perceptual responses were assessed every 10 minutes, while blood lactate concentration ([La]) and neuromuscular fatigue were assessed at timepoints 0 (baseline), 20, 60 min, and post-TTF. Participant were allowed to drink water at 20 and 60 minutes (when the mask was removed), and the intake volume was reproduced in each trial.

Blood volume assessment and manipulation

Hemoglobin mass and blood volume

Hbmass was measured in duplicate on separate days before phlebotomy to calculate the required volume (7% of total) for blood withdrawal. The measurement was performed as previously reported by our laboratory, using the same techniques and assumptions (Kontro et al., 2022). Blood volume was calculated from measured [Hb] and Hct values as follows: BV = 109.9 ·

Hbmass / [Hb] (assuming 0.91 as the whole body-to-venous Hct correction factor (Chaplin et al., 1953).

Phlebotomy

In a separate laboratory, participants sat in an upright position on a plinth wearing sound-blocking headphones with their arm extended through a plastic curtain to prevent unblinding. An antecubital arm vein was catheterized and flushed with saline. In the phlebotomy condition, a blood bag (Macopharma, Mouvaux, France) placed on a scale was attached to the line and filled until reaching the required mass (calculated as 7% of measured blood volume, assuming a blood density of 0.994 g/L (Vitello et al., 2015)). In the sham condition, the nurse waited an equal time (~10 minutes) with the line clamped. On average, 496 [106] mL of blood was removed to reduce blood volume by 7%. Approximately 5-7 mL of blood was withdrawn from participants in the sham group through the catheterization/flushing procedure and for hematocrit measurements.

To collect data on the effectiveness of blinding, the participants and the primary researcher completed surveys before and after exercise on the experimental day to state whether they believed the volunteer had undergone phlebotomy or sham. A visual-analog scale was used to indicate the certainty of the answer and space was provided to explain their rationale (i.e., procedural issues vs. physiological responses and perceived changes in well-being or performance).

Data collection

Pulmonary gas exchange variables

In all exercise tests, ventilatory and gas exchange variables were measured with a metabolic cart (Quark CPET, Cosmed, Rome, Italy) using a physical mixing chamber and 10-s data averaging. The flowmeter was calibrated using a 3-L syringe as per manufacturer’s instructions. Gas analyzers were calibrated using a precision-analyzed gas mixture of known concentration (15.90% O_2, 4.95% CO_2, and balanced N_2). HR was monitored and recorded continuously using a chest strap monitor (Polar Electro, Kempele, Finland).

Body composition

Whole-body dual energy X-ray absorptiometry (DXA) scans (Lunar iDXA, GE Healthcare, Chicago, IL, USA) were used to quantify FFM.

Blood lactate, blood pressure, and pulse oximetry

Blood lactate was measured as single measurement by finger prick using a handheld device (Lactate Plus, Nova Biomedical, Waltham, MA). Blood pressure was measured at rest before exercise, and pulse oximetry was measured from a finger continuously during exercise (Masimo Rad-97, Cardiac Direct, San Diego, CA, USA).

Neuromuscular fatigue

All participants were familiarized and instrumented prior to any neuromuscular function (NMF) testing. The stimulating cathode electrode (Ag-AgCl disks, 20mm diameter, Kendall MediTrace foam electrodes, Mansfield, MA, USA) was placed adjacent to the femoral nerve in the inguinal triangle of the right groin, and the anode electrode (Durastick Plus, DJO Global, Vista, CA, USA) was placed between the ipsilateral greater trochanter and the gluteal fold. Electromyography (EMG) data was collected via electrodes placed on the rectus femoris, vastus lateralis, and hamstring muscles. A custom-built chair stationed directly behind the cycle ergometer was used for performing maximal voluntary contractions (MVC) of the quadriceps and hamstrings along with femoral nerve stimulation (Kontro et al., 2022). The participants were secured in the chair by two cross-body belts and one waist belt, with their hips and knees positioned at 90˚ of flexion, and their right leg secured into the S-beam load cell apparatus (OMEGA Engineering, Norwalk, CT, USA) to assess isometric kicking force.

Warm up and stimulus strength determination were conducted, as described before (Azevedo et al., 2021). The baseline NMF trial included two assessments, whereas NMF trials completed at 20 minutes, 60 minutes, and post-TTF included one assessment. For each NMF assessment, a 5-s MVC was performed, with a 100Hz doublet stimulus delivered at the force plateau. The peak force resulting from this 100Hz doublet, above the force plateau, is the superimposed twitch (SIT) force. Three automatic stimulations were administered 5s following the SIT (~2 s post-MVC), including 100Hz and 10Hz doublets and a single stimulus, each separated by 3 seconds. The time delay between cessation of cycling and MVC protocols was <30 s. Participants were instructed to “kick as hard as they could” during NMF assessments and were given verbal encouragement throughout the kicks.

Data analysis

NMF data

All NMF data were analysed in Labchart 8 (ADInstruments). Peripheral fatigue was assessed by examining the changes in peak potentiated single-twitch (PT) force, and LFF measured as the ratio between peak force production from low frequency (10Hz) and high frequency (100Hz) stimulation. Voluntary activation, a measure of central fatigue, was calculated using Equation 3.2.

Cardiorespiratory variables

The highest 30-sec rolling averages from the ramp incremental tests, and constant-load TTF trials, were considered $\dot{V}O_2$max. The same criterion was used to determine other ventilatory peak values (i.e., $\dot{V}_E$, fB, and VT). The highest $\dot{V}O_2$ achieved in the 60-min + TTF trial was considered $\dot{V}O_2$peak. Submaximal, constant work rate data were analyzed by averaging the last 5 minutes of the 20-, 40-, and 60-min blocks.

Statistics

Submaximal data (mean of 20-, 40-, and 60-min measures) were compared with two-way ANOVAs (group [SHAM or PHLE] x trial [control or experimental ride]). Maximal responses were analyzed by comparing the peak values recorded from TTF trials using a two-way ANOVA (group x trial). Ordinal (RPE) data were analyzed using the Mann-Whitney U test and the 60-min timepoint (in submaximal) and post-TTF (in maximal) as a point difference between trials. NMF data were analysed as a two-way ANOVA with the 60-min change from baseline as the measure of submaximal fatigue and the post-TTF change from baseline as the measure of maximal fatigue (group x trial). When no interaction effects were found, the effects of time within groups were analyzed with a repeated measures ANOVA. The differences between group characteristics were analyzed with a student’s t test. Alpha was set to 0.05 for all analyses. Reliability measures were calculated from visits 2 and 3. Typical error (TE) was calculated as TE = SD/$\sqrt{2}$, where SD is the standard deviation of the difference score between trials. Coefficient of variation (CV) was calculated as SD/$\bar{x}$) * 100, where $\bar{x}$ is the mean of the two visits.

Sample size justification

Based on previous TTF data in a within-subject study (Burnley et al., 2006), we estimated an effect size of d = 1.2 for a between-subject design. This gave us an estimate of n=20 (10 per group) to be sufficient to demonstrate a significant effect on exercise capacity in the severe domain. While 20 participants were recruited, three were withdrawn after visit 1 due to inadequate fitness (n=2) and prior to the post-phlebotomy exercise bout due to an adverse reaction (n=1).

4.3 Results

Participants

Data from 17 participants were used for the analysis of submaximal responses and from 16 participants for the analysis of maximal responses. The exclusion of one participant from the maximal data was due to the failure of this participant (who was in the SHAM group) to reach maximal levels of physical effort in the experimental trial, as indicated by the values (e.g., peak HR 154 vs. 177 bpm, peak lactate 4.5 vs. 8.2 mmol/L, peak $\dot{V}_E$ 99 vs. 134 L/min, peak RER 0.89 vs. 1.02, and TTF 90 vs. 282 s in the experimental and control trials, respectively). Such large deviation from expected values suggests premature cessation of exercise unrelated to physiological limiters and was not evident in any of the other 16 participants. Except for body mass (BM), the PHLE and SHAM groups were not different at baseline (**Table 4.1**).

Table 4.1 Participant characteristics at baseline

	SHAM (n=9, 3 F)		PHLE (n=8, 2 F)		
	Mean	**SD**	**Mean**	**SD**	**P value**
Age (y)	34.2	8.1	36.4	8.3	0.60
Body mass (kg)	68.0	8.0	79.0	11.3	**0.034**
Fat-free mass (kg)	54.7	8.3	62.2	9.9	0.11
% Body fat (%)	20%	5%	21%	6%	0.54
$\dot{V}O_2max$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	50.6	9.5	50.0	6.4	0.87
$Hb_{\mathbf{mass}}$ ($g \cdot kg^{-1}$)	11.9	1.6	12.3	1.9	0.59
BV ($mL \cdot kg^{-1}$)	85.0	10.5	89.4	12.6	0.44
Resting HR ($b \cdot min^{-1}$)	60	12	57	7	0.56
$HR_{\mathbf{peak}}$ ($b \cdot min^{-1}$)	179	12	178	12	0.77
SBP (mmHg)	122	9	123	8	0.99
DBP (mmHg)	72	7	75	7	0.35
MAP (mmHg)	89	6	91	6	0.47
PPO ($W \cdot kg^{-1}$)	4.70	0.83	4.67	0.59	0.93

Notes: $\dot{V}O_2max$ = maximal oxygen uptake; Hbmass = hemoglobin mass; BV = Blood Volume; HR = heart rate; SBP = systolic blood pressure; DBP = diastolic blood pressure; PPO = Peak Power Output

Blinding success

Blinding of the participants was successful, but blinding of the researcher during testing was not. Before exercise on the experimental day, 59% (10/17) of the participants correctly identified the condition (SHAM or PHLE). After exercise, 53% (9/17) of the participants correctly identified the condition; 18% (3/17) changed their answer from pre to post. Despite no improvement in the fraction of correct answers, the average certainty of the answer grew from 67% to 78% pre- to post-exercise. The blinded researcher correctly identified 82% (14/17) of the conditions before and 76% (13/17) after exercise, changing their mind in 18% (3/17) of the cases, also with a growing confidence in their answer from a mean of 66% to 75% certainty.

Submaximal exercise

Mean $\dot{V}O_2$ in the submaximal exercise bout was 51 [13] % of the difference between RCP and GET or 71 [6] % of $\dot{V}O2max$ without any differences between the groups (P=0.20 and P=0.42, respectively). Based on significant interaction effects (**Table 4.2**), relative to control, $\dot{V}_E$ (6 [7]%), $\dot{V}_E/\dot{V}O_2$ (8 [8]%), HR (5 [4]%), and [La] (25 [32]%) were significantly increased while O2 pulse decreased (-5 [6]%) in response to phlebotomy, whereas these variables did not change in SHAM. $\dot{V}O_2$, RER, substrate oxidation, and RPE were not affected by phlebotomy (**Table 4.2**).

Maximal exercise

Time-to-task failure was reduced by 24% in PHLE relative to the control trial while it was improved by 9% in SHAM, resulting in a difference between groups of 33% (P=0.018; **Table 4.4**). $\dot{V}O_2$max was not significantly affected by phlebotomy (P=0.089), although this effect was largely driven by one individual who demonstrated a 7% increase in measured $\dot{V}O_2$max after phlebotomy despite a similar TTF. Of the remaining participants, five showed a reduction of ≥10% in $\dot{V}O_2$max, while two showed no change (0 and 1.5%) (**Figure 4.3**). Except for O_2 pulse, which decreased by 5% (P=0.034), and $\dot{V}_E/\dot{V}O_2$, which increased by 6% (P=0.001), other peak physiological variables were not affected by phlebotomy (**Table 4.4**). In the phlebotomized participants, peak $\dot{V}_E$ in TTF was negatively correlated with fitness, while other peak variables were not (**Figure 4.3**).

Neuromuscular fatigue

NMF was evident in response to the exercise with MVC decreasing from 767 [226] N to 670 [211] N during the 60-min submaximal bout (P<0.001) and further to 643 [206] N after TTF (P=0.003). Similar responses were evident for PT, LFF, but not for VA (**Figure 4.4**). However, NMF was not affected by phlebotomy, as no significant group x trial interaction effects were observed for MVC, VA, LFF, or PT in either submaximal exercise from baseline to 60 min (**Table 4.3**) or in TTF (**Table 4.5**).

Table 4.2. Submaximal responses to 60 min of heavy exercise for participants in the SHAM and phlebotomy (PHLE) groups in control and experimental trials.

Variable	**Trial**	**Group**				**Statistics**			**Effect size**
		SHAM (n=9)		PHLE (n=8)		P value			Partial Eta Squared
		Mean	SD	Mean	SD	Group	Trial	Interaction	
$\dot{V}O_2$ (L·min^{-1})	Control	2.44	0.76	2.84	0.54	0.248	0.143	0.251	0.137
	Experimental	2.43	0.74	2.80	0.53				
	Δ	-0.01	0.08	-0.05	0.06				
$\dot{V}_E$ (L·min^{-1})	Control	76.5	20.8	87.2	12.1	0.092	0.328	**0.005**	0.064
	Experimental	73.5	19.1	92.9	15.6				
	Δ	-3.0	4.5	5.7*	6.5				
RER	Control	0.87	0.05	0.84	0.05	0.695	0.274	0.205	0.079
	Experimental	0.86	0.04	0.87	0.04				
	Δ	0.00	0.04	0.03	0.05				
HR (b·min^{-1})	Control	151	18	153	21	0.421	0.165	**0.001**	0.124
	Experimental	147	20	160	18				
	Δ	-3	4	7*	5				
O_2 pulse (mL·b^{-1})	Control	16.6	6.6	19.0	4.6	0.585	**0.036**	**0.001**	0.260
	Experimental	17.0†	6.7	17.7†	3.9				
	Δ	0.4	0.5	-1.3*	1.1				
Lactate (mmol·L^{-1})	Control	4.0	2.3	3.7	1.5	0.733	0.560	**0.014**	0.023
	Experimental	3.5	2.0	4.4	1.6				
	Δ	-0.5	0.8	0.8*	1.1				
RPE (6-20)	Control	13.6	1.5	14.4	1.0	0.423	N/A	N/A	N/A
	Experimental	13.5	1.3	14.7	1.1				
	Δ	-0.1	1.1	0.3	0.9				
CHO Ox. (g·min^{-1})	Control	1.64	0.63	1.71	0.90	0.545	0.26	0.305	0.084
	Experimental	1.65	0.70	1.99	0.67				
	Δ	0.01	0.42	0.28	0.59				

Note: * Significantly different between SHAM and PHLE. † Significantly different between control and experimental trials. Effect size is for interaction. SD, standard deviation; $\dot{V}O_2$; oxygen uptake; $\dot{V}_E$, minute ventilation; RER: respiratory exchange ratio; HR: heart rate; RPE, rating of perceived exertion; CHO Ox: Carbohydrate oxidation

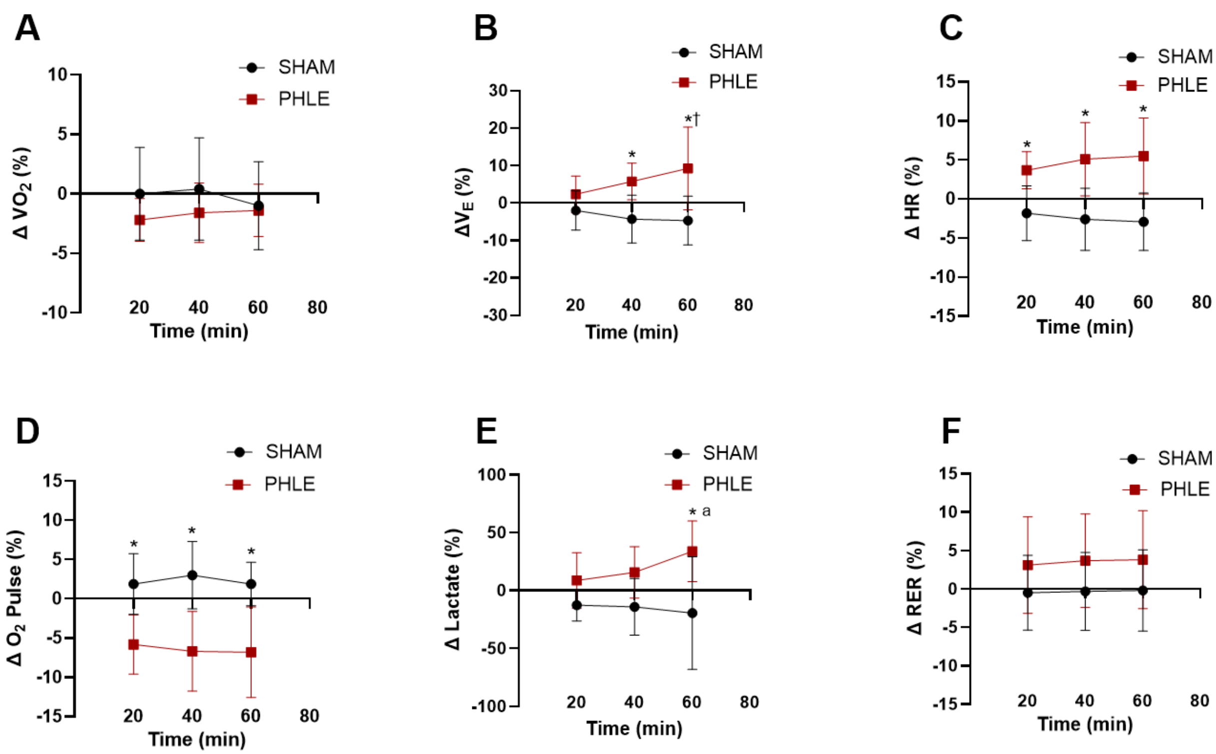

Figure 4.2. Relative changes to physiological responses from the control trial to the experimental trial, phlebotomy (PHLE) or sham phlebotomy (SHAM), over 60 minutes of exercise. Values are percent changes from the control ride at 20, 40, and 60 minutes. * Significantly different between SHAM and PHLE. † Significantly different between 20 min and 60 min. *a*: P=0.053 between 20 min and 60 min. Data are mean + SD.

Table 4.3. Percent changes in neuromuscular fatigue variables during 60 min of heavy exercise relative to baseline for participants in the SHAM and phlebotomy (PHLE) groups in control and experimental trials.

Variable	Trial	Submaximal Fatigue (60-bsln)				Statistics			Effect Size
		SHAM		PHLE		P Value			Partial η^2
		Mean	SD	Mean	SD	Group	Trial	Interaction	
MVC (%Δ)	Control	-13.7	14.5	-7.8	9.3	0.543	0.290	0.959	0.001
	Experimental	-15.0	11.9	-8.8	11.5				
	Δ	-1.3	-2.6	-1.1	2.2				
VA (%Δ)	Control	-5.2	19.6	-14.3	35.9	0.647	0.640	0.906	0.001
	Experimental	-6.3	8.3	-15.9	34.1				
	Δ	-1.1	-11.3	-1.6	-1.8				
LFF (10 Hz/100Hz) (%Δ)	Control	-29.4	13.8	-39.1	28.4	0.661	0.389	0.299	0.089
	Experimental	-32.6	11.1	-32.6	28.7				
	Δ	-3.1	-2.8	6.5	0.3				
PT (%Δ)	Control	-26.7	14.4	-28.3	19.6	0.809	0.426	0.533	0.004
	Experimental	-20.5	10.8	-28.0	27.6				
	Δ	6.2	-3.5	0.3	8.0				

Note: MVC = Maximal Voluntary Contraction; VA = Voluntary Activation; LFF = Low-Frequency Fatigue; PT = Single Potentiated Twitch. Values are the difference between SHAM and PHLE (timepoint change from baseline). For MVC, n=9 in SHAM and n=8 in PHLE. Absolute data are reported in Figure 4. For VA, LFF, and PT, n=7 in both.

Table 4.4. Maximal responses to the time-to-failure task trials following 60 min of heavy exercise relative to baseline for participants in the SHAM and phlebotomy (PHLE) groups in control and experimental trials.

Variable	**Trial**	**Group**				**Statistics**			**Effect Size**
		SHAM (n=8)		PHLE (n=8)		P value			Partial η^2
		Mean	SD	Mean	SD	Group	Trial	Interaction	
$\dot{V}O_2$ ($L{\cdot}min^{-1}$)	Control	3.47	1.01	3.83	0.79	0.572	**0.048**	0.089	0.193
	Experimental	3.45†	0.96	3.59†	0.69				
	Δ	-0.02	0.11	-0.24	0.32				
$\dot{V}_E$ ($L{\cdot}min^{-1}$)	Control	147.0	29.1	167.1	33.9	0.206	0.671	0.729	0.009
	Experimental	145.1	29.3	166.9	35.1				
	Δ	-2.0	5.4	-0.2	13.1				
RER	Control	1.02	0.07	0.98	0.06	0.375	0.241	0.614	0.019
	Experimental	1.03	0.09	1.01	0.06				
	Δ	0.01	0.07	0.03	0.09				
HR ($b{\cdot}min^{-1}$)	Control	180.3	13.3	176.1	12.5	0.575	0.131	0.708	0.010
	Experimental	178.6	14.0	175.1	13.8				
	Δ	-2	4	-1.0	2.4				
O_2 pulse ($mL{\cdot}b^{-1}$)	Control	19.6	6.9	21.9	4.9	0.572	0.055	**0.043**	0.261
	Experimental	19.6	6.6	20.7*	4.2				
	Δ	0.0	0.5	-1.2	1.6				
Lactate ($mmol{\cdot}L^{-1}$)	Control	10.1	2.6	9.6	2.4	0.533	**0.028**	0.725	0.009
	Experimental	9.2†	2.0	8.4†	2.0				
	Δ	-0.9	1.6	-1.2	1.9				
RPE (6-20)	Control	19.3	1.0	19.4	0.7	0.522	N/A	N/A	N/A
	Experimental	19.1	0.8	19.5	0.8				
	Δ	-0.1	1.0	0.1	0.4				
TTF (s)	Control	287	134	231	95	0.114	0.274	**0.018**	0.336
	Experimental	313	171	167	70				
	Δ	26	74	-64*	59				
$VE/\dot{V}O_2$	Control	43.4	6.5	44.0	6.0	0.395	0.273	**0.003**	0.536
	Experimental	42.1	7.1	46.7	5.3				
	Δ	-0.5	1.3	2.7*	1.7				

Note: *Significantly different between SHAM and PHLE. † Significantly different between control and experimental trials. Effect size is for interaction. SD, standard deviation; $\dot{V}O_2$; oxygen uptake; $\dot{V}_E$, minute ventilation; RER: respiratory exchange ratio; HR: heart rate; RPE, rating of perceived exertion.

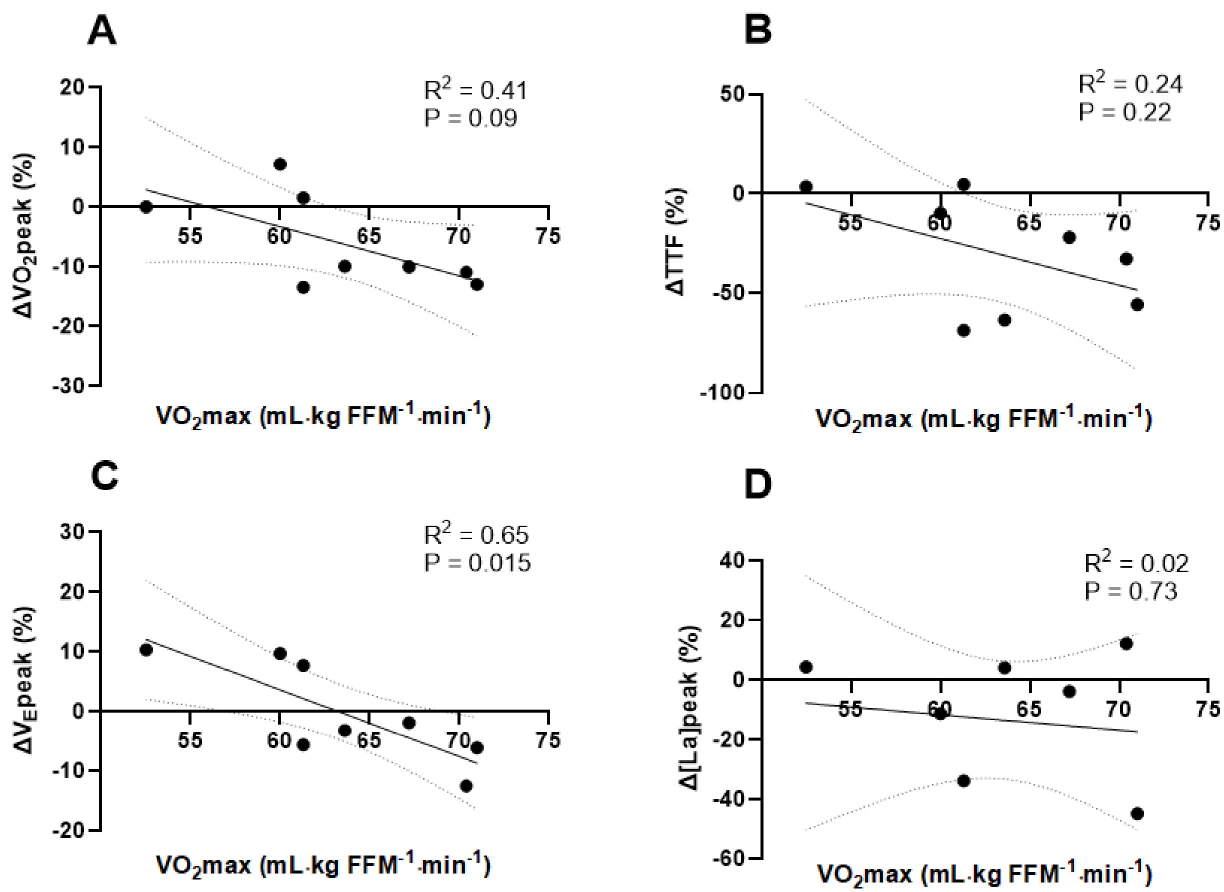

Figure 4.3. Relationships between change in (A) $\dot{V}O_2$peak; (B) time to task failure; (C) peak ventilation; and D) peak lactate following phlebotomy, relative to participant's fitness. FFM = Fat-free mass

Table 4.5. Percent changes in neuromuscular fatigue variables the time-to-failure task trials following 60 min of heavy exercise relative to baseline for participants in the SHAM and phlebotomy (PHLE) groups in control and experimental trials.

Variable	**Trial**	**Maximal Fatigue (TTF-bsln)**				**Statistics**			**Effect Size**
		SHAM		PHLE		P Value			Partial Eta Squared
		Mean	SD	Mean	SD	Group	Trial	Interaction	
MVC (%Δ)	Control	-20.2	13.0	-13.1	13.8	0.579	0.067	0.746	0.001
	Experimental	-17.4	18.1	-11.6	16.9				
	Δ	2.7	5.1	1.5	3.1				
VA (%Δ)	Control	-6.2	16.9	-5.0	9.9	0.853	0.904	0.824	<0.001
	Experimental	-5.5	9.9	-4.9	9.3				
	Δ	0.7	-7.0	0.1	-0.6				
LFF (10 Hz/100Hz) (%Δ)	Control	-39.4	18.0	-19.6	25.2	0.285	**0.047**	0.866	<0.001
	Experimental	-32.9†	8.2	-16.9†	14.4				
	Δ	6.4	-9.8	2.7	-10.8				
PT (%Δ)	Control	-46.2	12.9	-39.7	33.3	0.279	0.458	0.182	0.132
	Experimental	-28.9	11.7	-41.4	33.4				
	Δ	17.3	-1.1	-1.7	0.1				

Note: † Significantly different between control and experimental trials. Effect size is for interaction. MVC = Maximal Voluntary Contraction; VA = Voluntary Activation; LFF = Low-Frequency Fatigue; PT = Single Potentiated Twitch. Values are the difference between SHAM and PHLE (timepoint change from baseline). Absolute data are reported in Figure 4. For MVC, n=9 in SHAM and n=8 in PHLE; for VA, n=7 in SHAM and n=8 in PHLE; for LFF and PT, n=8 in SHAM and n=7 in PHLE.

Table 4.6. Measures of reliability for selected variables

Submaximal variables														
	VO_2	VCO_2	RER	V_E	F_B	HR	O_2 pulse	Cadence	Lactate	SpO_2	EE	CHO ox	Fat ox	GE
	$L \cdot min^{-1}$	$L \cdot min^{-1}$		$L \cdot min^{-1}$	$br \cdot min^{-1}$	$b \cdot min^{-1}$	$mL \cdot b^{-1}$	rpm	$mmol \cdot L^{-1}$	%	$kcal \cdot min^{-1}$	$g \cdot min^{-1}$	$g \cdot min^{-1}$	%
Typical Error	0.06	0.08	0.03	2.4	1.6	3.2	0.42	1.8	0.4	0.7	0.38	0.32	0.14	0.01
CV (%)	1.6	2.9	3.1	2.5	3.5	1.9	1.9	1.7	8.8	0.6	2.5	18.7	20.8	2.5

Maximal variables											
	VO_2	VCO_2	RER	V_E	F_B	HR	O_2 pulse	Cadence	Lactate	SpO_2	TTF
	$L \cdot min^{-1}$	$L \cdot min^{-1}$		$L \cdot min^{-1}$	$br \cdot min^{-1}$	$b \cdot min^{-1}$	$mL \cdot b^{-1}$	rpm	$mmol \cdot L^{-1}$	%	s
Typical Error	0.11	0.18	0.05	8.6	4.0	3.2	0.60	4.1	1.2	2.1	41.5
CV	2.7	4.4	3.5	4.7	5.9	1.2	2.2	3.0	8.1	1.9	13.2

Note: Variables related to substrate use are only reported for submaximal exercise, and time to task failure only for maximal exercise. EE: Energy expenditure; GE: Gross efficiency.

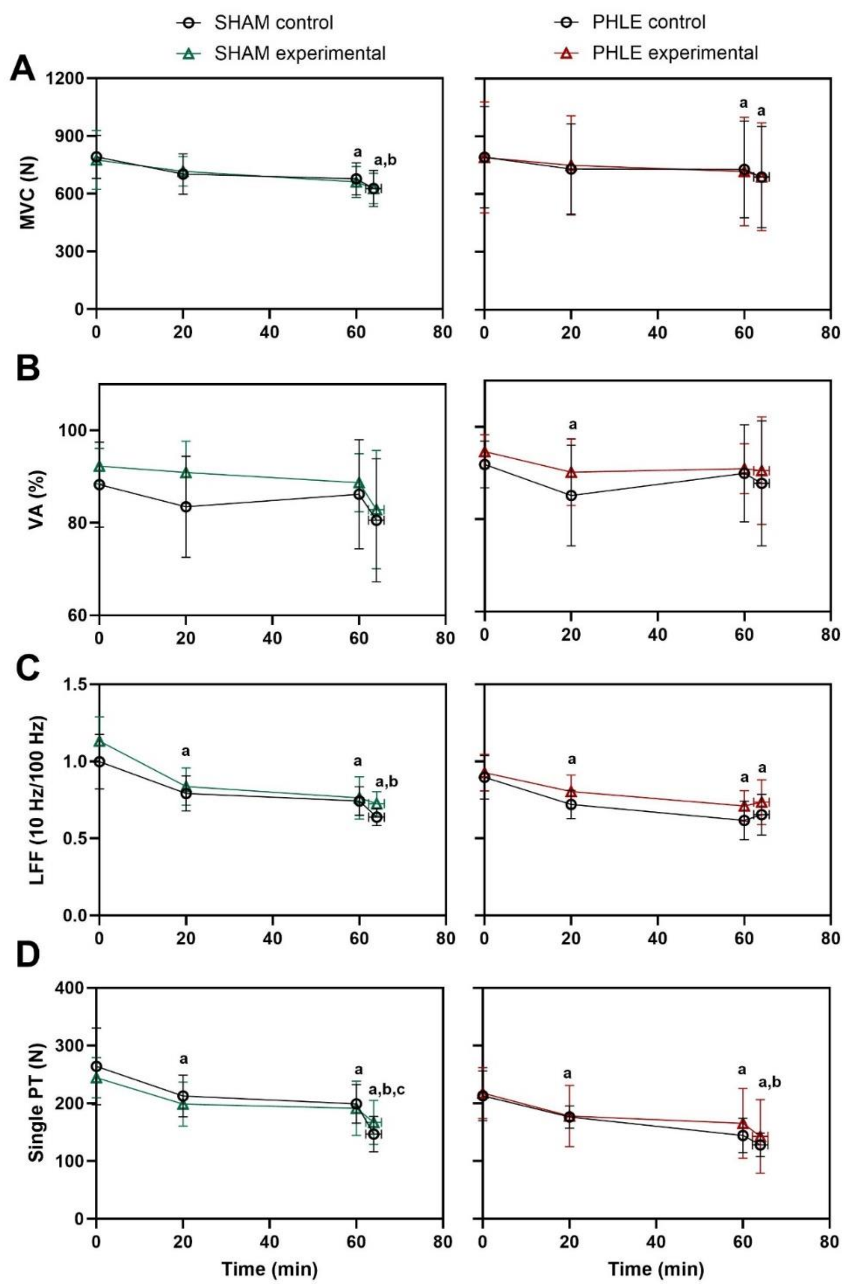

Figure 4.4. Neuromuscular fatigue variables in the sham group (SHAM) and phlebotomy group (PHLE) for each timepoint in the control trial and the experimental trial: baseline, 20 min, 60 min, and post time-to-failure; *a*: different from baseline; *b*: different from 20 min, *c*: different from 60 min. A) MVC: Maximal voluntary contraction; B) VA: Voluntary activation; C) LFF: Low-frequency fatigue; D) P_T: Potentiated twitch. MVC, n=9 in SHAM and n=8 in PHLE. For VA, LFF, and PT, n=7 in both.

4.4 Discussion

Withdrawing 7% of blood volume affected acute responses to prolonged, submaximal exercise. Sham-controlled phlebotomy increased $\dot{V}_E$, [La], and HR during 60 min of heavy-intensity cycling without affecting $\dot{V}O_2$, RER or RPE. Although NMF was unaffected by phlebotomy, the reduction in blood volume impaired subsequent severe intensity exercise performance (i.e., TTF), indicating the stress of the overall trial was likely greater following blood loss. Other peak physiological responses to exercise ($\dot{V}_E$, HR, [La], and $\dot{V}O_2$) were unaffected by phlebotomy. This is the first study to investigate acute post-phlebotomy responses in prolonged exercise without added heat stress, revealing that blood loss has systemic effects also at intensities well below $\dot{V}O_2$max.

Phlebotomy decreased the primary outcome, TTF at 80% of PPO following 60 min of heavy-intensity exercise, an indicator of the cumulative effects of exercising after blood loss The TTF decreased by 24% in PHLE compared to increasing by 9% in SHAM; therefore, if the apparent learning/training effect were considered, the overall decline in TTF performance was >30%. This is a greater impairment than reported by Burnley et al. (2006) who found a 14% decrement, and by Hill et al. (2013) who reported a 19% reduction in a severe-intensity TTF after a standard blood donation. The present study differs from those experiments by the incorporation of a demanding 60-min preload task that elevated several indicators of stress (**Table 4.2**, **Figure 4.3**), likely leaving the phlebotomized participants more compromised than the sham group prior to the TTF task. Together, the submaximal and maximal performance results indicate that the ability to resist time-dependent performance decrements (i.e., durability) was impaired (Maunder et al., 2021).

Phlebotomy impacted several cardiorespiratory and metabolic responses to prolonged submaximal exercise. Firstly, the same $\dot{V}O_2$ was required for the task but was achieved with a higher HR, resulting in a lower O_2 pulse, an outcome also demonstrated by others, albeit in the heat (Mora-Rodriguez et al., 2012). An unchanged $\dot{V}O_2$ at submaximal intensity is consistent with other studies that manipulated oxygen delivery (Celsing & Ekblom, 1986; Ekblom et al., 1976). The lower O_2 pulse is explained by a 7% reduction in Hb_{mass}, which is expected to require an increase in cardiac output to compensate for the reduction in oxygen carrying capacity or cardiac filling (secondary to a reduction in blood volume). Indeed, oxygen delivery appears to be tightly regulated and not sensitive to even large changes in [Hb] at submaximal intensities, as demonstrated by Koskolou et al. (1997). They showed that leg oxygen delivery and $\dot{V}O_2$ could be maintained in submaximal exercise by increasing cardiac output and limb blood flow after a 20% loss in total Hb, when blood volume was restored by plasma expansion. As plasma expansion did not allow maximal blood flow to be increased beyond values achieved in the control condition, $\dot{V}O_2$max was compromised post-phlebotomy (Koskolou et al., 1997). Secondly, $\dot{V}_E$ and [La] were each progressively exacerbated during the 60-minute exercise bout (**Figure 4.2**), with the greatest deviations compared with the control trial observed at the end of exercise. That the magnitude of this effect was dependent on the duration of exercise may indicate that resilience against time-dependent homeostatic disruptions is partly mediated by circulating vascular volumes. As an example, impaired thermoregulation has previously been reported during acute submaximal exercise in the heat after a standard blood donation (Mora-Rodriguez et al., 2012). Although the present study did not impose additional heat stress and provided a fan for cooling, the temperature of the laboratory was 23 ºC, and rises in core temperatures (not measured) could partly explain the observed time course. In agreement with the present study, Mora-Rodriguez et al. (2012) also reported higher [La] in steady state exercise. They also found an increased RER leading to a higher calculated CHO oxidation rate, which were numerically but not significantly greater in the present study, possibly due to insufficient statistical power.

Most studies that investigated the effects of acute blood withdrawal on submaximal exercise employed short submaximal exercise loads, often concluding that submaximal exercise performance is not impaired by phlebotomy (Janetzko et al., 1998; Panebianco et al., 1995). For example, after a standard blood donation in cyclists, Panebianco reported no difference in the submaximal $\dot{V}O_2$ values for given fixed heart rate values (130, 150, 170 bpm) in a step incremental test; however, translating oxygen pulse from an incremental test to constant-load may be problematic due to delayed HR responses (Coyle & González-Alonso, 2001), and the large $\dot{V}O_2$ variability reported therein. In our study, we fixed the work rate and assessed physiological responses to 60 min of steady exercise. Our findings generally agreed with previous studies that examined physiological responses to short submaximal exercise bouts with fixed work rates when intensities were higher (~heavy), whereas, at lower (~moderate) intensities, changes in physiological responses were small or not observed (Celsing & Ekblom, 1986; Celsing et al., 1986; Ekblom et al., 1972; Kanstrup & Ekblom, 1982). Defining exercise intensity as a percentage of $\dot{V}O_2max$ rather than relative to thresholds may potentially obscure the interpretation of reported results (Iannetta et al., 2020), as some, but not all, individuals may be pushed into the severe domain at 75% of pre-phlebotomy $\dot{V}O_2max$.

No difference in NMF progression was found between groups. While no previous studies have investigated NMF in exercise post-phlebotomy, we have previously demonstrated that the inhalation of carbon monoxide (CO) prior to exercise (an alternative method of decreasing oxygen-carrying capacity without affecting blood volume) led to greater peripheral fatigue of the quadriceps muscle for the same workload, compared to a sham condition (Kontro et al., 2022). The exercise intensity in that study was higher, as the participants exercised at their individual MLSS (82% of baseline $\dot{V}O_2max$ vs. 71% in the present study), likely placing them closer to the limit of tolerance for the chosen durations after the reduction in oxygen-carrying capacity. This difference in relative intensity may explain why increases in peripheral fatigue were observed for that intervention but not in the present study, where the participants were still demonstrably in a steady state despite similar reductions in effective [Hb] for the two studies.

We chose to include a sham condition to limit the effect of the perception of impaired performance on the outcomes. Blinding of the participants was successful with the likelihood of correctly identifying the condition not different than random guessing. In most cases, the reasons given for the answer chosen by the participants were ascribed to bodily sensations such as feeling weak or lightheaded, and in a minority of the answers, to the procedure itself (such as nurse behavior or time spent with equipment); however, no accidental unblinding was reported. Post-exercise, the reasons given were 100% related to the sensations and perception of effort during exercise. The researcher, who was blinded but able to correctly identify the condition more often than chance would predict, indicated that their answers were based on procedural clues and participant appearance or behaviour before exercise. Post-exercise, participant’s physical/perceptional responses or performance was the main reason for the answer chosen, but their success rate was effectively unchanged.

In summary, blood loss equal to 7% of total blood volume (i.e., ~1 unit donation) increased $\dot{V}_E$, [La], and HR during submaximal exercise without affecting $\dot{V}O_2$ or substrate oxidation, resulting in a lowered oxygen pulse. Subsequent maximal exercise performance was impaired by over 30% when the response of the SHAM group was considered. While shorter submaximal exercise bouts at comparable intensities have shown similar effects, this investigation focused on prolonged submaximal exercise, revealing progressive exacerbation of ventilatory and lactate responses during a 60-minute exercise bout, indicating a potential role for circulating vascular volumes in mediating resilience against time-dependent disturbances in homeostasis. As NMF was not exacerbated at iso-time during submaximal exercise or post-TTF, these altered responses seem to have limited effects on muscle function.

Chapter V: Sex-based differences in hematological values after normalization to body mass or fat-free mass in adults matched for aerobic fitness

5.1 Introduction

Blood volume (BV) plays a key role in exercise performance, particularly through the delivery of oxygen to tissues. The relationship between BV—and its close correlate hemoglobin mass (Hb_{mass})—and maximal oxygen consumption ($\dot{V}O_2max$) is well-established, with higher BV and Hb_{mass} associated with higher $\dot{V}O_2max$ (Gore et al. 1997, Heinicke et al. 2001). Furthermore, manipulations of BV and RBCV have been shown to induce acute changes in $\dot{V}O_2max$ (Kanstrup and Ekblom 1982, Celsing et al. 1987, Coyle et al. 1990, Krip et al. 1997), indicating a causal relationship between aerobic capacity and these hematological variables and highlighting the role of convective oxygen delivery as a limiting factor for whole-body $\dot{V}O_2max$ (Calbet et al. 2006, Lundby et al. 2017). To better understand variability in exercise performance between individuals, it is important to understand factors that influence hematological variables.

Biological sex contributes to the interindividual variability in hematological variables (Murphy 2014), but there is uncertainty in the existence and magnitude of sex differences in these variables, independent of anthropometrics and aerobic fitness. Males are taller and heavier than females on average, and BV and Hb_{mass} are strongly dependent on body size (Retzlaff et al. 1969), explaining much of the absolute differences in Hb_{mass} and intravascular volumes between sexes. As males maintain a higher [Hb] and hematocrit after the onset of puberty due to greater testosterone concentrations, they are expected to have a greater mass-specific Hb_{mass}, RBCV, and oxygen-carrying capacity than females (Åstrand 1952, Hero et al. 2005) as well as better absolute endurance performance (Joyner 2017). Nevertheless, the magnitude of these differences are unclear. Furthermore, there is equivocal evidence regarding whether normalizing BV (and plasma volume (PV)) to body mass (BM) (Falz et al. 2019, Oberholzer et al. 2023) or to fat-free mass (FFM) (Falz et al. 2019, Oberholzer et al. 2023) eliminates sex differences, possibly because unequal aerobic fitness between male and female samples has obscured interpretations in previous studies. Specifically, because adipose tissue, which does not contribute to $\dot{V}O_2max$, represents a higher percentage of body mass in females, sexes must be matched for $\dot{V}O_2max$ normalized to FFM to reduce the impact of sex differences in body size and composition on the variable of interest (Cureton and Sparling 1980). Applied to hematological data, this experimental approach minimizes the effect of aerobic fitness on comparisons between males and females.

Given the lack of studies in which sexes were matched for aerobic fitness, this study aimed to compare hematological values, normalized to BM and FFM, between males and females with (approximately) equal FFM-normalized $\dot{V}O_2max$ (Sparling and Cureton 1983). We hypothesised that normalization to FFM would eliminate the sex difference in BV but that Hb_{mass} and RBCV would remain greater in males, meaning that females would have greater FFM-normalized PV compared to males.

5.2 Methods

<u>*Study design*</u>

This study had a cross-sectional design and involved healthy volunteers of mixed training status. Data from four separate studies completed in our laboratory during a 4-year period and involving body composition scans, ramp incremental cycling tests, and Hb_{mass} measurements were pooled for this analysis. **Figure 5.1** illustrates the methods common to these studies. The resulting dataset included 79 unique individuals (34 female and 45 male) of similar average age and fitness (**Table 5.1**). All participants were post-pubertal, non-obese (BMI < 30 kg/m^2), non-smokers, and free of any known conditions or medications affecting cardiovascular responses to exercise. All females were pre-menopausal and either eumenorrheic (n=15), oral contraceptive users (n=10), or intra-uterine device users (n=9). The participants were habitually residing at mild altitude in Calgary, Alberta (~1100 m above sea level, with a barometric pressure of ~660 mmHg).

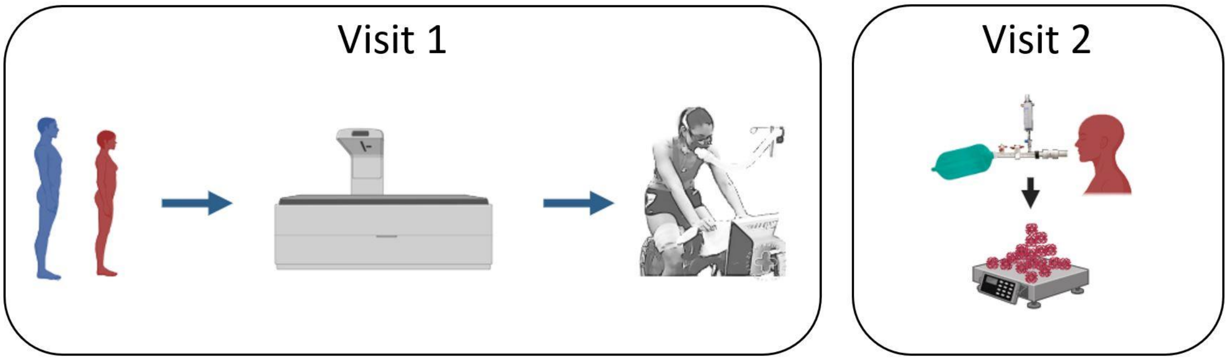

Figure 5.1. A schematic representation of the methods. The participants underwent a dual-energy x-ray absorptiometry (DXA) scan to measure body composition, a maximal incremental cycling test to measure ventilatory thresholds and maximal oxygen uptake ($\dot{V}O_2max$), and a carbon monoxide rebreathing test to measure hemoglobin mass and calculate vascular volumes.

Table 5.1. Participant characteristics disaggregated by sex.

Variable	Units	Total		Female (n=34)		Male (n=45)		P value	d	Δ (%)
		Mean	SD	Mean	SD	Mean	SD			
Age	y	29.3	7.2	27.7	7.2	30.2	7.5	0.14	0.3	9
Height	cm	173.4	9.2	166.1	5.2	177.3	7.7	**<0.0001**	1.7	7
BM	kg	71.0	12.2	61.7	6.9	76.6	10.6	**<0.0001**	1.6	22
FFM	kg	56.4	11.2	45.8	4.4	63.5	8.5	**<0.0001**	2.5	32
Body fat	%	20.7	6.7	25.6	4.9	16.9	5.9	**<0.0001**	1.6	-41
Hct	%	45.8	3.9	43.5	2.0	48.3	3.5	**<0.0001**	1.9	10
[Hb]	g/dL	15.0	1.3	14.2	0.7	15.9	1.1	**<0.0001**	1.6	11

Note: BM: Body mass; FFM: Fat-free mass; Hct: Hematocrit; [Hb]: Hemoglobin; d: Cohen's effect size; Δ, relative difference between sexes

Ethical Approval

All participants provided written, informed consent prior to enrollment in the studies. The studies were approved by the University of Calgary Conjoint Health Research Ethics Board (REB19-

1761, REB20-2005, REB21-0215, REB22-0501) and conformed to the standards set by the Declaration of Helsinki, except for pre-trial registration.

Body composition

Whole-body dual energy X-ray absorptiometry (DXA) scans by the Horizon DXA system (n=17) (Hologic, Marlborough, MA) and Lunar iDXA (n=62) (GE Healthcare, Chicago, IL, USA) were used to quantify FFM.

Ramp incremental tests

All exercise tests were conducted on an electromagnetically braked cycle ergometer (Velotron; Dynafit Pro, Racer Mate, Seattle, WA, USA) using an externally controlled work rate that was independent of cadence. Slightly different protocols were used in the four studies. In two studies, a simple 30 W/min ramp following a 4-min warm-up at 50 W was used. In the two remaining studies, a step-ramp-step protocol was applied, adapted from Iannetta et al. (2021), using a ramp rate of either 25 or 30 W/min after 18 min of cycling in the moderate-intensity domain and followed by a heavy domain step (12 or 20 min) after ~15 min of recovery. In both types of ramps, participants cycled until they reached volitional exhaustion or were unable to maintain a cadence within 20 rpm of their self-selected cadence. Perceptual responses were recorded during warm-up and after task failure. Despite differences in protocol, no differences in the $\dot{V}O_2$ at gas exchange threshold (GET), respiratory compensation point (RCP), or $\dot{V}O_2max$ were expected between protocols (Iannetta, de Almeida Azevedo et al. 2019). In three studies, the ramp incremental test was performed after the DXA scan; in one study, it was performed on a separate day.

Pulmonary gas exchange variables

Ventilatory and gas exchange variables were measured with a metabolic cart (Quark CPET, Cosmed, Rome, Italy) using a mixing chamber and 10-s data averaging. The flowmeter and gas analyzers were calibrated prior to each test following manufacturer's instructions. Heart rate (HR) was monitored and recorded continuously using a chest strap HR monitor (Polar Electro, Kempele, Finland). The GET and RCP were identified from blinded data to the nearest 0.1 L/min independently by at least two researchers, using previously published methods (Beever et al. 2020, Beaver et al. 1986). Briefly, the GET was defined as the first non-linear increase in ventilation relative to $\dot{V}O_2$, coinciding with a levelling off in mixed-expired CO_2 partial pressure ($PeCO_2$) and a turn point in mixed-expired O_2 partial pressure (PeO_2). The RCP was defined as the decline in $PeCO_2$, which is associated with a second non-linear increase in ventilation vs. VO_2. Any discrepancies were discussed until agreement was achieved.

Hb_{mass} and vascular volumes

Hb_{mass} was measured using a modified carbon monoxide (CO) rebreathing procedure, as described by Schmidt & Prommer (2005). Briefly, 1.0 to 1.3 mL/kg body mass (BM) of CO (determined based on sex and habitual physical activity) was injected into a closed respirometer system (Blood tec GmbH, Bayreuth, Germany) with a 3-L anesthetic bag filled with 100 % O_2, and this gas mixture was rebreathed for 2 min. Before the rebreathing, and 7 minutes after the onset of rebreathing, venous blood samples were drawn from a forearm vein in the antecubital area. Carboxyhemoglobin (COHb, %) and [Hb] were measured using a blood gas analyzer (ABL80 FLEX; Radiometer, Brea, CA) in duplicate and quadruplicate, respectively. The CO from exhaled air was measured using a hand-held CO detector (Dräeger Pac 7000, Dräeger AG, Lübeck, Germany) connected to a mouthpiece. Blood volume (BV), plasma volume (PV), and red blood cell volume (RBCV) were calculated (Keiser et al. 2017) using 0.91 as the whole-body correction factor for venous hematocrit (Hct) (Chaplin et al. 1953).

Statistical analysis

Male and female values for all variables were compared using independent sample t tests after confirmation of the data being normally distributed (Kolmogorov-Smirnov test and visual inspection of histograms). Relationships between variables were assessed using Pearson correlations. To analyze differences in regression slopes, an analysis of covariance (ANCOVA) was used with sex as a covariate. Statistical significance was set to $\alpha=0.05$ for all analyses. Data analysis was performed using GraphPad Prism version 10.

5.3 Results

Data from 34 females and 45 males (n=79) were included in the analysis. Males and females were similar in age and matched for aerobic fitness, as defined by $\dot{V}O_2max$ per kg FFM, but males were taller, heavier, and had a lower percent body fat than females (**Table 5.1**). Ventilatory thresholds were not significantly different between females and males when expressed in ml·kg FFM^{-1}·min^{-1} (**Table 5.2**). When expressed as a percentage of $\dot{V}O_2$max, GET in females was higher compared to males but RCP was not different between sexes (Table 5.2).

Absolute and relative Hb_{mass} (**Figure 5.2**, A-C) and RBCV (**Figure 5.2**, D-F) were higher in males than females. After normalization to BM, the 40% difference in absolute Hb_{mass} was reduced to 19%. With normalization to FFM, this difference was further reduced to 9%, similar to the sex differences in [Hb] (11%) and Hct (10%). When normalized to BM, BV was 7% higher in males but PV was similar between sexes. Normalization to FFM resulted in females having 4% and 13% greater BV and PV, respectively (**Table 5.3**).

Hb_{mass} and BV were positively correlated with aerobic fitness in both sexes with no evident sex differences when absolute values were correlated to absolute $\dot{V}O_2$max or when normalized values were correlated to normalized $\dot{V}O_2$max (either mL·kg BM^{-1}·min^{-1} or mL·kg FFM^{-1}·min), with r values between 0.50 and 0.71 (P<0.01 for all) (**Table 5.2**, **Figure 5.3**). [Hb] and Hct were not correlated with fitness in females, but Hct was negatively correlated with relative $\dot{V}O_2$max in males (P=0.04) (**Table 5.4**, **Figure 5.4**). Regression slopes for sexes were not significantly different for any of the reported correlations (0.28 < P < 0.46 for all).

Table 5.2. Indices of aerobic fitness disaggregated by sex.

Variable	**Units**	**Total**		**Female (n=34)**		**Male (n=45)**		**P value**	**d**	**Δ (%)**
		Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**			
$\dot{V}O_2$max	mL·min^{-1}	3434	833	2776	545	3931	649	**<0.0001**	1.9	34
$\dot{V}O_2$max	mL·kg BM^{-1}·min^{-1}	49.1	9.7	45.2	8.8	52.0	9.3	**0.0015**	0.8	14
$\dot{V}O_2$max	mL·kg FFM^{-1}·min^{-1}	61.6	9.9	60.5	10.2	62.4	9.7	0.405	0.2	3
RCP	mL·min^{-1}	2882	730	2356	520	3246	638	**<0.0001**	1.5	32
RCP	mL·kg BM^{-1}·min^{-1}	41.2	8.9	38.4	8.7	42.9	8.8	**0.0266**	0.5	11
RCP	mL·kg FFM^{-1}·min^{-1}	51.8	9.6	51.4	10.3	51.5	9.2	0.970	0	0
RCP	% of $\dot{V}O_2$max	83.9	5.4	84.7	6.2	82.3	5.5	0.070	-0.4	-3
GET	mL·min^{-1}	2027	526	1684	370	2241	530	**<0.0001**	1	28
GET	mL·kg BM^{-1}·min^{-1}	29.1	6.8	27.4	6.3	29.7	7.4	0.161	0.3	8
GET	mL·kg FFM^{-1}·min^{-1}	36.6	7.7	36.8	7.9	35.7	8.2	0.525	-0.2	-3
GET	% of $\dot{V}O_2$max	59.3	7.2	60.9	7.9	56.8	7.4	**0.0207**	-0.5	-7

Note: RCP: Respiratory Compensation Point; GET: Gas Exchange Threshold; d: Cohen's effect size; Δ, relative difference between sexes.

Table 5.3. Hb_{mass} and vascular volumes for the total sample and disaggregated by sex

Variable	Units	Total (n=79)		Female (n=34)		Male (n=45)		P value	d	Δ (%)
		Mean	SD	Mean	SD	Mean	SD			
Hb_{mass}	g	824	203	640	86	964	146	**<0.001**	2.6	40
Hb_{mass}	$g \cdot kg^{-1}$	11.7	1.7	10.4	1.2	12.6	1.4	**<0.001**	1.7	20
Hb_{mass}	$g \cdot kg\ FFM^{-1}$	14.7	1.5	14.0	1.3	15.2	1.4	**<0.001**	0.9	9
BV	mL	5890	1212	4966	627	6587	1074	**<0.001**	1.8	28
BV	$mL \cdot kg^{-1}$	84.0	10.3	80.8	8.8	86.4	10.7	0.16	0.6	7
BV	$mL \cdot kg\ FFM^{-1}$	105.9	10.4	108.5	9.6	103.9	10.7	**0.05**	-0.5	-4
RBCV	mL	2508	611	1986	265	2925	443	**<0.001**	2.6	40
RBCV	$mL \cdot kg^{-1}$	35.6	5.2	31.8	3.6	38.4	4.3	**<0.001**	1.6	19
RBCV	$mL \cdot kg\ FFM^{-1}$	44.7	4.6	42.7	3.9	46.2	4.5	**<0.001**	0.8	8
PV	mL	3381	649	3009	385	3663	669	**<0.001**	1.2	20
PV	$mL \cdot kg^{-1}$	48.4	6.4	49.0	5.6	48.0	7.0	0.51	-0.2	-2
PV	$mL \cdot kg\ FFM^{-1}$	61.2	7.9	65.8	6.3	57.7	7.1	**<0.001**	-1.2	-13

Note: BM = Body mass; FFM = Fat-free mass; Hb_{mass} = Hemoglobin mass; BV = Blood volume; RBCV = Red blood cell volume; PV =Plasma volume.

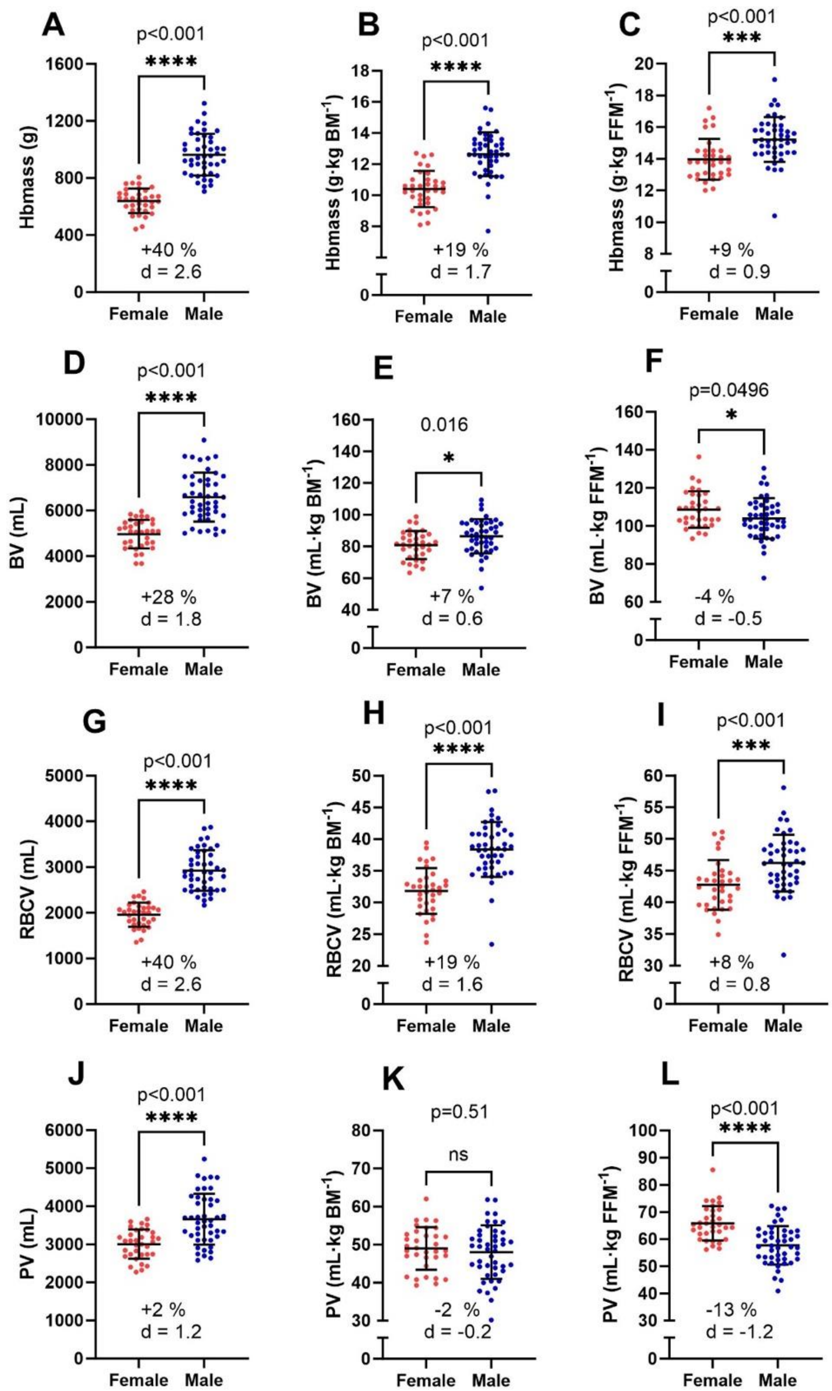

Figure 5.2. Individual and group data for females (red) and males (blue) for hemoglobin mass (Hb_{mass}; panels A-C), red blood cell volume (RBCV; panels D-F), blood volume (BV; panels G-I), and plasma volume (PV; panels J-L). All variables are expressed as absolute values, values normalized to body mass (BM), and values normalized to fat-free mass (FFM). Black lines and error bars represent means and standard deviations. Individual data are plotted for females (red) and males (blue). n=79.

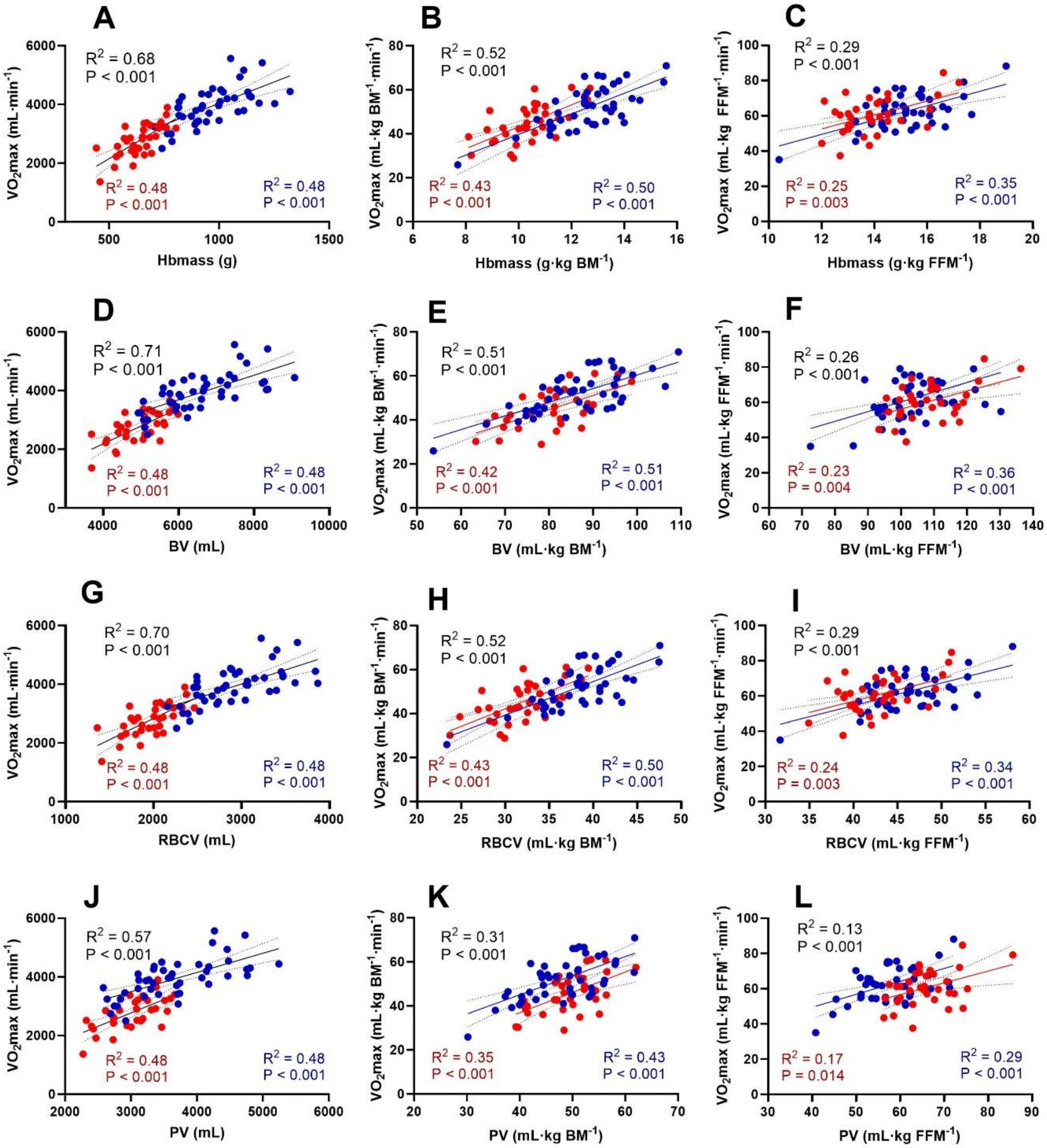

Figure 5.3. Relationships between maximal oxygen uptake ($\dot{V}O_2max$) and various hematological variables expressed in absolute units, units per body mass (BM), and units per fat-free mass (FFM). Correlations in the overall population are shown in black text and the same data analyzed for female (red) and male (blue) samples separately in red and blue text, respectively. Hb_{mass} = hemoglobin mass; BV = blood volume; RBCV = red blood cell volume, PV = plasma volume.

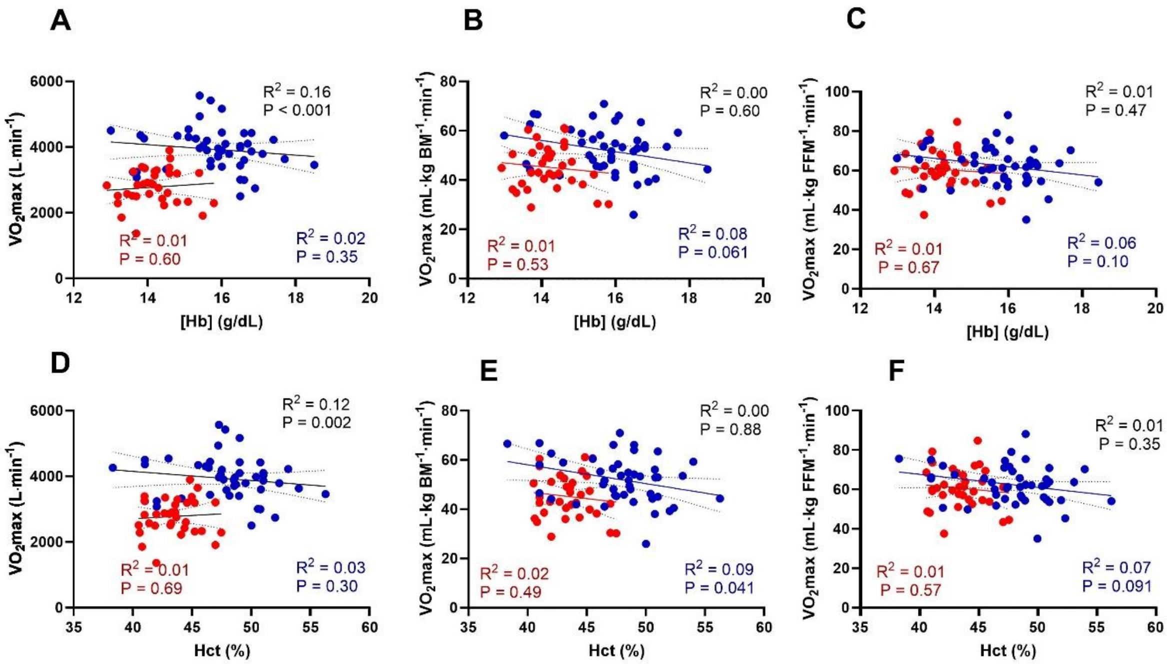

Figure 5.4. Relationships between maximal oxygen uptake ($\dot{V}O_2max$) and hemoglobin [Hb] and hematocrit (Hct) expressed in absolute units, units per body mass (BM), and units per fat-free mass (FFM). Correlations in the overall population are shown in black text and the same data analyzed for female (red) and male (blue) samples separately in red and blue text, respectively.

5.4 Discussion

The present study demonstrates that when males and females are of similar average aerobic fitness, normalizing hematological variables to FFM a) reduces, but does not abolish, sex differences in Hb_{mass} and RBCV and b) yields higher calculated BV and PV in females than males. These findings suggest that a similar aerobic capacity may be achieved by different oxygen delivery and extraction strategies in each sex. That our comparisons were made between groups of males and females matched for aerobic fitness provides confidence that the differences observed herein are related to biological sex rather than differences in fitness.

Sex differences in hematological variables

The magnitudes of the differences in Hb_{mass} and RBCV between males and females were greatly reduced but not eliminated by normalizing to BM and FFM, which agrees with previous studies.

While sex differences in Hb_{mass} and RBCV are well established, few studies have matched aerobic fitness between the male and female volunteers to eliminate—or at least reduce—the confounding effect of aerobic fitness on the sex-based differences in hematological variables. Sjöstrand (1948) was among the first to report a sex difference in Hb_{mass} independent of body size (i.e., g per kg of BM). Kjellberg et al. (1949) further expanded on this finding by demonstrating that not only did males have greater Hb_{mass}, but trained individuals exhibited higher values compared to untrained individuals. In his thesis, Åstrand (1952) described the development of a sex difference in relative Hb_{mass}, observing that such disparity emerges during puberty, an effect directly attributable to the rise in androgens affecting the setpoint of [Hb] (Hero et al. 2005, Murphy et al. 2010). Like these early studies, contemporary studies have not explicitly controlled for aerobic fitness between sexes, resulting in equivocal reported sex differences in Hb_{mass} (e.g., 13.6 vs. 16.1; 14.0 vs. 14.9; and 14.5 vs. 15.3 g·kg FFM^{-1} (or lean body mass [LBM])) (Falz et al. 2019, Diaz-Canestro et al. 2021, Oberholzer et al. 2023) and RBCV (e.g., 43.2 vs. 45.1; 49.5 vs. 51.6 mL·kg FFM^{-1} (or LBM)) for females and males, respectively (Diaz-Canestro et al. 2021, Oberholzer et al. 2023). The present study demonstrated that Hb_{mass} and RBCV respectively were ~9% and ~8% greater in males compared to females matched for aerobic fitness.

When aerobic fitness is matched between sexes, females seem to have higher BV and PV normalized to FFM. We reported 13% greater PV in females, offsetting the 8% difference in RBCV and yielding a 4% greater BV in females. In a recent multi-center cross-sectional study (n=582), females had higher BV and PV after normalization to LBM. While this study did not report the same fitness-matching strategy as our study, the $\dot{V}O_2max$ normalized to LBM from the average values reported results seems to yield close matching between sexes (60.6 and 62.0 mL·kg LBM^{-1}·min^{-1} for males and females, respectively) (Oberholzer et al. 2023). Other studies have reported no difference in BV when normalized this way (Diaz-Canestro et al. 2021). Another study by Diaz-Canestro (2021b) examining middle-aged and older individuals found no differences in BV or PV when normalized to BM; however, the fitness level of the female participants was potentially higher as suggested by a similar $\dot{V}O_2max$ of 35 mL·kg BM^{-1}·min^{-1} in both sexes. In a study on Hb_{mass} in adolescent boys and girls (Ulrich et al. 2011), BV and PV were not calculated, but using the mean values reported for Hb_{mass}, [Hb], and Hct to estimate these values based on the same assumptions we used, it appears that females likely had a higher numerical value for FFM-normalized BV (95.1 vs. 92.3 mL·kg FFM^{-1}) and PV (62.4 vs. 58.1 mL·kg FFM^{-1}). Overall, inconsistencies exist in prior literature regarding sex differences in BV and PV; however, when using body composition to normalize aerobic fitness, as in the present study, results indicate that both values are higher in females than males.

Females and males showed similar positive relationships between aerobic fitness and BV, PV, RBCV, and Hb_{mass}, and a lack of (or in males, a weak negative) relationship between aerobic fitness and [Hb] or Hct. The relationships remained when normalizing fitness and vascular volumes or Hb_{mass} to FFM instead of BM, which contrasts with data published by Oberholzer et al. (2023) who found no correlation between LBM-normalized $\dot{V}O_2max$ and LBM-normalized BV (supplementary data). The reason for this discrepancy is unknown but might be related to the heterogeneity of both the populations and the variable methods applied by Oberholzer and colleagues when compiling a large dataset from several laboratories. In the present study, data were collected in the same laboratory using the same instruments and methods to measure $\dot{V}O_2max$ (although two devices were used for DXA), and the population was relatively homogenous with non-obese, primarily moderately trained individuals of 18-49 years of age.

Normalization approaches

Several studies have explored the association between BV and body size and body composition and found a strong correlation between FFM and BV, indicating that BV may be more precisely estimated from FFM or lean body mass than from body mass or size (Hunt et al. 1998, Goodrich et al. 2020, Oberholzer et al. 2023). As demonstrated by the moderate-to-strong correlations between vascular volumes and $\dot{V}O_2max$ both in the male and female samples, failure to account for aerobic fitness would skew, if not invalidate, any conclusions drawn on intrinsic sex-based differences in these variables. Still, it is necessary to choose a normalization strategy.

Absolute $\dot{V}O_2max$ is better related to performance than relative $\dot{V}O_2max$ in some sports due to the greater importance of absolute power output; however, this variable is strongly related to body size, and differences in body size between sexes preclude matching based on this variable; therefore, normalizing to body size is reasonable. While BM is often used to determine relative $\dot{V}O_2max$, sex differences in body composition challenge this approach. Given the role of adipose tissue as an energy store rather than a tissue participating in oxygen consumption at maximal exercise, FFM is a more appropriate surrogate for the structures and tissues facilitating oxygen uptake and FFM-specific aerobic capacity reveals their level of aerobic conditioning (Cureton 1981). While it is possible that randomly selected males and females could be matched for aerobic fitness, males and females volunteering for studies with invasive methods may differ with respect to anthropometric variables, physical activity, and fitness status. For example, women are less likely than men to volunteer in studies involving pain, discomfort, and medical procedures, which may lead to gender-related volunteer bias (Nuzzo 2021). Therefore, equal aerobic fitness must be ensured to make valid conclusions about biological sex differences and to describe the association between fitness and blood-based parameters appropriately.

In contrast to aerobic fitness, it is unclear whether normalizing BV and PV to FFM instead of BM is appropriate. Given that our results for BV and PV depend on the normalization method, our approach could explain the finding of higher BV and PV in females. Adipose tissue is vascular and requires its share of perfusion, which could contribute to the total blood volume (Rosell and Belfrage 1979). Blood flow to adipose tissue at rest has been reported to be approximately 20-30 mL/ kg adipose tissue / min (~5-7% of cardiac output in normal weight individuals) and several times higher in adipose tissue adjacent to active muscles during exercise, but the relative perfusion rate is reduced with increasing body fatness to ~15 mL/kg adipose tissue / min in individuals with body fat percentages >40% (Larsen et al. 1966, Lesser and Deutsch 1967, Rosell and Belfrage 1979, Stallknecht et al. 2007).

Integrative consequences of differences in RBCV/Hb_{mass}

While the components of the oxygen delivery cascade are well understood, little research exists on the relative importance of these components in each sex. Females in the present study were matched with males for FFM-normalized $\dot{V}O_2max$ yet had lower FFM-normalized Hb_{mass} and RBCV. That females achieve a similar $\dot{V}O_2max$ with a lower capacity for whole-body oxygen delivery could be explained by a sex-difference in capillary hematocrit. In a very large study of blood donors, females were reported to maintain a higher capillary hematocrit, which may facilitate oxygen delivery at the level of muscle tissue (Murphy, Tong et al. 2010). This estrogen-mediated effect on capillary hematocrit would reduce the sex-difference observed in arterial oxygen content when blood reaches the capillaries for gas-exchange (Murphy 2014). This phenomenon could be explained by sex differences in RBC rheology, which changes in response to estrogen and increases the deformability of RBC (Grau et al. 2018). Maintenance of tissue oxygenation may be further augmented by the higher nitric oxide-mediated vasodilatory capacity associated with higher estrogen (Grau et al. 2018). In addition, females possess, on average, a higher percentage of type I fibers (Simoneau and Bouchard 1989), which, according to emerging evidence, maintain a higher capillary hematocrit in the capillaries supplying them, facilitating greater oxidative energy production relative to type II fibers (Colburn et al. 2020).

Regardless of the mechanism, according to the Fick equation, achieving a similar FFM-normalized $\dot{V}O_2max$ with lower arterial oxygen content indicates either a higher level of oxygen extraction or a higher mass-specific cardiac output/leg blood flow in females. Evidence exists that in equally trained males and females, BM-normalized stroke volume is similar (Hutchinson et al. 1991, Wiebe et al. 1998, Giraldeau et al. 2015). At given absolute workloads in single leg exercise, females demonstrate higher blood flow (Parker et al., 2007). Whether differences in vasodilatory function could allow for higher limb blood flow during maximal large muscle mass exercise is not well understood. In a study measuring peak femoral blood flow in young and old males and females, when normalized by either quadriceps muscle mass or perfusion pressure, peak femoral blood flow at maximal exercise tended to be higher in females, and peak blood flow was not correlated with cardiac output in females unlike in males (Ridout et al. 2010). Invasive studies in fitness-matched males and females are needed to ascertain whether sex-differences in oxygen extraction exist.

Experimental Considerations

The absence of habitual physical activity data from participants prevents us from assessing whether the sexes were equally trained as well as equally fit; however, we are not aware of a sex difference in the dose-response ratio of training in males and females and consider it unlikely to influence the outcomes reported given that each sex experiences similar improvements in cardiovascular fitness in training studies (Drinkwater 1984).

The inability of the CO-rebreathing method to directly measure vascular volumes must also be considered. Using this method, BV, RBCV, and PV are calculated from Hb_{mass}. While 0.91 has been widely adopted as a valid correction factor for *f*, the whole body-to-venous conversion (Chaplin et al. 1953), many studies have found a lower value for this correction factor (~0.86) (Retzlaff et al. 1969, Fairbanks et al. 1996). While most studies do not report a sex difference in *f* (Chaplin et al. 1953, Fairbanks and Tauxe 1967, Balga et al. 2000), greater (Karlson and Senn

1963, Retzlaff et al. 1969) and lower *f* values (Fairbanks et al. 1996) have been reported in females, which would overestimate or underestimate, respectively, the BV and PV of females relative to males in our study. Whether endurance training may change the *f* value in males or females has not been reported in the literature to our knowledge. Notably, considerable interindividual variation in venous-to-whole body hematocrit exist within each sex (Retzlaff et al. 1969, Wright et al. 1975), which is a reminder that vascular volume measurements obtained through the CO-rebreathe technique, although producing comparable results (Thomsen et al. 1991), are still surrogates of perhaps more accurate direct isotope-labelled measurements of PV and RBCV (Mayerson et al. 1948).

Chapter VI: A proof of principle method to measure fractional synthetic rate of hemoglobin in humans

6.1 Introduction

Deuterated water (^2H_2O) is a relatively low cost and practical method to investigate protein synthesis compared to isotope-labelled amino acids (Miller et al., 2020; Wilkinson et al., 2017). With ^2H_2O, amino acids are labelled in vivo through transamination reactions, and the principle of measuring protein turnover rates with ^2H_2O is the same as with labelled amino acids: the rate of incorporation of the label into the protein of interest over time, relative to the concentration of the label in the "precursor" pool, is used to determine the rate of protein synthesis. The abundance of the labelled alanine, chosen because of its abundance and four available hydrogens, can be determined by collecting tissues of interest at specific time points. The precursor pool is often more challenging to measure; however, previous research has demonstrated that using body water ^2H enrichment is a valid surrogate for ^2H-labelled alanine in the precursor pool, and that stable enrichment of ^2H in plasma and tissue free alanine is achieved in ~20 min after the first dose (Belloto et al., 2007; Dufner et al., 2005). While extensive studies in muscle and plasma protein synthesis have been conducted, no studies in humans have attempted to measure the fractional synthetic rate (FSR) in red blood cell (RBC) protein.

Comprising 97% of all RBC proteins, hemoglobin (Hb) is a heme protein that binds and transports oxygen throughout the body (Weed et al., 1963). The Hb protein is critically important to health and fitness (Calbet et al., 2006).There are few methods to assess the rate at which Hb (or RBCs) are produced, but characteristics of Hb make it feasible to assess with the ^2H_2O method. Firstly, blood is easy to collect and Hb is easy to isolate in blood samples (Bain, 2021). Secondly, although bone marrow is difficult to access in humans, body water ^2H enrichment is a reasonable surrogate for the ^2H enrichment in the bone marrow precursor pool. For example, experiments with ^{13}C-glycine in rats have shown that the enrichment of the labelled amino acid in the circulating RBC approximates the enrichment of bone marrow free glycine (Hibbert et al., 2001). On the other hand, as mature RBCs lack nuclei and are incapable of protein synthesis, almost all of their proteins are synthesized during their maturation in the bone marrow (Palis, 2014). As such, there is a delay between the synthesis of Hb and its appearance in the blood, which could result in errors in Hb synthesis measurements; however, if this delay is properly considered, it is likely possible to calculate the FSR of Hb using a $^{2}H_2O$ method. Specifically, Hb FSR can theoretically be measured when a linear increase in alanine-bound ^{2}H is observed in Hb in conjunction with stable ^{2}H enrichment in body water. Finally, for Hb synthesis, it is possible to measure the entire mass of Hb in circulation (Hb_{mass}), facilitating measurements of the absolute Hb synthetic rate (ASR).

The aim of the present pilot study was to apply the oral tracer method to establish a stable isotope tracer method to measure Hb FSR and ASR by oral administration of $^{2}H_2O$ and subsequent determination of ^{2}H enrichment in body water and RBC protein for 28 days.

6.2 Methods

Ethical Approval

All participants provided written, informed consent prior to enrollment in the study. The study was approved by the University of Calgary Conjoint Health Research Ethics Board (REB19-0215) and conformed to the standards set by the Declaration of Helsinki, except for registration in a database.

Study design

This proof of principle investigation aimed to determine Hb (i.e., RBC) and albumin synthesis in five healthy individuals during 4 weeks of habitual living. Participants completed an aerobic fitness test and a Hb_{mass} assessment prior to baseline sample collection and $^{2}H_2O$ ingestion. Thereafter, regular blood and saliva samples were collected. Hb_{mass} was assessed again at the end of the study.

Participants

Five young volunteers (2M, 3F), sedentary but free of any known medical conditions, were recruited to the experiment. Participant characteristics are reported in **Table 6.1**.

Table 6.1. Participant characteristics at baseline

Age (y)	BM (kg)	FFM (kg)	Hb_{mass} (g)	Hb_{mass} ($g\cdot kg^{-1}$)	VO_2max ($L\cdot min^{-1}$)	[Hb] ($g\cdot dL^{-1}$)
24 (4)	62.7 (12.1)	45.5 (7.2)	659 (151)	10.5 (0.8)	2.4 (0.6)	15.1 (1.4)

Note: BM = Body mass; Hb_{mass} = Hemoglobin mass. Data are reported as mean (SD).

<u>^2H_2O protocol and blood sampling</u>

On Day 0, baseline blood samples and saliva were collected, and the baseline Hb_{mass} was determined using the CO rebreathing technique (Schmidt & Prommer, 2005). Thereafter, a loading dose of 8 servings of ^2H_2O (0.625 mL/kg FFM per serving) was provided for the participants to ingest at 90 min intervals during the day. On the subsequent 28 days, one daily serving of deuterated water was prescribed for the participants to consume each morning. On Days 1, 3, 7, 14, 21, and 28, the participants returned to the laboratory for fasted morning blood samples. On either day 1 or 3, additional blood samples were obtained to measure markers of iron status and to obtain a complete blood count. The study protocol is illustrated in **Figure 6.1**.

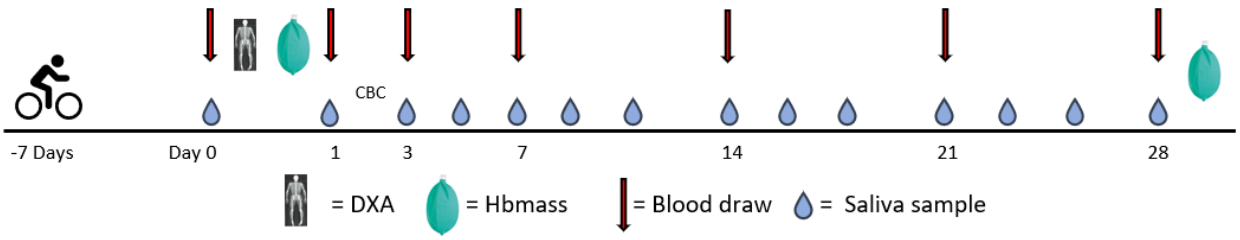

Figure 6.1. Timeline of measurements and sampling of blood and saliva. 1 dose of ^2H_2O was ingested daily after the loading phase (i.e., 8 doses) on Day 0.

<u>Sample processing</u>

Blood was drawn into a 2.7-mL Na^+-citrate plastic blood collection tube (BD Vacutainer, BD, Franklin Lakes, NJ, USA) using standard procedures and centrifuged at 1500×g for 10 min at +4 °C. Plasma was aliquoted into tubes and frozen in -30 °C until sample preparation.

Hemoglobin isolation

After the initial centrifugation, 400 µl from the RBC fraction was pipetted into a 1.5 mL tube and gently washed with an equal volume of cold phosphate buffered saline (PBS). After each wash, the solution was centrifuged at 10000×g for 5 min at 4°C and the supernatant was discarded, and the remaining cells were stirred with the pipette tip and aspirated slowly several times until the suspension appeared homogenous. After repeating this procedure three times, the washed RBCs were lysed by subjecting them to two cycles of freezing (15 min, -80 °C, followed by thawing on ice), and then homogenized by sonication in 4 °C (20 min, 17 W). The lysed cells were centrifuged at 10000×g for 15 min at 4 °C to pellet solids. The Hb-containing supernatant was collected and used for HCl hydrolysis.

Albumin isolation

Plasma proteins were precipitated from 500 µL plasma by adding 1 mL 10% trichloroacetic acid (TCA) (wt:vol), then centrifuge at 4500×g for 5 min. The supernatant was discarded, and the resultant pellet resuspended in 500 µL ddH_2O. Albumin was solubilized by adding 2.5 mL 1% TCA (wt:vol) in 90% ethanol and centrifuged at 4500×g for 5 min. The supernatant fluid was collected, and the albumin precipitated by adding 1 mL 26.8% ammonium sulfate (wt:vol). After centrifugation at 4500 rpm for 5 min, the supernatant was removed and discarded, and the resultant pellet washed once more with 1 mL 26.8% ammonium sulfate. To remove any residual free amino acids, the albumin pellet was washed twice with 1 mL 0.2 M perchloric acid. The pellet was used directly for HCl hydrolysis. The purity of both Hb and albumin fractions was confirmed by SDS-PAGE in a 4-15% TGX stain-free gel (Bio-Rad Laboratories, CA, USA) followed by transfer to a nitrocellulose membrane and Ponceau S staining (**Figure 6.2**).

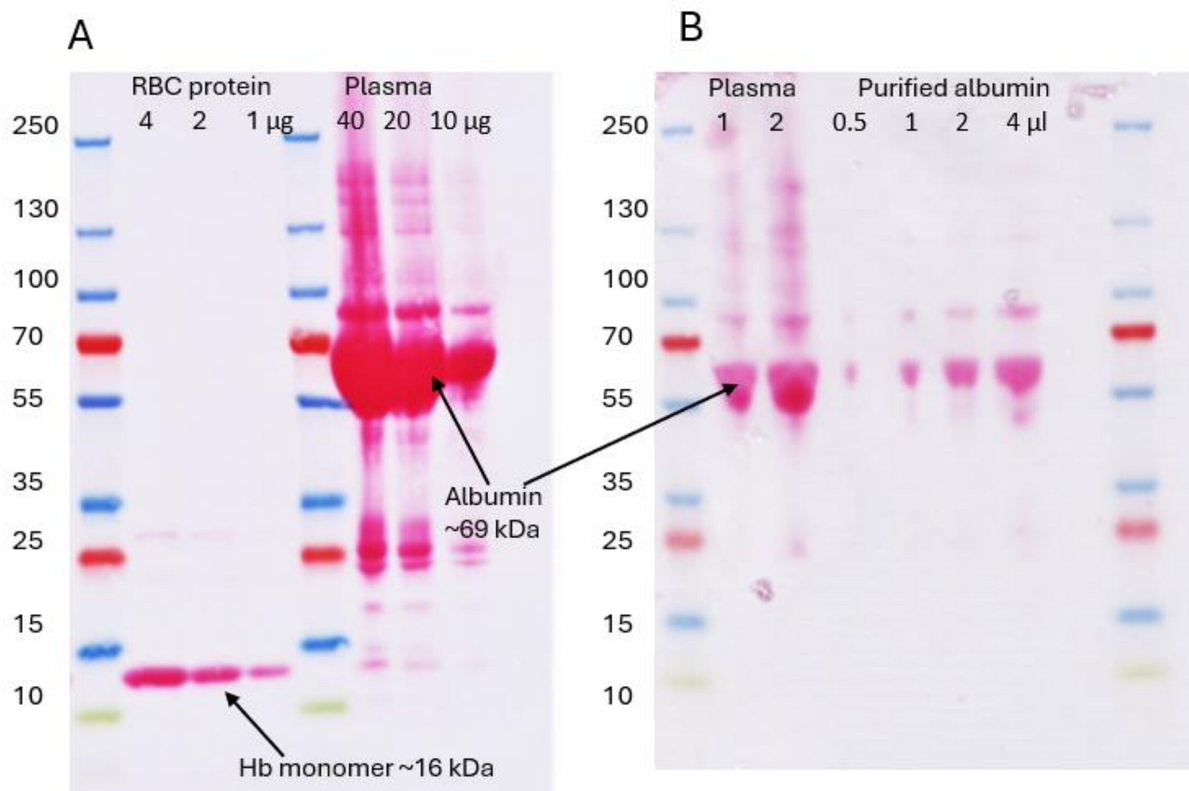

Figure 6.2. Gel electrophoresis of (A) washed and hydrolysed red blood cells compared vs. plasma, and (B) plasma vs. purified albumin. The proteins from a 4-15% TGX stain-free gel were transferred to a nitrocellulose membrane and stained with Ponceau S for imaging.

HCl hydrolysis and sample purification

The protein-bound amino acids in both albumin and Hb samples were liberated by HCl hydrolysis. Briefly, 2 mL of Dowex in 1 M HCl (constantly under magnetic stirring) was added to the samples, which were then vortexed and left in an oven set to 110 °C for 72h and vortexed every 24h. Ion exchange was performed by binding the samples to columns of Dowex, preconditioned with 2M NH_4OH and 1M HCl, then washed with H_2O until neutral, and eluted with 2M NH_4OH into glass tubes. The eluted samples were dried in a SpeedVac SPD1030 (Thermo Scientific, USA) at 60 °C over 18-24h and the resultant amino acid residue reconstituted in 0.5M HCl. The final samples were derivatized to their N-methoxycarbonyl methyl esters and analyzed using gas chromatography combustion isotope ratio mass spectrometry by Metabolic Solutions Inc. (Nashua, NH, USA).

Measurement of ^{2}H in body water

Saliva samples were taken independently by the participants using Salivette cotton swab tubes (Sarstedt, Nümbrecht, Germany). The frozen samples were thawed at RT and centrifuged at 1500×g for 10 minutes at 4°C to collect the fluid, then frozen at -30°C until analysis. The samples were prepared for ^{2}H enrichment analysis by diluting them 1:35 in ddH_2O and vortexed. ^{2}H enrichment of saliva was determined by cavity ring-down spectroscopy with the use of a liquid isotope analyser (Picarro L2130-I analyser, Picarro, Santa Clara, CA) with an automated injection system.

Hbmass and vascular volumes

Hbmass was measured using a modified CO rebreathing procedure, as described by (Schmidt & Prommer, 2005). Briefly, 1.0 to 1.5 mL/kg body mass (BM) of CO (determined based on sex and habitual physical activity) was injected into a closed respirometer system (Blood tec GmbH, Bayreuth, Germany) connected to a 3-L anesthetic bag filled with 100 % O_2, and this gas mixture was rebreathed for 2 min. Before the rebreathing and 7 minutes after the start of rebreathing, blood samples were drawn from a forearm vein. COHb and [Hb] were measured using a blood gas analyzer (ABL80 FLEX; Radiometer, Brea, CA). CO from exhaled air was measured using a hand-held CO detector (Dräeger Pac 7000, Dräeger AG, Lübeck, Germany) connected to a mouthpiece. Blood volume (BV), plasma volume (PV), and red blood cell volume (RBCV) were calculated as described by Keiser et al. (2017) using 0.91 as the correction factor for venous Hct.

Ramp test

A ramp incremental test for fitness evaluation was performed on an electromagnetically braked cycle ergometer (Velotron; Dynafit Pro, Racer Mate, Seattle, WA, USA) using a 30 W/min ramp after a 5-min warm up at 50 W. Ventilatory and gas exchange variables were measured with a metabolic cart (Quark CPET, Cosmed, Rome, Italy) using a mixing chamber and 10-sec data averaging. The flowmeter was calibrated using a 3-L syringe as per manufacturer's instructions. Gas analyzers were calibrated using a precision-analyzed gas mixture of known concentration (15.90% O_2, 4.95% CO_2, and balanced N_2). Heart rate (HR) was monitored and recorded continuously using a chest strap HR monitor (Polar Electro, Kempele, Finland).

Data analysis

Calculation of FSR and ASR

FSR is the rate at which ^2H-labeled alanine is incorporated into the protein when normalized to the total abundance of the tracer in the precursor pool per unit of time. FSR and ASR were calculated using the following equations:

$$FSR\ (\%/day) = [(APE_{Ala})]/[(APE_{BW}) \times t] \times 100 \qquad (Eq.\ 6.1)$$

where APE_{Ala} = deuterium enrichment of protein-bound alanine, APE_{BW} = mean precursor enrichment over the time period, and t is the time between blood samples. A ^2H exchange ratio of 3.7 between body water and free alanine was assumed.

The average ^2H enrichment in body water of the first 14 days was used for the calculation of Hb FSR, assuming that ^2H in body water is in equilibrium with ^2H in alanine present in bone marrow (Belloto et al., 2007). Albumin FSR was determined from days 0, 1, 3 only due to the faster turnover of this protein.

$$ASR = FSR \times Protein\ pool\ (g) \qquad (Eq.\ 6.2)$$

where the "protein pool" refers to Hbmass for Hb (measured, as described above) or the total amount of albumin in circulation, which was calculated using PV and an assumed plasma albumin concentration of 40 g/L in all individuals.

To obtain the number of days needed for a complete turnover of the body's Hb stores, red blood cell lifespan was calculated as

$$RBC\ lifespan = 100\ \% / FSR\ (\%/d) \qquad (Eq.\ 6.3)$$

6.3 Results

All participants complied with the prescribed ^2H_2O ingestion over the 28 days, which was confirmed by analyzing the enrichment of ^2H in body water from the saliva samples (**Figure 6.3A**). An increase in RBC APE was detected from day 7 onwards, and the increase was linear from day 14 onwards (**Figure 6.3B**). The linearity of the increase was assessed by comparing the slope of ΔAPD between days 14 and 21 to the slope between days 21 and 28, which was not statistically different (P=0.52). Raw values for APD and APE are reported in **Table 6.2**.

Table 6.2. Enrichment of deuterium in RBC protein

Day	APD (%)	APE (%)	ΔAPD (%·day^{-1})	ΔAPD (of APD_{max})
0	0.0132 [0.0001]	0.0000 [0.0000]		0 [0] %
1	0.0133 [0.0001]	0.0001 [0.0000]	0.0001 [0.0002]	9 [13] %
3	0.0135 [0.0002]	0.0003 [0.0001]	0.0001 [0.0001]	7 [7] %
7	0.0156 [0.0013]	0.0023 [0.0012]	0.0005 [0.0003]	44 [12] %
14	0.0202 [0.0017]	0.0070 [0.0016]	0.0007 [0.0003]	56 [32] %
21	0.0284 [0.0031]	0.0151 [0.0030]	0.0012 [0.0004]	100 [16] %
28	0.0358 [0.0040]	0.0226 [0.0038]	0.0011 [0.0002]	91 [17] %

Note. Values are mean [SD]. APD: Atom Percent Deuterium; APE: Atom Percent Excess.

FSR values were derived from changes in APE from day 14 to 28. As an approximation of precursor availability at the time of synthesis, saliva ^2H enrichment from 14 days prior was assumed. Calculation of Hb FSR for each participant for the linear phase (days 14 to 28) resulted in a range of values between 0.47% and 0.95%/day. Values for protein FSR and associated variables are reported in **Table 6.3**.

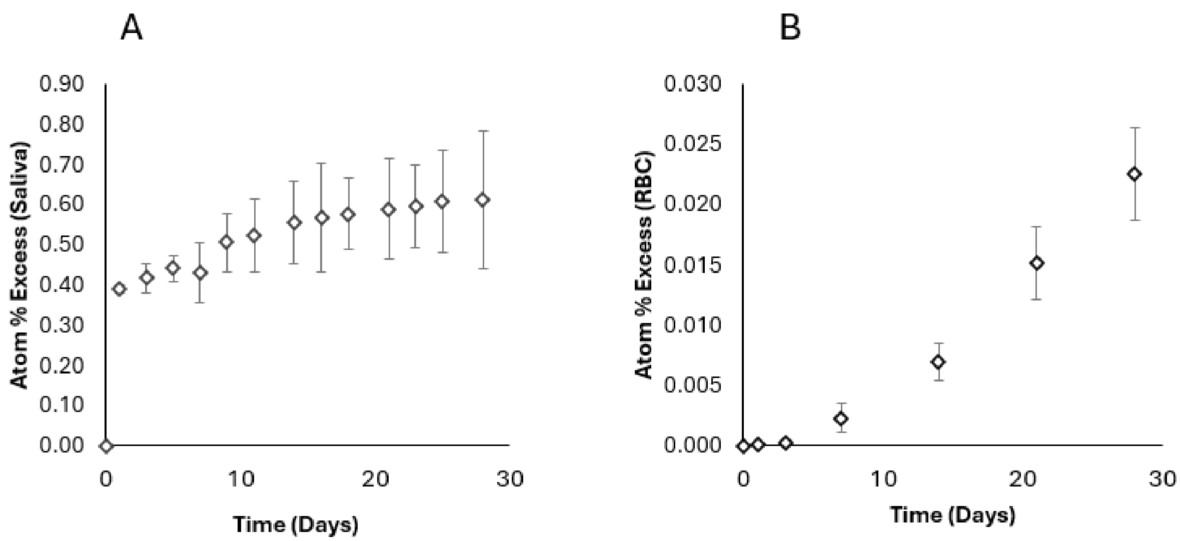

Figure 6.3. Determination of atom percent deuterium (APD) increase in Hb. A) Atom % excess ^{2}H in body water over the study period. B) Atom % excess ^{2}H in RBC over the study period. Data are presented as the mean ± SD. n=5 for both panels.

Table 6.3. Synthetic rates of Hb and albumin

Hb FSR (%/d)	Hb ASR (g/d)	Hb ASR (mg/d/kg)	Hb ASR (mg/d/kg FFM)	Alb FSR (%/d)	Hb breakdown (%/d)	RBC lifespan (days)
0.71 [0.18]	4.7 [1.9]	74 [21]	102 [31]	3.1 [0.3]	0.88 [0.20]	149 [41]

Note: Values are mean [SD]. FSR: Fractional Synthetic Rate; ASR: Absolute Synthetic Rate. Hb, hemoglobin; Alb, albumin.

6.4 Discussion

The purpose of the present study was to develop a method to measure Hb protein turnover in young healthy individuals using $^{2}H_2O$ as a metabolic label. Based on the successfully detected ^{2}H in RBC-derived alanine and the enrichment patterns of it, this method is feasible, and the delayed but steady appearance rate of the metabolic label is consistent with the erythropoietic process. The average Hb FSR of 0.71% suggests an average lifespan of 149 days for Hb protein. The observed range was considerable, from 105 to 213 days. Whether this finding is true interindividual variation in RBC lifespan or a result of the methodology employed is uncertain. This method has promise for application in human biology and medicine.

The RBC lifespan reported herein is longer than dogma suggests. The first stable isotope experiments by Shemin and Rittenberg in the 1940's (Shemin & Rittenberg, 1945, 1946), and more recently Khera and colleagues (Khera et al., 2015), employed labelled ^{15}N-glycine to demonstrate how a metabolic label incorporated into heme may be used instead of cell labelling to determine RBC lifespan. The average RBC lifespan based on these experiments was 127 (Shemin) and 106 ± 21 (Khera) days. Other investigations using radioactive di-iso-propyl-fluorophosphonate ($DP^{32}F$), ^{51}Cr, or biotin labelling, or endogenous carbon monoxide production, have resulted in mean life spans of 110 to 126 days (Bentley et al., 1974; Bratteby & Wadman, 1968; Mock et al., 2011; Zhang et al., 2018); however, interindividual variation is considerable with standard deviations often exceeding 20 days. In the large sample size study by Zhang and colleagues measuring CO production, the range of RBC life span in healthy individuals was from 82 to 215 days. Of the 109 volunteers, 15 had RBC life spans of 150 days or more. Some of this variation might be explained by great day-to-day variation in the CO breath test: in a more recent study employing multiple measurements in the same subjects, the standard deviation of within-subjects RBC life span measurements exceeded 30 days in most volunteers (Ye et al., 2021). Werre et al. (2004) have argued that methods utilizing cell-membrane labelling (by biotin or ^{51}Cr) underestimate the true lifespan of RBC due to membrane shedding, which causes the cells to shrink in size, losing 20% of their membranes, including associated proteins, and Hb content during their lifespan. Thus, they suggest the true lifespan to be ~150 instead of ~120 days.

A benefit of using ^{2}H to measure RBC turnover instead of a ^{15}N-labeled glycine bolus, or labeling of mature RBCs, is that the measurement can be completed in several weeks rather than ~150 days, which makes it more feasible for most research questions. This is even more important when selecting methods for an intervention, in which participant commitment and compliance over several months is a crucial factor to consider.

Some methodological concerns should be considered. In order to calculate protein synthesis from metabolic tracer data, a few assumptions must be made. First, we need to measure atom percent excess in the correct precursor pool. Since the measurement period in the present study was weeks rather than hours, it is almost certain that the ^{2}H-labelled free alanine pool present in bone marrow was in equilibrium with ^{2}H-labelled body water. While we were unable to confirm this assumption, we are not aware of any mechanism allowing the discrimination of isotope-labelled amino acids from unlabelled ones in any tissue; therefore, this assumption is likely valid. Second, we must assume that no recycling of the precursor took place. We are unable to rule out the possibility of this assumption being violated. This could happen by the means of 1) ineffective erythropoiesis (Sannolo et al., 1992) or 2) significant (early) shedding of Hb-containing vesicles from circulating RBCs (Werre et al., 2004). Ineffective erythropoiesis is the destruction of developing RBC in the marrow and can be significant in disorders of Hb synthesis (e.g., folate or iron deficiency or thalassemia). As we recruited participants with no known conditions affecting erythropoiesis, ineffective erythropoiesis is unlikely to be a major factor contributing to precursor recycling. Additionally, none of the participants were deficient in iron based on the screening of biomarkers associated with iron status (ferritin, transferrin saturation, and total iron). As mentioned, Hb loss from mature RBCs through membrane shedding would also result in potential precursor recycling. However, the reduction in RBC size happens first through a reduction in water content (up to 25 days of age) and only later through loss of membrane and Hb, which becomes apparent in older cells (>80 days). Hence loss of Hb through vesiculation is unlikely to affect the FSR measurement in the first 28 days of Hb synthesis (i.e., the present study).

Albumin FSR was also measured to allow a comparison against the second most abundant blood protein (i.e., an internal control). The mean FSR of 3.1% for albumin is similar or slightly lower than previously reported in sedentary adults. Previous studies report 5.0-7.3% · day^{-1} (varying by precursor used for calculation and subject group) (Fu & Nair, 1998), 4.0-6.0%· day^{-1} (Sheffield-Moore et al., 2005), and 3.4-4.0%· day^{-1} (Gersovitz et al., 1980). While 3.1%· day^{-1} is not a major deviation from previously published values, it is ~20-25% lower than could be expected. It is possible that the low Hb FSR measured is reflective of a methodological issues or systematic error also present in the albumin FSR.

A limitation of this pilot study was the lack of cross-validation using previously established methods for RBC life span determination (e.g., ^{15}N-glycine, ^{51}Cr or biotin labels, or the CO breath test). A future study comparing Levitt's CO breath test with the turnover rate obtained by ^2H_2O-labelling would give valuable data to compare against.

In summary, we have successfully showed that the deuterated water method for measuring FSR in RBC-derived protein (primarily Hb) is a promising technique to establish rates of erythropoiesis in humans. With this method, we calculated a FSR for Hb of ~0.7% and an ASR of Hb of ~5 g/day. Future experiments should consider employing greater sample sizes and validation methods. Using this technique, it could be possible to quantify how various exercise, nutritional, pharmacological, or environmental interventions affect Hb turnover in athletes and healthy individuals as well as in clinical populations.

Chapter VII: Summary, discussion, and future directions

The importance of oxygen delivery to exercise is well documented by plentiful studies in exercise physiology, encompassing several decades of evidence from direct manipulations of [Hb], RBCV, or BV; however, most of this work focused on maximal exercise capacity. One of the main focuses of this thesis was to expand on the limited data existing on the role of the central capacity for oxygen delivery, specifically its surrogates Hb_{mass} and BV, in submaximal exercise performance. This chapter will summarize and discuss the main findings of the original work presented in this thesis and their implications.

7.1 Reduction in [Hb] or Hb_{mass} exacerbates submaximal exercise responses

The aims of Chapters III and IV were to assess the effect of impaired capacity for whole-body oxygen delivery on submaximal exercise responses and indicators of performance. In both experiments, reducing the Hb that was available to bind oxygen—using CO inhalation to reduce effective [Hb] or phlebotomy to decrease Hb_{mass}—resulted in elevated blood lactate ([La]), ventilation ($\dot{V}_E$), and heart rate (HR), compared with the control condition. In Chapter III, perceived exertion was also increased, as well as neuromuscular fatigue, as evidenced by an increase in peripheral fatigue measured in isometric knee-extension. Why these effects on fatiguability were not observed in Chapter IV may be related to the lower intensity used (avg. intensity 71% vs. 82% of $\dot{V}O_2$max), which, despite a longer duration (60 vs. 30 min), likely allowed for greater compensations to mitigate potential reductions in oxygen delivery to the locomotor muscles throughout the exercise bout.

7.2 MLSS is sensitive to oxygen carrying capacity

In Chapter III, the intensity was selected so that the participants were exercising at their individual MLSS. As 9 out of 16 participants were, by definition, exercising above their MLSS after CO inhalation (indicated by non-steady state blood lactate concentration), it suggests that this boundary delineating the heavy and severe exercise domains is, at least partly, reliant on oxygen delivery. Indeed, evidence exists to suggest that leg blood flow does not significantly differ between exercise intensities at slightly below CP (~MLSS) and maximal knee extension exercise (Dorff et al., 2024). If these findings can be generalized to whole-body exercise, it means that small reductions in CaO_2 may not be compensated for by increasing leg blood flow when approaching this threshold, thus disrupting the balance of lactate production and clearance. A small reduction in oxygen carrying capacity may increase lactate production: intracellular PO_2 has been shown to influence lactate production mediated by changes in the cellular [ADP]/[ATP] ratio (Ferguson et al., 2018).

7.3 Trained individuals are more sensitive to Hb_{mass} manipulation

As hypothesized based on existing literature, the effect of reduced effective [Hb] and Hb_{mass} were more severe in individuals with higher $\dot{V}O_2max$. In Chapter III, both maximal exercise capacity, ($\Delta\dot{V}O_2peak$ and ΔPPO), and submaximal exercise capacity (MLSS) were impaired more in fitter individuals than their less fit peers. In Chapter IV, a significant negative relationship between $\Delta\dot{V}_Epeak$ and fitness, and a non-significant (P=0.09) negative relationship between $\Delta\dot{V}O_2peak$ and fitness were demonstrated. While the sample size (n=8) in the phlebotomy group may have been insufficient to make definitive conclusions, the trend was similar to what was observed with CO inhalation. This evidence further supports the paradigm of changing bottlenecks for $\dot{V}O_2max$ with improved training status, meaning that oxygen delivery becomes more limiting with increasing fitness. A novel finding in this thesis was that this phenomenon extends to MLSS. [La] kinetics are believed to be more dependent on peripheral qualities than central oxygen delivery (Gollnick & Saltin, 1982; Saltin et al., 1976); however, as demonstrated in this thesis, at higher intensities (in the heavy domain), oxygen delivery becomes a significant factor, elevating lactate concentrations in steady-state exercise and reducing the MLSS.

7.4 BV plays a role in prolonged exercise

While neuromuscular fatigue was not impacted during 60 minutes of cycling after phlebotomy, the ventilatory and lactate responses showed time-course dependent increases, suggesting that vascular volumes are important for the maintenance of a homeostatic environment also during steady state exercise. Previous studies have in fact reported impaired thermoregulation after blood donation during low-intensity exercise in the heat (Fortney et al., 1981; Mora-Rodriguez et al., 2012). Novel results from Chapter IV extend these finding by showing similar effects in room temperature at a slightly higher intensity, which was set by individually determined thresholds to place all participants in a similar region of the heavy intensity domain for a more homogenous exercise stimulus. Future research should investigate the mechanisms of this apparent temporal buffering by vascular volumes. This could include measurements of hemoconcentration, plasma albumin and ion concentrations, blood gases, and catecholamines, for example, to help explain increased stimulation of ventilation and altered [La] kinetics.

7.5 The relationship between Hb_{mass} and fitness differs between sexes

In Chapter V, i) the relationships between fitness and hematological parameters, and ii) the sex-specific values of Hb_{mass} and vascular volumes normalized in different ways, were explored by pooling data from several studies. The resulting dataset was comprised of 79 fitness-matched males and females. While these relationships were positive and similarly strong in both sexes, as evidenced by the similar slopes, the intercepts of the regressions differed, meaning that a given level of fitness was achieved with different oxygen carrying capacity and Hb_{mass} per kg BM and per kg FFM in females vs. males. Specifically, Hb_{mass} and RBCV were greater in males regardless of the normalization method (absolute values or divided by either BM or FFM). However, PV and BV were greater in females after normalization to FFM. The strength of this study was the appropriate matching of aerobic fitness, which meant including males and females of similar VO_2max per kg FFM. If fitness is not accounted for, any potential differences found may be confounded by aerobic conditioning, as fitness also affects Hb_{mass} and vascular volumes. This unmeasured or disregarded effect of aerobic fitness is a limitation obscuring previously published research on the topic.

7.6 Deuterated water can be used to measure Hb FSR

In Chapter VI, a novel method to measure Hb synthesis rates in humans was described. Preliminary data obtained from a study involving 28 days of oral administration of ^2H_2O showed that i) this metabolic label is detectable in blood-derived RBC protein; ii) the rate of appearance of ^2H follows a pattern of a slower phase followed by a steady increase after ~2 weeks; iii) the atom percent excess values measured during the steady phase result in a Hb FSR of ~0.5-0.9%/day. In the future, this method may be applied to study Hb turnover in healthy and clinical populations to better understand the erythropoietic process and how various interventions may affect it. Compared with existing methods to study RBC turnover, this shorter time frame of weeks rather than months, and the technique's relatively low cost and safety, are obvious advantages.

7.7 Conclusion

The main findings and some implications deserving to be highlighted from the present work include the following points:

- Reduced effective [Hb] by CO inhalation did not only limit maximal exercise capacity, but also submaximal exercise capacity as MLSS was destabilized. This reduction in MLSS was accompanied by increased peripheral fatigue of the quadriceps, exacerbated cardiorespiratory and perceptual responses in all participants, and the inability to complete the 30-min exercise bout in two of eight participants. Thus, oxygen delivery is a limiting factor for exercise at the MLSS.
- Acute reduction in BV led to exacerbated responses to 60 min of exercise in the heavy domain, inducing increases in ventilation, [La], and HR in a time-dependent manner. This observation indicates that PV and/or RBCV play a role in the regulation of homeostasis in prolonged steady state exercise. The mechanisms of how vascular volumes may buffer against reductions in durability remain to be explored.
- Like demonstrated for maximal exercise performance, high-intensity submaximal performance also suffered more in trained individuals when the maximum capacity for oxygen delivery was acutely reduced. No sex difference was observed in this phenomenon, and females were as likely as males to demonstrate an unstable [La] response after CO inhalation.
- Sex-based differences in hematological parameters and endurance performance were reduced, diminished, or even reversed when participants are matched for fitness ($\dot{V}O_2$max per kg FFM). When fitness-matched males and females were compared, males possessed higher Hb_{mass} for a given $\dot{V}O_2$max. Females possessed higher FFM-normalized PV and BV for a given $\dot{V}O_2$max. These values revealed sex differences in how a given $\dot{V}O_2$max were achieved.
- Finally, the synthetic rate of hemoglobin may be measured using deuterated water. The technique requires oral administration of ^2H_2O of over two weeks in order to establish a linear increase in the atom percent excess of ^2H in RBC protein. This method is

promising for future investigations in RBC turnover as a low-risk and minimally invasive technique.

References

Allen, D. G., Lamb, G. D., & Westerblad, H. (2008). Skeletal muscle fatigue: cellular mechanisms. *Physiol Rev*, *88*(1), 287-332. https://doi.org/10.1152/physrev.00015.2007

Altehoefer, C., Schmid, A., Büchert, M., Ghanem, N. A., Heinrich, L., & Langer, M. (2002). Characterization of hematopoietic bone marrow in male professional cyclists by magnetic resonance imaging of the lumbar spine. *J Magn Reson Imaging*, *16*(3), 284-288. https://doi.org/10.1002/jmri.10157

Amann, M. (2011). Central and peripheral fatigue: interaction during cycling exercise in humans. *Med Sci Sports Exerc*, *43*(11), 2039-2045. https://doi.org/10.1249/MSS.0b013e31821f59ab

Amann, M., & Calbet, J. A. (2008). Convective oxygen transport and fatigue. In *J Appl Physiol (1985)* (Vol. 104, pp. 861-870). https://doi.org/10.1152/japplphysiol.01008.2007

Arbab-Zadeh, A., Perhonen, M., Howden, E., Peshock, R. M., Zhang, R., Adams-Huet, B., . . . Levine, B. D. (2014). Cardiac remodeling in response to 1 year of intensive endurance training. *Circulation*, *130*(24), 2152-2161. https://doi.org/10.1161/circulationaha.114.010775

Ashenden, M. J., Schumacher, Y. O., Sharpe, K., Varlet-Marie, E., & Audran, M. (2007). Effects of Hemopure on maximal oxygen uptake and endurance performance in healthy humans. *Int J Sports Med*, *28*(5), 381-385. https://doi.org/10.1055/s-2006-924365

Azevedo, R. A., Forot, J., Iannetta, D., MacInnis, M. J., Millet, G. Y., & Murias, J. M. (2021). Slight power output manipulations around the maximal lactate steady state have a similar impact on fatigue in females and males. *J Appl Physiol (1985)*, *130*(6), 1879-1892. https://doi.org/10.1152/japplphysiol.00892.2020

Azevedo, R. D. A., J, E. B., Inglis, E. C., Iannetta, D., & Murias, J. M. (2020). Hypoxia equally reduces the respiratory compensation point and the NIRS-derived [HHb] breakpoint during a ramp-incremental test in young active males. *Physiol Rep*, *8*(12), e14478. https://doi.org/10.14814/phy2.14478

Bain. (2021). *Blood Cells: A Practical Guide* (6th ed.). John Wiley & Sons.

Balke, B., Grillo, G. P., Konecci, E. B., & Luft, U. C. (1954). Work capacity after blood donation. *J Appl Physiol*, *7*(3), 231-238. https://doi.org/10.1152/jappl.1954.7.3.231

Bang, O. (1936). The lactate content of the blood during and after muscular exercise in man. *Skandinavisches Archiv Für Physiologie*, *74*(S10), 51-82. https://doi.org/https://doi.org/10.1111/j.1748-1716.1936.tb00435.x

Barcroft, J., & Uyeno, K. (1923). The effect of concentration of the red blood corpuscles on the dissociation curve of blood. *J Physiol*, *57*(3-4), 200-202. https://doi.org/10.1113/jphysiol.1923.sp002057

Bassett, D. R., Jr., & Howley, E. T. (2000). Limiting factors for maximum oxygen uptake and determinants of endurance performance. *Med Sci Sports Exerc*, *32*(1), 70-84. https://doi.org/10.1097/00005768-200001000-00012

Bauer, A., Tronche, F., Wessely, O., Kellendonk, C., Reichardt, H. M., Steinlein, P., . . . Beug, H. (1999). The glucocorticoid receptor is required for stress erythropoiesis. *Genes Dev*, *13*(22), 2996-3002. https://doi.org/10.1101/gad.13.22.2996

Beaver, W. L., Wasserman, K., & Whipp, B. J. (1986). A new method for detecting anaerobic threshold by gas exchange. *J Appl Physiol (1985)*, *60*(6), 2020-2027. https://doi.org/10.1152/jappl.1986.60.6.2020

Beever, A. T., Tripp, T. R., Zhang, J., & MacInnis, M. J. (2020). NIRS-derived skeletal muscle oxidative capacity is correlated with aerobic fitness and independent of sex. *J Appl Physiol (1985)*, *129*(3), 558-568. https://doi.org/10.1152/japplphysiol.00017.2020

Bejder, J., Breenfeldt Andersen, A., Solheim, S. A., Gybel-Brask, M., Secher, N. H., Johansson, P. I., & Nordsborg, N. B. (2019). Time Trial Performance Is Sensitive to Low-Volume Autologous Blood Transfusion. *Med Sci Sports Exerc*, *51*(4), 692-700. https://doi.org/10.1249/mss.0000000000001837

Belloto, E., Diraison, F., Basset, A., Allain, G., Abdallah, P., & Beylot, M. (2007). Determination of protein replacement rates by deuterated water: validation of underlying assumptions. *Am J Physiol Endocrinol Metab*, *292*(5), E1340-1347. https://doi.org/10.1152/ajpendo.00488.2006

Beneke, R. (1995). Anaerobic threshold, individual anaerobic threshold, and maximal lactate steady state in rowing. *Med Sci Sports Exerc*, *27*(6), 863-867.

Beneke, R., & von Duvillard, S. P. (1996). Determination of maximal lactate steady state response in selected sports events. *Med Sci Sports Exerc*, *28*(2), 241-246. https://doi.org/10.1097/00005768-199602000-00013

Bentley, S. A., Glass, H. I., Lewis, S. M., & Szur, L. (1974). Elution correction in 51Cr red cell survival studies. *Br J Haematol*, *26*(2), 179-184. https://doi.org/10.1111/j.1365-2141.1974.tb00461.x

Berger, N. J., Campbell, I. T., Wilkerson, D. P., & Jones, A. M. (2006). Influence of acute plasma volume expansion on VO2 kinetics, VO2 peak, and performance during high-intensity cycle exercise. *J Appl Physiol (1985)*, *101*(3), 707-714. https://doi.org/10.1152/japplphysiol.00154.2006

Bergman, B. C., Wolfel, E. E., Butterfield, G. E., Lopaschuk, G. D., Casazza, G. A., Horning, M. A., & Brooks, G. A. (1999). Active muscle and whole body lactate kinetics after endurance training in men. *J Appl Physiol (1985)*, *87*(5), 1684-1696. https://doi.org/10.1152/jappl.1999.87.5.1684

Bhoopalan, S. V., Huang, L. J., & Weiss, M. J. (2020). Erythropoietin regulation of red blood cell production: from bench to bedside and back. *F1000Res*, *9*. https://doi.org/10.12688/f1000research.26648.1

Billat, V., Sirvent, P., Lepretre, P. M., & Koralsztein, J. P. (2004). Training effect on performance, substrate balance and blood lactate concentration at maximal lactate steady state in master endurance-runners. *Pflugers Arch*, *447*(6), 875-883. https://doi.org/10.1007/s00424-003-1215-8

Billat, V. L., Sirvent, P., Py, G., Koralsztein, J. P., & Mercier, J. (2003). The concept of maximal lactate steady state: a bridge between biochemistry, physiology and sport science. *Sports Med*, *33*(6), 407-426. https://doi.org/10.2165/00007256-200333060-00003

Blomqvist, C. G., & Saltin, B. (1983). Cardiovascular adaptations to physical training. *Annu Rev Physiol*, *45*, 169-189. https://doi.org/10.1146/annurev.ph.45.030183.001125

Bodary, P. F., Pate, R. R., Wu, Q. F., & McMillan, G. S. (1999). Effects of acute exercise on plasma erythropoietin levels in trained runners. *Med Sci Sports Exerc*, *31*(4), 543-546. https://doi.org/10.1097/00005768-199904000-00008

Bonne, T. C., Doucende, G., Flück, D., Jacobs, R. A., Nordsborg, N. B., Robach, P., . . . Lundby, C. (2014). Phlebotomy eliminates the maximal cardiac output response to six weeks of exercise training. *Am J Physiol Regul Integr Comp Physiol*, *306*(10), R752-760. https://doi.org/10.1152/ajpregu.00028.2014

Borg, G. A. (1982). Psychophysical bases of perceived exertion. *Med Sci Sports Exerc*, *14*(5), 377-381.

Bratteby, L. E., & Wadman, B. (1968). Labelling of red blood cells in vitro with small amounts of di-iso-propyl-flurophosphonate (DF32P). *Scand J Clin Lab Invest*, *21*(3), 197-201. https://doi.org/10.3109/00365516809076985

Brien, A. J., & Simon, T. L. (1987). The effects of red blood cell infusion on 10-km race time. *JAMA*, *257*(20), 2761-2765.

Brotherhood, J., Brozović, B., & Pugh, L. G. (1975). Haematological status of middle- and long-distance runners. *Clin Sci Mol Med*, *48*(2), 139-145. https://doi.org/10.1042/cs0480139

Broxterman, R. M., Ade, C. J., Craig, J. C., Wilcox, S. L., Schlup, S. J., & Barstow, T. J. (2015). Influence of blood flow occlusion on muscle oxygenation characteristics and the parameters of the power-duration relationship. In *J Appl Physiol (1985)* (Vol. 118, pp. 880-889). 2015 the American Physiological Society. https://doi.org/10.1152/japplphysiol.00875.2014

Buick, F. J., Gledhill, N., Froese, A. B., Spriet, L., & Meyers, E. C. (1980). Effect of induced erythrocythemia on aerobic work capacity. *J Appl Physiol Respir Environ Exerc Physiol*, *48*(4), 636-642. https://doi.org/10.1152/jappl.1980.48.4.636

Burge, C. M., & Skinner, S. L. (1995). Determination of hemoglobin mass and blood volume with CO: evaluation and application of a method. *J Appl Physiol (1985)*, *79*(2), 623-631. https://doi.org/10.1152/jappl.1995.79.2.623

Burnley, M., Roberts, C. L., Thatcher, R., Doust, J. H., & Jones, A. M. (2006). Influence of blood donation on O2 uptake on-kinetics, peak O2 uptake and time to exhaustion during severe-intensity cycle exercise in humans. *Exp Physiol*, *91*(3), 499-509. https://doi.org/10.1113/expphysiol.2005.032805

Burnley, M., Vanhatalo, A., & Jones, A. M. (2012). Distinct profiles of neuromuscular fatigue during muscle contractions below and above the critical torque in humans. *J Appl Physiol (1985)*, *113*(2), 215-223. https://doi.org/10.1152/japplphysiol.00022.2012

Calbet, J. A., Boushel, R., Radegran, G., Sondergaard, H., Wagner, P. D., & Saltin, B. (2003). Why is VO2 max after altitude acclimatization still reduced despite normalization of arterial O2 content? In *Am J Physiol Regul Integr Comp Physiol* (Vol. 284, pp. R304-316). https://doi.org/10.1152/ajpregu.00156.2002

Calbet, J. A., Jensen-Urstad, M., van Hall, G., Holmberg, H. C., Rosdahl, H., & Saltin, B. (2004). Maximal muscular vascular conductances during whole body upright exercise in humans. *J Physiol*, *558*(Pt 1), 319-331. https://doi.org/10.1113/jphysiol.2003.059287

Calbet, J. A., & Joyner, M. J. (2010). Disparity in regional and systemic circulatory capacities: do they affect the regulation of the circulation? *Acta Physiol (Oxf)*, *199*(4), 393-406. https://doi.org/10.1111/j.1748-1716.2010.02125.x

Calbet, J. A., Losa-Reyna, J., Torres-Peralta, R., Rasmussen, P., Ponce-González, J. G., Sheel, A. W., . . . Lundby, C. (2015). Limitations to oxygen transport and utilization during sprint exercise in humans: evidence for a functional reserve in muscle O2 diffusing capacity. *J Physiol*, *593*(20), 4649-4664. https://doi.org/10.1113/jp270408

Calbet, J. A., Lundby, C., Koskolou, M., & Boushel, R. (2006). Importance of hemoglobin concentration to exercise: acute manipulations. *Respir Physiol Neurobiol*, *151*(2-3), 132-140. https://doi.org/10.1016/j.resp.2006.01.014

Calbet, J. A., Rådegran, G., Boushel, R., & Saltin, B. (2009). On the mechanisms that limit oxygen uptake during exercise in acute and chronic hypoxia: role of muscle mass. *J Physiol*, *587*(2), 477-490. https://doi.org/10.1113/jphysiol.2008.162271

Caldemeyer, K. S., Smith, R. R., Harris, A., Williams, T., Huang, Y., Eckert, G. J., & Slemenda, C. W. (1996). Hematopoietic bone marrow hyperplasia: correlation of spinal MR findings, hematologic parameters, and bone mineral density in endurance athletes. *Radiology*, *198*(2), 503-508. https://doi.org/10.1148/radiology.198.2.8596857

Cardinale, D. A., Larsen, F. J., Schiffer, T. A., Morales-Alamo, D., Ekblom, B., Calbet, J. A. L., . . . Boushel, R. (2018). Superior Intrinsic Mitochondrial Respiration in Women Than in Men. *Front Physiol*, *9*, 1133. https://doi.org/10.3389/fphys.2018.01133

Cardús, J., Marrades, R. M., Roca, J., Barberà, J. A., Diaz, O., Masclans, J. R., . . . Wagner, P. D. (1998). Effects of F(I)O2 on leg VO2 during cycle ergometry in sedentary subjects. *Med Sci Sports Exerc*, *30*(5), 697-703. https://doi.org/10.1097/00005768-199805000-00009

Celsing, F., & Ekblom, B. (1986). Anemia causes a relative decrease in blood lactate concentration during exercise. *Eur J Appl Physiol Occup Physiol*, *55*(1), 74-78. https://doi.org/10.1007/bf00422897

Celsing, F., Nystrom, J., Pihlstedt, P., Werner, B., & Ekblom, B. (1986). Effect of long-term anemia and retransfusion on central circulation during exercise. *J Appl Physiol (1985)*, *61*(4), 1358-1362. https://doi.org/10.1152/jappl.1986.61.4.1358

Celsing, F., Svedenhag, J., Pihlstedt, P., & Ekblom, B. (1987). Effects of anaemia and stepwise-induced polycythaemia on maximal aerobic power in individuals with high and low haemoglobin concentrations. *Acta Physiol Scand*, *129*(1), 47-54. https://doi.org/10.1111/j.1748-1716.1987.tb08038.x

Chaplin, H., Jr., Mollison, P. L., & Vetter, H. (1953). The body/venous hematocrit ratio: its constancy over a wide hematocrit range. *J Clin Invest*, *32*(12), 1309-1316. https://doi.org/10.1172/jci102859

Christensen, T., & Christensen, G. (1978). The effects of blood loss on the performance of physical exercise. *Eur J Appl Physiol Occup Physiol*, *39*(1), 17-25. https://doi.org/10.1007/bf00429675

Convertino, V. A. (1991). Blood volume: its adaptation to endurance training. *Med Sci Sports Exerc*, *23*(12), 1338-1348.

Convertino, V. A. (2007). Blood volume response to physical activity and inactivity. *Am J Med Sci*, *334*(1), 72-79. https://doi.org/10.1097/MAJ.0b013e318063c6e4

Coviello, A. D., Kaplan, B., Lakshman, K. M., Chen, T., Singh, A. B., & Bhasin, S. (2008). Effects of graded doses of testosterone on erythropoiesis in healthy young and older men. *J Clin Endocrinol Metab*, *93*(3), 914-919. https://doi.org/10.1210/jc.2007-1692

Coyle, E. F. (1995). Integration of the physiological factors determining endurance performance ability. *Exerc Sport Sci Rev*, *23*, 25-63.

Coyle, E. F., Coggan, A. R., Hopper, M. K., & Walters, T. J. (1988). Determinants of endurance in well-trained cyclists. *J Appl Physiol (1985)*, *64*(6), 2622-2630. https://doi.org/10.1152/jappl.1988.64.6.2622

Coyle, E. F., Feltner, M. E., Kautz, S. A., Hamilton, M. T., Montain, S. J., Baylor, A. M., . . . Petrek, G. W. (1991). Physiological and biomechanical factors associated with elite endurance cycling performance. *Med Sci Sports Exerc*, *23*(1), 93-107.

Coyle, E. F., & González-Alonso, J. (2001). Cardiovascular drift during prolonged exercise: new perspectives. *Exerc Sport Sci Rev*, *29*(2), 88-92. https://doi.org/10.1097/00003677-200104000-00009

Cureton, K. J. (1981). Matching of Male and Female Subjects Using VO2 max. *Research Quarterly for Exercise and Sport*, *52*(2), 264-268. https://doi.org/10.1080/02701367.1981.10607865

de Back, D. Z., Kostova, E. B., van Kraaij, M., van den Berg, T. K., & van Bruggen, R. (2014). Of macrophages and red blood cells; a complex love story. *Front Physiol*, *5*, 9. https://doi.org/10.3389/fphys.2014.00009

Dekerle, J., Baron, B., Dupont, L., Vanvelcenaher, J., & Pelayo, P. (2003). Maximal lactate steady state, respiratory compensation threshold and critical power. *Eur J Appl Physiol*, *89*(3-4), 281-288. https://doi.org/10.1007/s00421-002-0786-y

Dempsey, J. A., & Wagner, P. D. (1999). Exercise-induced arterial hypoxemia. *J Appl Physiol (1985)*, *87*(6), 1997-2006. https://doi.org/10.1152/jappl.1999.87.6.1997

Diaz-Canestro, C., & Montero, D. (2022). Sex and age interaction in fundamental circulatory volumetric variables at peak working capacity. *Biol Sex Differ*, *13*(1), 1. https://doi.org/10.1186/s13293-021-00409-9

Diaz-Canestro, C., Pentz, B., Sehgal, A., & Montero, D. (2021). Sex Differences In Cardiorespiratory Fitness Are Explained By Blood Volume And Oxygen Carrying Capacity. *Cardiovasc Res*. https://doi.org/10.1093/cvr/cvab028

Diaz-Canestro, C., Siebenmann, C., & Montero, D. (2021). Blood Oxygen Carrying Capacity Determines Cardiorespiratory Fitness in Middle-Age and Older Women and Men. *Med Sci Sports Exerc*, *53*(11), 2274-2282. https://doi.org/10.1249/mss.0000000000002720

Dorff, A., Bradford, C., Hunsaker, A., Atkinson, J., Rhees, J., Leach, O. K., & Gifford, J. R. (2024). Vascular dysfunction and the age-related decline in critical power. *Exp Physiol*, *109*(2), 240-254. https://doi.org/10.1113/ep091571

Dufner, D. A., Bederman, I. R., Brunengraber, D. Z., Rachdaoui, N., Ismail-Beigi, F., Siegfried, B. A., . . . Previs, S. F. (2005). Using 2H2O to study the influence of feeding on protein synthesis: effect of isotope equilibration in vivo vs. in cell culture. *Am J Physiol Endocrinol Metab*, *288*(6), E1277-1283. https://doi.org/10.1152/ajpendo.00580.2004

Durussel, J., Daskalaki, E., Anderson, M., Chatterji, T., Wondimu, D. H., Padmanabhan, N., . . . Pitsiladis, Y. P. (2013). Haemoglobin mass and running time trial performance after recombinant human erythropoietin administration in trained men. *PLoS One*, *8*(2), e56151. https://doi.org/10.1371/journal.pone.0056151

Dziembowska, I., Wójcik, M., Bukowski, J., & Żekanowska, E. (2021). Physical Training Increases Erythroferrone Levels in Men. *Biology (Basel)*, *10*(11). https://doi.org/10.3390/biology10111215

Díaz, V., Lombardi, G., Ricci, C., Jacobs, R. A., Montalvo, Z., Lundby, C., & Banfi, G. (2011). Reticulocyte and haemoglobin profiles in elite triathletes over four consecutive seasons. *Int J Lab Hematol*, *33*(6), 638-644. https://doi.org/10.1111/j.1751-553X.2011.01348.x

Eastwood, A., Bourdon, P. C., Snowden, K. R., & Gore, C. J. (2012). Detraining decreases Hb(mass) of triathletes. *Int J Sports Med*, *33*(4), 253-257. https://doi.org/10.1055/s-0031-1291184

Ekblom, B., Goldbarg, A. N., & Gullbring, B. (1972). Response to exercise after blood loss and reinfusion. *J Appl Physiol*, *33*(2), 175-180. https://doi.org/10.1152/jappl.1972.33.2.175

Ekblom, B., & Huot, R. (1972). Response to submaximal and maximal exercise at different levels of carboxyhemoglobin. *Acta Physiol Scand*, *86*(4), 474-482. https://doi.org/10.1111/j.1748-1716.1972.tb05350.x

Ekblom, B., Huot, R., Stein, E. M., & Thorstensson, A. T. (1975). Effect of changes in arterial oxygen content on circulation and physical performance. *J Appl Physiol*, *39*(1), 71-75. https://doi.org/10.1152/jappl.1975.39.1.71

Ekblom, B., Wilson, G., & Astrand, P. O. (1976). Central circulation during exercise after venesection and reinfusion of red blood cells. *J Appl Physiol*, *40*(3), 379-383. https://doi.org/10.1152/jappl.1976.40.3.379

Enoka, R. M., & Duchateau, J. (2016). Translating Fatigue to Human Performance. *Med Sci Sports Exerc*, *48*(11), 2228-2238. https://doi.org/10.1249/mss.0000000000000929

Falz, R., Fikenzer, S., Hoppe, S., & Busse, M. (2019). Normal Values of Hemoglobin Mass and Blood Volume in Young, Active Women and Men. *Int J Sports Med*, *40*(4), 236-244. https://doi.org/10.1055/a-0826-9235

Faude, O., Hecksteden, A., Hammes, D., Schumacher, F., Besenius, E., Sperlich, B., & Meyer, T. (2017). Reliability of time-to-exhaustion and selected psycho-physiological variables during constant-load cycling at the maximal lactate steady-state. *Appl Physiol Nutr Metab*, *42*(2), 142-147. https://doi.org/10.1139/apnm-2016-0375

Faude, O., Kindermann, W., & Meyer, T. (2009). Lactate threshold concepts: how valid are they? *Sports Med*, *39*(6), 469-490. https://doi.org/10.2165/00007256-200939060-00003

Fellmann, N. (1992). Hormonal and plasma volume alterations following endurance exercise. A brief review. *Sports Med*, *13*(1), 37-49. https://doi.org/10.2165/00007256-199213010-00004

Ferguson, B. S., Rogatzki, M. J., Goodwin, M. L., Kane, D. A., Rightmire, Z., & Gladden, L. B. (2018). Lactate metabolism: historical context, prior misinterpretations, and current understanding. *Eur J Appl Physiol*, *118*(4), 691-728. https://doi.org/10.1007/s00421-017-3795-6

Ferretti, G., Kayser, B., Schena, F., Turner, D. L., & Hoppeler, H. (1992). Regulation of perfusive O2 transport during exercise in humans: effects of changes in haemoglobin concentration. *J Physiol*, *455*, 679-688. https://doi.org/10.1113/jphysiol.1992.sp019322

Fortney, S. M., Nadel, E. R., Wenger, C. B., & Bove, J. R. (1981). Effect of acute alterations of blood volume on circulatory performance in humans. *J Appl Physiol Respir Environ Exerc Physiol*, *50*(2), 292-298. https://doi.org/10.1152/jappl.1981.50.2.292

Freedson, P. S. (1981). The influence of hemoglobin concentration on exercise cardiac output. *Int J Sports Med*, *2*(2), 81-86. https://doi.org/10.1055/s-2008-1034587

Friedmann, B., Bauer, T., Menold, E., & Bärtsch, P. (2004). Exercise with the intensity of the individual anaerobic threshold in acute hypoxia. *Med Sci Sports Exerc*, *36*(10), 1737-1742. https://doi.org/10.1249/01.mss.0000142307.62181.37

Fu, A., & Nair, K. S. (1998). Age effect on fibrinogen and albumin synthesis in humans. *Am J Physiol*, *275*(6), E1023-1030. https://doi.org/10.1152/ajpendo.1998.275.6.E1023

Fulco, C. S., Lewis, S. F., Frykman, P. N., Boushel, R., Smith, S., Harman, E. A., . . . Pandolf, K. B. (1996). Muscle fatigue and exhaustion during dynamic leg exercise in normoxia and hypobaric hypoxia. *J Appl Physiol (1985)*, *81*(5), 1891-1900. https://doi.org/10.1152/jappl.1996.81.5.1891

Galbo, H., Holst, J. J., Christensen, N. J., & Hilsted, J. (1976). Glucagon and plasma catecholamines during beta-receptor blockade in exercising man. *J Appl Physiol*, *40*(6), 855-863. https://doi.org/10.1152/jappl.1976.40.6.855

Gandevia, S. C. (2001). Spinal and supraspinal factors in human muscle fatigue. *Physiol Rev*, *81*(4), 1725-1789. https://doi.org/10.1152/physrev.2001.81.4.1725

Garby, L., Sjolin, S., & Vuille, J. C. (1962). Studies on erythro-kinetics in infancy. II. The relative rate of synthesis of haemoglobin F and haemoglobin A during the first months of life. *Acta Paediatr (Stockh)*, *51*, 245-254. https://doi.org/10.1111/j.1651-2227.1962.tb06538.x

Gersovitz, M., Munro, H. N., Udall, J., & Young, V. R. (1980). Albumin synthesis in young and elderly subjects using a new stable isotope methodology: response to level of protein intake. *Metabolism*, *29*(11), 1075-1086. https://doi.org/10.1016/0026-0495(80)90219-x

Gifford, J. R., Garten, R. S., Nelson, A. D., Trinity, J. D., Layec, G., Witman, M. A., . . . Richardson, R. S. (2016). Symmorphosis and skeletal muscle V̇O2 max : in vivo and in vitro measures reveal differing constraints in the exercise-trained and untrained human. *J Physiol*, *594*(6), 1741-1751. https://doi.org/10.1113/jp271229

Gollnick, P. D., & Saltin, B. (1982). Significance of skeletal muscle oxidative enzyme enhancement with endurance training. *Clin Physiol*, *2*(1), 1-12. https://doi.org/10.1111/j.1475-097x.1982.tb00001.x

Gonzalez-Alonso, J., Richardson, R. S., & Saltin, B. (2001). Exercising skeletal muscle blood flow in humans responds to reduction in arterial oxyhaemoglobin, but not to altered free oxygen. *J Physiol*, *530*(Pt 2), 331-341. https://doi.org/10.1111/j.1469-7793.2001.0331l.x

González-Alonso, J., Mora-Rodríguez, R., & Coyle, E. F. (1999). Supine exercise restores arterial blood pressure and skin blood flow despite dehydration and hyperthermia. *Am J Physiol*, *277*(2), H576-583. https://doi.org/10.1152/ajpheart.1999.277.2.H576

Goodrich, J. A., Frisco, D. J., Kim, S., Holliday, M., Rueda, M., Poddar, S., & Byrnes, W. C. (2020). The importance of lean mass and iron deficiency when comparing hemoglobin mass in male and female athletic groups. *J Appl Physiol (1985)*, *129*(4), 855-863. https://doi.org/10.1152/japplphysiol.00391.2020

Goran, M., Fields, D. A., Hunter, G. R., Herd, S. L., & Weinsier, R. L. (2000). Total body fat does not influence maximal aerobic capacity. *Int J Obes Relat Metab Disord*, *24*(7), 841-848. https://doi.org/10.1038/sj.ijo.0801241

Gordon, D., Marshall, K., Connell, A., & Barnes, R. J. (2010). Influence of blood donation on oxygen uptake kinetics during moderate and heavy intensity cycle exercise. *Int J Sports Med*, *31*(5), 298-303. https://doi.org/10.1055/s-0030-1248321

Gore, C. J., Hahn, A. G., Burge, C. M., & Telford, R. D. (1997). VO2max and haemoglobin mass of trained athletes during high intensity training. *Int J Sports Med*, *18*(6), 477-482. https://doi.org/10.1055/s-2007-972667

Gore, C. J., Little, S. C., Hahn, A. G., Scroop, G. C., Norton, K. I., Bourdon, P. C., . . . Emonson, D. L. (1997). Reduced performance of male and female athletes at 580 m altitude. *Eur J Appl Physiol Occup Physiol*, *75*(2), 136-143. https://doi.org/10.1007/s004210050138

Gough, C. E., Eastwood, A., Saunders, P. U., Anson, J. M., & Gore, C. J. (2012). Spurious Hb mass increases following exercise. *Int J Sports Med*, *33*(9), 691-695. https://doi.org/10.1055/s-0031-1295441

Goulding, R. P., Roche, D. M., & Marwood, S. (2020). Effect of Hyperoxia on Critical Power and V˙O2 Kinetics during Upright Cycling. *Med Sci Sports Exerc*, *52*(5), 1041-1049. https://doi.org/10.1249/mss.0000000000002234
Hampson, N. B. (1998). Pulse oximetry in severe carbon monoxide poisoning. *Chest*, *114*(4), 1036-1041. https://doi.org/10.1378/chest.114.4.1036
Hardy, C. J., & Rejeski, W. J. (1989). Not What, but How One Feels: The Measurement of Affect during Exercise. *Journal of Sport and Exercise Psychology*, *11*(3), 304-317. https://doi.org/10.1123/jsep.11.3.304
Harms, C. A., Wetter, T. J., McClaran, S. R., Pegelow, D. F., Nickele, G. A., Nelson, W. B., . . . Dempsey, J. A. (1998). Effects of respiratory muscle work on cardiac output and its distribution during maximal exercise. *J Appl Physiol (1985)*, *85*(2), 609-618. https://doi.org/10.1152/jappl.1998.85.2.609
Harnish, C. R., Swensen, T. C., & Pate, R. R. (2001). Methods for estimating the maximal lactate steady state in trained cyclists. *Med Sci Sports Exerc*, *33*(6), 1052-1055. https://doi.org/10.1097/00005768-200106000-00027
Hee, J., Callais, F., Momas, I., Laurent, A. M., Min, S., Molinier, P., . . . Festy, B. (1995). Smokers' behaviour and exposure according to cigarette yield and smoking experience. *Pharmacol Biochem Behav*, *52*(1), 195-203. https://doi.org/10.1016/0091-3057(95)00089-f
Hellsten, Y., & Nyberg, M. (2015). Cardiovascular Adaptations to Exercise Training. *Compr Physiol*, *6*(1), 1-32. https://doi.org/10.1002/cphy.c140080
Hero, M., Wickman, S., Hanhijärvi, R., Siimes, M. A., & Dunkel, L. (2005). Pubertal upregulation of erythropoiesis in boys is determined primarily by androgen. *J Pediatr*, *146*(2), 245-252. https://doi.org/10.1016/j.jpeds.2004.09.002
Heuberger, J., Rotmans, J. I., Gal, P., Stuurman, F. E., van 't Westende, J., Post, T. E., . . . Cohen, A. F. (2017). Effects of erythropoietin on cycling performance of well trained cyclists: a double-blind, randomised, placebo-controlled trial. *Lancet Haematol*, *4*(8), e374-e386. https://doi.org/10.1016/s2352-3026(17)30105-9
Hibbert, J. M., Sutherland, G. B., Jr., Wright, L. L., Jr., Wolfe, L. G., Wolfe, K. A., Gao, S. P., . . . Abd-Elfattah, A. S. (2001). Measurement of hemoglobin synthesis rate in vivo using a stable isotope method. *Anal Biochem*, *291*(1), 118-123. https://doi.org/10.1006/abio.2001.4997
Hill, D. W., McFarlin, B. K., & Vingren, J. L. (2021). Exercise above the maximal lactate steady state does not elicit a $\dot{V}$O(2) slow component that leads to attainment of $\dot{V}$O(2max). *Appl Physiol Nutr Metab*, *46*(2), 133-140. https://doi.org/10.1139/apnm-2020-0261
Hill, D. W., & Rowell, A. L. (1997). Responses to exercise at the velocity associated with VO2max. *Med Sci Sports Exerc*, *29*(1), 113-116. https://doi.org/10.1097/00005768-199701000-00016
Hill, D. W., Williams, C. S., & Burt, S. E. (1997). Responses to exercise at 92% and 100% of the velocity associated with VO2max. *Int J Sports Med*, *18*(5), 325-329. https://doi.org/10.1055/s-2007-972641
Hinton, P. S., Giordano, C., Brownlie, T., & Haas, J. D. (2000). Iron supplementation improves endurance after training in iron-depleted, nonanemic women. *J Appl Physiol (1985)*, *88*(3), 1103-1111. https://doi.org/10.1152/jappl.2000.88.3.1103

Houtman, S., Oeseburg, B., & Hopman, M. T. (2000). Blood volume and hemoglobin after spinal cord injury. *Am J Phys Med Rehabil*, *79*(3), 260-265. https://doi.org/10.1097/00002060-200005000-00008
Iannetta, D., Fontana, F. Y., Maturana, F. M., Inglis, E. C., Pogliaghi, S., Keir, D. A., & Murias, J. M. (2018). An equation to predict the maximal lactate steady state from ramp-incremental exercise test data in cycling. *J Sci Med Sport*, *21*(12), 1274-1280. https://doi.org/10.1016/j.jsams.2018.05.004
Iannetta, D., Inglis, E. C., Fullerton, C., Passfield, L., & Murias, J. M. (2018). Metabolic and performance-related consequences of exercising at and slightly above MLSS. *Scand J Med Sci Sports*, *28*(12), 2481-2493. https://doi.org/10.1111/sms.13280
Iannetta, D., Inglis, E. C., Mattu, A. T., Fontana, F. Y., Pogliaghi, S., Keir, D. A., & Murias, J. M. (2020). A Critical Evaluation of Current Methods for Exercise Prescription in Women and Men. *Med Sci Sports Exerc*, *52*(2), 466-473. https://doi.org/10.1249/mss.0000000000002147
Iannetta, D., Ingram, C. P., Keir, D. A., & Murias, J. M. (2021). Methodological Reconciliation of CP and MLSS and Their Agreement with the Maximal Metabolic Steady State. *Med Sci Sports Exerc*. https://doi.org/10.1249/mss.0000000000002831
Iannetta, D., Passfield, L., Qahtani, A., MacInnis, M. J., & Murias, J. M. (2019). Interlimb differences in parameters of aerobic function and local profiles of deoxygenation during double-leg and counterweighted single-leg cycling. *Am J Physiol Regul Integr Comp Physiol*, *317*(6), R840-r851. https://doi.org/10.1152/ajpregu.00164.2019
Iannetta, D., Zhang, J., Murias, J. M., & Aboodarda, S. J. (2022). Neuromuscular and perceptual mechanisms of fatigue accompanying task failure in response to moderate-, heavy-, severe-, and extreme-intensity cycling. *J Appl Physiol (1985)*, *133*(2), 323-334. https://doi.org/10.1152/japplphysiol.00764.2021
Inglis, E. C., Iannetta, D., Keir, D. A., & Murias, J. M. (2019). Training-Induced Changes in the Respiratory Compensation Point, Deoxyhemoglobin Break Point, and Maximal Lactate Steady State: Evidence of Equivalence. *Int J Sports Physiol Perform*, 1-7. https://doi.org/10.1123/ijspp.2019-0046
Ivy, J. L., Withers, R. T., Van Handel, P. J., Elger, D. H., & Costill, D. L. (1980). Muscle respiratory capacity and fiber type as determinants of the lactate threshold. *J Appl Physiol Respir Environ Exerc Physiol*, *48*(3), 523-527. https://doi.org/10.1152/jappl.1980.48.3.523
Janetzko, K., Bocher, R., Klotz, K. F., Kirchner, H., & Kluter, H. (1998). Effects of blood donation on the physical fitness and hemorheology of healthy elderly donors. *Vox Sang*, *75*(1), 7-11.
Johnson, D. M., Roberts, J., & Gordon, D. (2019). The acute effects of whole blood donation on cardiorespiratory and haematological factors in exercise: A systematic review. *PLoS One*, *14*(4), e0215346. https://doi.org/10.1371/journal.pone.0215346
Jonckheere, A. I., Smeitink, J. A., & Rodenburg, R. J. (2012). Mitochondrial ATP synthase: architecture, function and pathology. *J Inherit Metab Dis*, *35*(2), 211-225. https://doi.org/10.1007/s10545-011-9382-9
Jones, A. M., Burnley, M., Black, M. I., Poole, D. C., & Vanhatalo, A. (2019). The maximal metabolic steady state: redefining the 'gold standard'. *Physiol Rep*, *7*(10), e14098. https://doi.org/10.14814/phy2.14098

Jones, A. M., & Vanhatalo, A. (2017). The 'Critical Power' Concept: Applications to Sports Performance with a Focus on Intermittent High-Intensity Exercise. *Sports Med*, *47*(Suppl 1), 65-78. https://doi.org/10.1007/s40279-017-0688-0

Jones, H., George, K., Edwards, B., & Atkinson, G. (2007). Is the magnitude of acute post-exercise hypotension mediated by exercise intensity or total work done? *Eur J Appl Physiol*, *102*(1), 33-40. https://doi.org/10.1007/s00421-007-0562-0

Joyner, M. J. (2017). Physiological limits to endurance exercise performance: influence of sex. *J Physiol*, *595*(9), 2949-2954. https://doi.org/10.1113/jp272268

Kalliokoski, K. K., Oikonen, V., Takala, T. O., Sipilä, H., Knuuti, J., & Nuutila, P. (2001). Enhanced oxygen extraction and reduced flow heterogeneity in exercising muscle in endurance-trained men. *Am J Physiol Endocrinol Metab*, *280*(6), E1015-1021. https://doi.org/10.1152/ajpendo.2001.280.6.E1015

Kanstrup, I. L., & Ekblom, B. (1982). Acute hypervolemia, cardiac performance, and aerobic power during exercise. *J Appl Physiol Respir Environ Exerc Physiol*, *52*(5), 1186-1191. https://doi.org/10.1152/jappl.1982.52.5.1186

Kanstrup, I. L., & Ekblom, B. (1984). Blood volume and hemoglobin concentration as determinants of maximal aerobic power. *Med Sci Sports Exerc*, *16*(3), 256-262.

Kasumov, T., Dabkowski, E. R., Shekar, K. C., Li, L., Ribeiro, R. F., Jr., Walsh, K., . . . Stanley, W. C. (2013). Assessment of cardiac proteome dynamics with heavy water: slower protein synthesis rates in interfibrillar than subsarcolemmal mitochondria. *Am J Physiol Heart Circ Physiol*, *304*(9), H1201-1214. https://doi.org/10.1152/ajpheart.00933.2012

Katz, A. M. (2002). Ernest Henry Starling, His Predecessors, and the “Law of the Heart”. *Circulation*, *106*(23), 2986-2992. https://doi.org/doi:10.1161/01.CIR.0000040594.96123.55

Keir, D. A., Fontana, F. Y., Robertson, T. C., Murias, J. M., Paterson, D. H., Kowalchuk, J. M., & Pogliaghi, S. (2015). Exercise Intensity Thresholds: Identifying the Boundaries of Sustainable Performance. *Med Sci Sports Exerc*, *47*(9), 1932-1940. https://doi.org/10.1249/mss.0000000000000613

Keir, D. A., Pogliaghi, S., & Murias, J. M. (2018). The Respiratory Compensation Point and the Deoxygenation Break Point Are Valid Surrogates for Critical Power and Maximum Lactate Steady State. *Med Sci Sports Exerc*, *50*(11), 2375-2378. https://doi.org/10.1249/mss.0000000000001698

Keiser, S., Meinild-Lundby, A. K., Steiner, T., Trösch, S., Rauber, S., Krafft, A., . . . Lundby, C. (2017). Detection of blood volumes and haemoglobin mass by means of CO re-breathing and indocyanine green and sodium fluorescein injections. *Scand J Clin Lab Invest*, *77*(3), 164-174. https://doi.org/10.1080/00365513.2016.1271908

Khera, P. K., Smith, E. P., Lindsell, C. J., Rogge, M. C., Haggerty, S., Wagner, D. A., . . . Cohen, R. M. (2015). Use of an oral stable isotope label to confirm variation in red blood cell mean age that influences HbA1c interpretation. *Am J Hematol*, *90*(1), 50-55. https://doi.org/10.1002/ajh.23866

Klausen, K., Andersen, C., & Nandrup, S. (1983). Acute effects of cigarette smoking and inhalation of carbon monoxide during maximal exercise. *Eur J Appl Physiol Occup Physiol*, *51*(3), 371-379. https://doi.org/10.1007/bf00429074

Koike, A., Wasserman, K., Armon, Y., & Weiler-Ravell, D. (1991). The work-rate-dependent effect of carbon monoxide on ventilatory control during exercise. *Respir Physiol*, *85*(2), 169-183. https://doi.org/10.1016/0034-5687(91)90059-r

Koistinen, P., Takala, T., Martikkala, V., & Leppäluoto, J. (1995). Aerobic fitness influences the response of maximal oxygen uptake and lactate threshold in acute hypobaric hypoxia. *Int J Sports Med*, *16*(2), 78-81. https://doi.org/10.1055/s-2007-972968
Kontro, H., Bertagnolli, C., Murias, J. M., & MacInnis, M. J. (2022). Impairment in maximal lactate steady state after carbon monoxide inhalation is related to training status. *Exp Physiol*. https://doi.org/10.1113/ep090642
Koskolou, M. D., Roach, R. C., Calbet, J. A., Radegran, G., & Saltin, B. (1997). Cardiovascular responses to dynamic exercise with acute anemia in humans. *Am J Physiol*, *273*(4), H1787-1793. https://doi.org/10.1152/ajpheart.1997.273.4.H1787
Krip, B., Gledhill, N., Jamnik, V., & Warburton, D. (1997). Effect of alterations in blood volume on cardiac function during maximal exercise. *Med Sci Sports Exerc*, *29*(11), 1469-1476. https://doi.org/10.1097/00005768-199711000-00013
Lacombe, S. P., Goodman, J. M., Spragg, C. M., Liu, S., & Thomas, S. G. (2011). Interval and continuous exercise elicit equivalent postexercise hypotension in prehypertensive men, despite differences in regulation. *Appl Physiol Nutr Metab*, *36*(6), 881-891. https://doi.org/10.1139/h11-113
Lai, N., Gladden, L. B., Carlier, P. G., & Cabrera, M. E. (2008). Models of muscle contraction and energetics. *Drug Discov Today Dis Models*, *5*(4), 273-288. https://doi.org/10.1016/j.ddmod.2009.07.001
Lawler, J., Powers, S. K., & Thompson, D. (1988). Linear relationship between VO2max and VO2max decrement during exposure to acute hypoxia. *J Appl Physiol (1985)*, *64*(4), 1486-1492. https://doi.org/10.1152/jappl.1988.64.4.1486
Legaz-Arrese, A., Carranza-García, L. E., Serrano-Ostáriz, E., González-Ravé, J. M., & Terrados, N. (2011). The traditional maximal lactate steady state test versus the 5 × 2000 m test. *Int J Sports Med*, *32*(11), 845-850. https://doi.org/10.1055/s-0031-1283178
Lundby, C., Boushel, R., Robach, P., Møller, K., Saltin, B., & Calbet, J. A. (2008). During hypoxic exercise some vasoconstriction is needed to match O2 delivery with O2 demand at the microcirculatory level. *J Physiol*, *586*(1), 123-130. https://doi.org/10.1113/jphysiol.2007.146035
Lundby, C., & Jacobs, R. A. (2016). Adaptations of skeletal muscle mitochondria to exercise training. *Exp Physiol*, *101*(1), 17-22. https://doi.org/10.1113/ep085319
Lundby, C., Montero, D., & Joyner, M. (2017). Biology of VO(2) max: looking under the physiology lamp. *Acta Physiol (Oxf)*, *220*(2), 218-228. https://doi.org/10.1111/apha.12827
MacDonald, A. J., Small, A. C., Greig, C. A., Husi, H., Ross, J. A., Stephens, N. A., . . . Preston, T. (2013). A novel oral tracer procedure for measurement of habitual myofibrillar protein synthesis. *Rapid Commun Mass Spectrom*, *27*(15), 1769-1777. https://doi.org/10.1002/rcm.6622
Maehara, K., Riley, M., Galassetti, P., Barstow, T. J., & Wasserman, K. (1997). Effect of hypoxia and carbon monoxide on muscle oxygenation during exercise. *Am J Respir Crit Care Med*, *155*(1), 229-235. https://doi.org/10.1164/ajrccm.155.1.9001317
Mairbäurl, H. (2013). Red blood cells in sports: effects of exercise and training on oxygen supply by red blood cells. *Front Physiol*, *4*, 332. https://doi.org/10.3389/fphys.2013.00332
Mairbäurl, H. (2018). Neocytolysis: How to Get Rid of the Extra Erythrocytes Formed by Stress Erythropoiesis Upon Descent From High Altitude. *Front Physiol*, *9*, 345. https://doi.org/10.3389/fphys.2018.00345

Mancera-Soto, E. M., Ramos-Caballero, D. M., Rojas, J. J., Duque, L., Chaves-Gomez, S., Cristancho-Mejía, E., & Schmidt, W. F. (2022). Hemoglobin Mass, Blood Volume and VO(2)max of Trained and Untrained Children and Adolescents Living at Different Altitudes. *Front Physiol*, *13*, 892247. https://doi.org/10.3389/fphys.2022.892247

Martinasek, M. P., Calvanese, A. V., & Lipski, B. K. (2021). A Naturalistic Study of Carbon Monoxide, Heart Rate, Oxygen Saturation, and Perfusion Index in Hookah Lounge Patrons. *Respir Care*, *66*(2), 269-274. https://doi.org/10.4187/respcare.08056

Mattioni Maturana, F., Keir, D. A., McLay, K. M., & Murias, J. M. (2016). Can measures of critical power precisely estimate the maximal metabolic steady-state? *Appl Physiol Nutr Metab*, *41*(11), 1197-1203. https://doi.org/10.1139/apnm-2016-0248

Maunder, E., Seiler, S., Mildenhall, M. J., Kilding, A. E., & Plews, D. J. (2021). The Importance of 'Durability' in the Physiological Profiling of Endurance Athletes. *Sports Med*, *51*(8), 1619-1628. https://doi.org/10.1007/s40279-021-01459-0

Merino, C. F. (1950). Studies on blood formation and destruction in the polycythemia of high altitude. *Blood*, *5*(1), 1-31.

Messonnier, L. A., Emhoff, C. A., Fattor, J. A., Horning, M. A., Carlson, T. J., & Brooks, G. A. (2013). Lactate kinetics at the lactate threshold in trained and untrained men. *J Appl Physiol (1985)*, *114*(11), 1593-1602. https://doi.org/10.1152/japplphysiol.00043.2013

Micklewright, D., St Clair Gibson, A., Gladwell, V., & Al Salman, A. (2017). Development and Validity of the Rating-of-Fatigue Scale. *Sports Med*, *47*(11), 2375-2393. https://doi.org/10.1007/s40279-017-0711-5

Midgley, A. W., Mc Naughton, L. R., & Wilkinson, M. (2006). The relationship between the lactate turnpoint and the time at VO2max during a constant velocity run to exhaustion. *Int J Sports Med*, *27*(4), 278-282. https://doi.org/10.1055/s-2005-865668

Miller, B. F., Reid, J. J., Price, J. C., Lin, H. L., Atherton, P. J., & Smith, K. (2020). CORP: The use of deuterated water for the measurement of protein synthesis. *J Appl Physiol (1985)*, *128*(5), 1163-1176. https://doi.org/10.1152/japplphysiol.00855.2019

Millet, G. Y., Martin, V., & Temesi, J. (2018). The role of the nervous system in neuromuscular fatigue induced by ultra-endurance exercise. *Appl Physiol Nutr Metab*, *43*(11), 1151-1157. https://doi.org/10.1139/apnm-2018-0161

Minotti, J. R., Johnson, E. C., Hudson, T. L., Zuroske, G., Fukushima, E., Murata, G., . . . Icenogle, M. V. (1990). Training-induced skeletal muscle adaptations are independent of systemic adaptations. *J Appl Physiol (1985)*, *68*(1), 289-294. https://doi.org/10.1152/jappl.1990.68.1.289

Mitchell, E. A., Martin, N. R. W., Bailey, S. J., & Ferguson, R. A. (2018). Critical power is positively related to skeletal muscle capillarity and type I muscle fibers in endurance-trained individuals. *J Appl Physiol (1985)*, *125*(3), 737-745. https://doi.org/10.1152/japplphysiol.01126.2017

Mock, D. M., Matthews, N. I., Zhu, S., Strauss, R. G., Schmidt, R. L., Nalbant, D., . . . Widness, J. A. (2011). Red blood cell (RBC) survival determined in humans using RBCs labeled at multiple biotin densities. *Transfusion*, *51*(5), 1047-1057. https://doi.org/10.1111/j.1537-2995.2010.02926.x

Montero, D., Breenfeldt-Andersen, A., Oberholzer, L., Haider, T., Goetze, J. P., Meinild-Lundby, A. K., & Lundby, C. (2017). Erythropoiesis with endurance training: dynamics and mechanisms. *Am J Physiol Regul Integr Comp Physiol*, *312*(6), R894-r902. https://doi.org/10.1152/ajpregu.00012.2017

Montero, D., Cathomen, A., Jacobs, R. A., Flück, D., de Leur, J., Keiser, S., . . . Lundby, C. (2015). Haematological rather than skeletal muscle adaptations contribute to the increase in peak oxygen uptake induced by moderate endurance training. *J Physiol*, *593*(20), 4677-4688. https://doi.org/10.1113/jp270250

Mora-Rodriguez, R., Aguado-Jimenez, R., Del Coso, J., & Estevez, E. (2012). A standard blood bank donation alters the thermal and cardiovascular responses during subsequent exercise. *Transfusion*, *52*(11), 2339-2347. https://doi.org/10.1111/j.1537-2995.2012.03613.x

Murphy, W. G. (2014). The sex difference in haemoglobin levels in adults - mechanisms, causes, and consequences. *Blood Rev*, *28*(2), 41-47. https://doi.org/10.1016/j.blre.2013.12.003

Naef, N., Steiner, T., & Wehrlin, J. P. (2015). Replicate Measurements of Haemoglobin Mass during a Single Day are Feasible and Precise. *Int J Sports Med*, *36*(5), e19-e23. https://doi.org/10.1055/s-0034-1398583

Nicholson, J. P., & Case, D. B. (1983). Carboxyhemoglobin Levels in New York City Runners. *Phys Sportsmed*, *11*(3), 134-138. https://doi.org/10.1080/00913847.1983.11708486

Nixon, R. J., Kranen, S. H., Vanhatalo, A., & Jones, A. M. (2021). Steady-state [Formula: see text] above MLSS: evidence that critical speed better represents maximal metabolic steady state in well-trained runners. *Eur J Appl Physiol*, *121*(11), 3133-3144. https://doi.org/10.1007/s00421-021-04780-8

Oberholzer, L., Montero, D., Robach, P., Siebenmann, C., Ryrsøe, C. K., Bonne, T. C., . . . Lundby, C. (2023). Determinants and reference values for blood volume and total hemoglobin mass in women and men. *Am J Hematol*. https://doi.org/10.1002/ajh.27162

Okada, Y., & Kamatani, Y. (2012). Common genetic factors for hematological traits in humans. *J Hum Genet*, *57*(3), 161-169. https://doi.org/10.1038/jhg.2012.2

Ou, L. C., Kim, D., Layton, W. M., Jr., & Smith, R. P. (1980). Splenic erythropoiesis in polycythemic response of the rat to high-altitude exposure. *J Appl Physiol Respir Environ Exerc Physiol*, *48*(5), 857-861. https://doi.org/10.1152/jappl.1980.48.5.857

Palis, J. (2014). Primitive and definitive erythropoiesis in mammals. *Front Physiol*, *5*, 3. https://doi.org/10.3389/fphys.2014.00003

Pallarés, J. G., Morán-Navarro, R., Ortega, J. F., Fernández-Elías, V. E., & Mora-Rodriguez, R. (2016). Validity and Reliability of Ventilatory and Blood Lactate Thresholds in Well-Trained Cyclists. *PLoS One*, *11*(9), e0163389. https://doi.org/10.1371/journal.pone.0163389

Panebianco, R. A., Stachenfeld, N., Coplan, N. L., & Gleim, G. W. (1995). Effects of blood donation on exercise performance in competitive cyclists. *Am Heart J*, *130*(4), 838-840. https://doi.org/10.1016/0002-8703(95)90085-3

Parker, B. A., Smithmyer, S. L., Pelberg, J. A., Mishkin, A. D., Herr, M. D., & Proctor, D. N. (2007). "Sex differences in leg vasodilation during graded knee extensor exercise in young adults." J Appl Physiol (1985), 103(5): 1583-1591.

Paulson, R. F., Hariharan, S., & Little, J. A. (2020). Stress erythropoiesis: definitions and models for its study. *Exp Hematol*, *89*, 43-54.e42. https://doi.org/10.1016/j.exphem.2020.07.011

Pethick, J., Winter, S. L., & Burnley, M. (2020). Physiological Evidence That the Critical Torque Is a Phase Transition, Not a Threshold. *Med Sci Sports Exerc*, *52*(11), 2390-2401. https://doi.org/10.1249/mss.0000000000002389

Peyrard, A., Willis, S. J., Place, N., Millet, G. P., Borrani, F., & Rupp, T. (2019). Neuromuscular evaluation of arm-cycling repeated sprints under hypoxia and/or blood flow restriction. *Eur J Appl Physiol*, *119*(7), 1533-1545. https://doi.org/10.1007/s00421-019-04143-4

Pirnay, F., Dujardin, J., Deroanne, R., & Petit, J. M. (1971). Muscular exercise during intoxication by carbon monoxide. *J Appl Physiol*, *31*(4), 573-575. https://doi.org/10.1152/jappl.1971.31.4.573

Pittman. (2011a). *Regulation of Tissue Oxygenation. Chapter 2, The Circulatory System and Oxygen Transport.* San Rafael (CA): Morgan & Claypool Life Sciences.

Pittman. (2011b). *Regulation of Tissue Oxygenation. Chapter 4, Oxygen Transport*. San Rafael (CA): Morgan & Claypool Life Sciences.

Pluim, B. M., Zwinderman, A. H., van der Laarse, A., & van der Wall, E. E. (2000). The athlete's heart. A meta-analysis of cardiac structure and function. *Circulation*, *101*(3), 336-344. https://doi.org/10.1161/01.cir.101.3.336

Plumb, J. O. M., Kumar, S., Otto, J., Schmidt, W., Richards, T., Montgomery, H. E., & Grocott, M. P. W. (2018). Replicating measurements of total hemoglobin mass (tHb-mass) within a single day: precision of measurement; feasibility and safety of using oxygen to expedite carbon monoxide clearance. *Physiol Rep*, *6*(17), e13829. https://doi.org/10.14814/phy2.13829

Pollock, M. L., Lowenthal, D. T., Foster, C., Pels, A. E. I., Rod, J., Stoiber, J., & Schmidt, D. H. (1991). Acute and Chronic Responses to Exercise in Patients Treated With Beta Blockers. *Journal of Cardiopulmonary Rehabilitation and Prevention*, *11*(2), 132-144.

Poole, D. C., Burnley, M., Vanhatalo, A., Rossiter, H. B., & Jones, A. M. (2016). Critical Power: An Important Fatigue Threshold in Exercise Physiology. *Med Sci Sports Exerc*, *48*(11), 2320-2334.

Poole, D. C., Ward, S. A., Gardner, G. W., & Whipp, B. J. (1988). Metabolic and respiratory profile of the upper limit for prolonged exercise in man. *Ergonomics*, *31*(9), 1265-1279. https://doi.org/10.1080/00140138808966766

Pottgiesser, T., Specker, W., Umhau, M., Dickhuth, H. H., Roecker, K., & Schumacher, Y. O. (2008). Recovery of hemoglobin mass after blood donation. *Transfusion*, *48*(7), 1390-1397. https://doi.org/10.1111/j.1537-2995.2008.01719.x

Previs, S. F., Fatica, R., Chandramouli, V., Alexander, J. C., Brunengraber, H., & Landau, B. R. (2004). Quantifying rates of protein synthesis in humans by use of 2H2O: application to patients with end-stage renal disease. In *Am J Physiol Endocrinol Metab* (Vol. 286, pp. E665-672). https://doi.org/10.1152/ajpendo.00271.2003

Prommer, N., & Schmidt, W. (2007). Loss of CO from the intravascular bed and its impact on the optimised CO-rebreathing method. *Eur J Appl Physiol*, *100*(4), 383-391. https://doi.org/10.1007/s00421-007-0439-2

Quinn, C. P., McDougall, R. M., Aboodarda, S. J., Murias, J. M., & MacInnis, M. J. (2024). Effects of carbohydrate availability on cycling endurance at the maximal lactate steady state. *Am J Physiol Regul Integr Comp Physiol*, *326*(3), R266-r275. https://doi.org/10.1152/ajpregu.00178.2023

Raes, A., Van Aken, S., Craen, M., Donckerwolcke, R., & Vande Walle, J. (2006). A reference frame for blood volume in children and adolescents. *BMC Pediatr*, *6*, 3. https://doi.org/10.1186/1471-2431-6-3

Raglin, J., Szabo, A., Lindheimer, J. B., & Beedie, C. (2020). Understanding placebo and nocebo effects in the context of sport: A psychological perspective. *Eur J Sport Sci*, *20*(3), 293-301. https://doi.org/10.1080/17461391.2020.1727021
Richardson, R. S., Grassi, B., Gavin, T. P., Haseler, L. J., Tagore, K., Roca, J., & Wagner, P. D. (1999). Evidence of O2 supply-dependent VO2 max in the exercise-trained human quadriceps. *J Appl Physiol (1985)*, *86*(3), 1048-1053. https://doi.org/10.1152/jappl.1999.86.3.1048
Richardson, R. S., Noyszewski, E. A., Saltin, B., & González-Alonso, J. (2002). Effect of mild carboxy-hemoglobin on exercising skeletal muscle: intravascular and intracellular evidence. *Am J Physiol Regul Integr Comp Physiol*, *283*(5), R1131-1139. https://doi.org/10.1152/ajpregu.00226.2002
Ridout, S. J., B. A. Parker, S. L. Smithmyer, J. U. Gonzales, K. C. Beck and D. N. Proctor (2010). "Age and sex influence the balance between maximal cardiac output and peripheral vascular reserve." J Appl Physiol (1985) 108(3): 483-489.
Robertson, R. J., Gilcher, R., Metz, K. F., Caspersen, C. J., Allison, T. G., Abbott, R. A., . . . Nixon, P. A. (1984). Hemoglobin concentration and aerobic work capacity in women following induced erythrocythemia. *J Appl Physiol Respir Environ Exerc Physiol*, *57*(2), 568-575. https://doi.org/10.1152/jappl.1984.57.2.568
Roca, J., Agusti, A. G., Alonso, A., Poole, D. C., Viegas, C., Barbera, J. A., . . . Wagner, P. D. (1992). Effects of training on muscle O2 transport at VO2max. *J Appl Physiol (1985)*, *73*(3), 1067-1076. https://doi.org/10.1152/jappl.1992.73.3.1067
Romer, L. M., Haverkamp, H. C., Lovering, A. T., Pegelow, D. F., & Dempsey, J. A. (2006). Effect of exercise-induced arterial hypoxemia on quadriceps muscle fatigue in healthy humans. *Am J Physiol Regul Integr Comp Physiol*, *290*(2), R365-375. https://doi.org/10.1152/ajpregu.00332.2005
Rossow, L., Yan, H., Fahs, C. A., Ranadive, S. M., Agiovlasitis, S., Wilund, K. R., . . . Fernhall, B. (2010). Postexercise hypotension in an endurance-trained population of men and women following high-intensity interval and steady-state cycling. *Am J Hypertens*, *23*(4), 358-367. https://doi.org/10.1038/ajh.2009.269
Rowell, L. B. (1988). Muscle blood flow in humans: how high can it go? *Med Sci Sports Exerc*, *20*(5 Suppl), S97-103. https://doi.org/10.1249/00005768-198810001-00001
Ruan, B., & Paulson, R. F. (2022). Metabolic regulation of stress erythropoiesis, outstanding questions, and possible paradigms. *Front Physiol*, *13*, 1063294. https://doi.org/10.3389/fphys.2022.1063294
Saito, F., Shimazu, T., Miyamoto, J., Maemura, T., & Satake, M. (2013). Interstitial fluid shifts to plasma compartment during blood donation. *Transfusion*, *53*(11), 2744-2750. https://doi.org/10.1111/trf.12120
Saltin, B., & Calbet, J. A. (2006). Point: in health and in a normoxic environment, VO2 max is limited primarily by cardiac output and locomotor muscle blood flow. *J Appl Physiol (1985)*, *100*(2), 744-745. https://doi.org/10.1152/japplphysiol.01395.2005
Saltin, B., Nazar, K., Costill, D. L., Stein, E., Jansson, E., Essén, B., & Gollnick, D. (1976). The nature of the training response; peripheral and central adaptations of one-legged exercise. *Acta Physiol Scand*, *96*(3), 289-305. https://doi.org/10.1111/j.1748-1716.1976.tb10200.x
Sannolo, N., Farina, V., & Fiorillo, A. (1992). Abnormal endogenous carbon monoxide production in children with ineffective erythropoiesis. *Ann Clin Biochem*, *29 (Pt 4)*, 397-399. https://doi.org/10.1177/000456329202900404

Santisteban, K. J., Lovering, A. T., Halliwill, J. R., & Minson, C. T. (2022). Sex Differences in VO(2max) and the Impact on Endurance-Exercise Performance. *Int J Environ Res Public Health*, *19*(9). https://doi.org/10.3390/ijerph19094946

Sawka, M. N., Convertino, V. A., Eichner, E. R., Schnieder, S. M., & Young, A. J. (2000). Blood volume: importance and adaptations to exercise training, environmental stresses, and trauma/sickness. *Med Sci Sports Exerc*, *32*(2), 332-348. https://doi.org/10.1097/00005768-200002000-00012

Schierbauer, J., Hoffmeister, T., Treff, G., Wachsmuth, N. B., & Schmidt, W. F. J. (2021). Effect of Exercise-Induced Reductions in Blood Volume on Cardiac Output and Oxygen Transport Capacity. *Front Physiol*, *12*, 679232. https://doi.org/10.3389/fphys.2021.679232

Schmidt, W., Eckardt, K. U., Hilgendorf, A., Strauch, S., & Bauer, C. (1991). Effects of maximal and submaximal exercise under normoxic and hypoxic conditions on serum erythropoietin level. *Int J Sports Med*, *12*(5), 457-461. https://doi.org/10.1055/s-2007-1024713

Schmidt, W., Maassen, N., Trost, F., & Böning, D. (1988). Training induced effects on blood volume, erythrocyte turnover and haemoglobin oxygen binding properties. *Eur J Appl Physiol Occup Physiol*, *57*(4), 490-498. https://doi.org/10.1007/bf00417998

Schmidt, W., & Prommer, N. (2005). The optimised CO-rebreathing method: a new tool to determine total haemoglobin mass routinely. *Eur J Appl Physiol*, *95*(5-6), 486-495. https://doi.org/10.1007/s00421-005-0050-3

Schmidt, W., & Prommer, N. (2008). Effects of various training modalities on blood volume. *Scand J Med Sci Sports*, *18 Suppl 1*, 57-69. https://doi.org/10.1111/j.1600-0838.2008.00833.x

Schmidt, W., & Prommer, N. (2010). Impact of alterations in total hemoglobin mass on VO 2max. In *Exerc Sport Sci Rev* (Vol. 38, pp. 68-75). https://doi.org/10.1097/JES.0b013e3181d4957a

Schwandt, H. J., Heyduck, B., Gunga, H. C., & Röcker, L. (1991). Influence of prolonged physical exercise on the erythropoietin concentration in blood. *Eur J Appl Physiol Occup Physiol*, *63*(6), 463-466. https://doi.org/10.1007/bf00868079

Sejersen, C., Fischer, M., Mattos, J. D., Volianitis, S., & Secher, N. H. (2021). Fluctuations in cardiac stroke volume during rowing. *Scand J Med Sci Sports*, *31*(4), 790-798. https://doi.org/10.1111/sms.13901

Shave, R. E., Lieberman, D. E., Drane, A. L., Brown, M. G., Batterham, A. M., Worthington, S., . . . Baggish, A. L. (2019). Selection of endurance capabilities and the trade-off between pressure and volume in the evolution of the human heart. *Proc Natl Acad Sci U S A*, *116*(40), 19905-19910. https://doi.org/10.1073/pnas.1906902116

Sheel, A. W., Boushel, R., & Dempsey, J. A. (2018). Competition for blood flow distribution between respiratory and locomotor muscles: implications for muscle fatigue. *J Appl Physiol (1985)*, *125*(3), 820-831. https://doi.org/10.1152/japplphysiol.00189.2018

Sheffield-Moore, M., Paddon-Jones, D., Sanford, A. P., Rosenblatt, J. I., Matlock, A. G., Cree, M. G., & Wolfe, R. R. (2005). Mixed muscle and hepatic derived plasma protein metabolism is differentially regulated in older and younger men following resistance exercise. *Am J Physiol Endocrinol Metab*, *288*(5), E922-929. https://doi.org/10.1152/ajpendo.00358.2004

Shellock, F. G., Morris, E., Deutsch, A. L., Mink, J. H., Kerr, R., & Boden, S. D. (1992). Hematopoietic bone marrow hyperplasia: high prevalence on MR images of the knee in asymptomatic marathon runners. *AJR Am J Roentgenol*, *158*(2), 335-338. https://doi.org/10.2214/ajr.158.2.1729795

Shemin, D., & Rittenberg, D. (1945). The utilization of glycine for the synthesis of a porphyrin. *J Biol Chem*, *159*(2), 567.

Shemin, D., & Rittenberg, D. (1946). The life span of the human red blood cell. *J Biol Chem*, *166*(2), 627-636.

Shepherd, J. R. A., Dominelli, P. B., Roy, T. K., Secomb, T. W., Hoyer, J. D., Oliveira, J. L., & Joyner, M. J. (2019). Modelling the relationships between haemoglobin oxygen affinity and the oxygen cascade in humans. *J Physiol*, *597*(16), 4193-4202. https://doi.org/10.1113/jp277591

Siebenmann, C., Keiser, S., Robach, P., & Lundby, C. (2017). CORP: The assessment of total hemoglobin mass by carbon monoxide rebreathing. *J Appl Physiol (1985)*, *123*(3), 645-654. https://doi.org/10.1152/japplphysiol.00185.2017

Silva, J. C. G., Domingos-Gomes, J. R., Freitas, E. D. S., Neto, G. R., Aniceto, R. R., Bemben, M. G., . . . Cirilo-Sousa, M. S. (2021). Physiological and Perceptual Responses to Aerobic Exercise With and Without Blood Flow Restriction. *J Strength Cond Res*, *35*(9), 2479-2485. https://doi.org/10.1519/jsc.0000000000003178

Simpson, L. P., Jones, A. M., Skiba, P. F., Vanhatalo, A., & Wilkerson, D. (2015). Influence of hypoxia on the power-duration relationship during high-intensity exercise. *Int J Sports Med*, *36*(2), 113-119. https://doi.org/10.1055/s-0034-1389943

Skattebo, Ø., Bjerring, A. W., Auensen, M., Sarvari, S. I., Cumming, K. T., Capelli, C., & Hallén, J. (2020). Blood volume expansion does not explain the increase in peak oxygen uptake induced by 10 weeks of endurance training. *Eur J Appl Physiol*, *120*(5), 985-999. https://doi.org/10.1007/s00421-020-04336-2

Skattebo, Ø., Calbet, J. A. L., Rud, B., Capelli, C., & Hallén, J. (2020). Contribution of oxygen extraction fraction to maximal oxygen uptake in healthy young men. *Acta Physiol (Oxf)*, *230*(2), e13486. https://doi.org/10.1111/apha.13486

Skattebo, Ø., Capelli, C., Rud, B., Auensen, M., Calbet, J. A. L., & Hallén, J. (2020). Increased oxygen extraction and mitochondrial protein expression after small muscle mass endurance training. *Scand J Med Sci Sports*, *30*(9), 1615-1631. https://doi.org/10.1111/sms.13707

Skattebo, Ø., Johansen, E. S., Capelli, C., & Hallén, J. (2021). Effects of 150- and 450-mL Acute Blood Losses on Maximal Oxygen Uptake and Exercise Capacity. *Med Sci Sports Exerc*, *53*(8), 1729-1738. https://doi.org/10.1249/mss.0000000000002618

Smith, J. A., Martin, D. T., Telford, R. D., & Ballas, S. K. (1999). Greater erythrocyte deformability in world-class endurance athletes. *Am J Physiol*, *276*(6), H2188-2193. https://doi.org/10.1152/ajpheart.1999.276.6.H2188

Sparling, P. B. (1980). A meta-analysis of studies comparing maximal oxygen uptake in men and women. *Res Q Exerc Sport*, *51*(3), 542-552. https://doi.org/10.1080/02701367.1980.10608077

Stangerup, I., Kramp, N. L., Ziegler, A. K., Dela, F., Magnussen, K., & Helge, J. W. (2017). Temporary impact of blood donation on physical performance and hematologic variables in women. *Transfusion*, *57*(8), 1905-1911. https://doi.org/10.1111/trf.14121

Stevenson, E. T., Davy, K. P., & Seals, D. R. (1994). Maximal aerobic capacity and total blood volume in highly trained middle-aged and older female endurance athletes. *J Appl Physiol (1985)*, *77*(4), 1691-1696. https://doi.org/10.1152/jappl.1994.77.4.1691
Sutehall, S., Martin-Rincon, M., Wang, G., Shurlock, J., Durussel, J., Mooses, M., . . . Pitsiladis, Y. P. (2018). The Performance Effects of Microdose Recombinant Human Erythropoietin Administration and Carbon Monoxide Rebreathing. *Curr Sports Med Rep*, *17*(12), 457-466. https://doi.org/10.1249/jsr.0000000000000551
Tarnopolsky, M. A. (2008). Sex differences in exercise metabolism and the role of 17-beta estradiol. *Med Sci Sports Exerc*, *40*(4), 648-654. https://doi.org/10.1249/MSS.0b013e31816212ff
Ulrich, G., Bärtsch, P., & Friedmann-Bette, B. (2011). Total haemoglobin mass and red blood cell profile in endurance-trained and non-endurance-trained adolescent athletes. *Eur J Appl Physiol*, *111*(11), 2855-2864. https://doi.org/10.1007/s00421-011-1920-5
van Baak, M. A., Verstappen, F. T., & Oosterhuis, B. (1986). Twenty-four hour effects of oxprenolol Oros and atenolol on heart rate, blood pressure, exercise tolerance and perceived exertion. *Eur J Clin Pharmacol*, *30*(4), 399-406. https://doi.org/10.1007/bf00607951
Van Remoortel, H., De Buck, E., Compernolle, V., Deldicque, L., & Vandekerckhove, P. (2017). The effect of a standard whole blood donation on oxygen uptake and exercise capacity: a systematic review and meta-analysis. *Transfusion*, *57*(2), 451-462. https://doi.org/10.1111/trf.13893
Vanhatalo, A., Fulford, J., DiMenna, F. J., & Jones, A. M. (2010). Influence of hyperoxia on muscle metabolic responses and the power-duration relationship during severe-intensity exercise in humans: a 31P magnetic resonance spectroscopy study. *Exp Physiol*, *95*(4), 528-540. https://doi.org/10.1113/expphysiol.2009.050500
Vogel, J. A., & Gleser, M. A. (1972). Effect of carbon monoxide on oxygen transport during exercise. *J Appl Physiol*, *32*(2), 234-239. https://doi.org/10.1152/jappl.1972.32.2.234
Vogelsang, T. W., Hanel, B., Kristoffersen, U. S., Petersen, C. L., Mehlsen, J., Holmquist, N., . . . Kjaer, A. (2008). Effect of eight weeks of endurance exercise training on right and left ventricular volume and mass in untrained obese subjects: a longitudinal MRI study. *Scand J Med Sci Sports*, *18*(3), 354-359. https://doi.org/10.1111/j.1600-0838.2007.00706.x
Vogt, S., Altehoefer, C., Bueltermann, D., Pottgiesser, T., Prettin, S., Schmid, A., . . . Heinrich, L. (2008). Magnetic resonance imaging of the lumbar spine and blood volume in professional cyclists. *Eur J Appl Physiol*, *102*(4), 411-416. https://doi.org/10.1007/s00421-007-0599-0
Wagner, P. D. (1996). A theoretical analysis of factors determining VO2 MAX at sea level and altitude. *Respir Physiol*, *106*(3), 329-343. https://doi.org/10.1016/s0034-5687(96)00086-2
Weed, R. I., Reed, C. F., & Berg, G. (1963). Is hemoglobin an essential structural component of human erythrocyte membranes? *J Clin Invest*, *42*(4), 581-588. https://doi.org/10.1172/jci104747
Weight, L. M., Byrne, M. J., & Jacobs, P. (1991). Haemolytic effects of exercise. *Clin Sci (Lond)*, *81*(2), 147-152. https://doi.org/10.1042/cs0810147

Werre, J. M., Willekens, F. L., Bosch, F. H., de Haans, L. D., van der Vegt, S. G., van den Bos, A. G., & Bosman, G. J. (2004). The red cell revisited--matters of life and death. *Cell Mol Biol (Noisy-le-grand)*, *50*(2), 139-145.

Wilkinson, D. J., Brook, M. S., Smith, K., & Atherton, P. J. (2017). Stable isotope tracers and exercise physiology: past, present and future. *J Physiol*, *595*(9), 2873-2882. https://doi.org/10.1113/jp272277

Williams, M. H., Lindhjem, M., & Schuster, R. (1978). The effect of blood infusion upon endurance capacity and ratings of perceived exertion. *Med Sci Sports*, *10*(2), 113-118.

Williams, M. H., Wesseldine, S., Somma, T., & Schuster, R. (1981). The effect of induced erythrocythemia upon 5-mile treadmill run time. *Med Sci Sports Exerc*, *13*(3), 169-175.

Willis, S. J., Millet, G. P., & Borrani, F. (2020). Insights for Blood Flow Restriction and Hypoxia in Leg Versus Arm Submaximal Exercise. *Int J Sports Physiol Perform*, *15*(5), 714-719. https://doi.org/10.1123/ijspp.2019-0168

Wilmore, J. H., Joyner, M. J., Freund, B. J., Ewy, G. A., & Morton, A. R. (1985). Beta-Blockade and Response to Exercise: Influence of Training. *Phys Sportsmed*, *13*(7), 60-69. https://doi.org/10.1080/00913847.1985.11708832

Ye, L., Ji, Y., Zhou, C., Luo, J., Zhang, L., Jing, L., . . . Zhang, F. (2021). Comparison of Levitt's CO breath test and the (15) N-glycine labeling technique for measuring the lifespan of human red blood cells. *Am J Hematol*, *96*(10), 1232-1240. https://doi.org/10.1002/ajh.26290

Young, D. (2010). *Control of Cardiac Output* (Vol. Chapter 2). Morgan & Claypool Life Sciences.

Zavorsky, G. S., Smoliga, J. M., Longo, L. D., Uhranowsky, K. A., Cadman, C. R., Duffin, J., & Fisher, J. A. (2012). Increased carbon monoxide clearance during exercise in humans. *Med Sci Sports Exerc*, *44*(11), 2118-2124. https://doi.org/10.1249/MSS.0b013e3182602a00

Zhang, H. D., Ma, Y. J., Liu, Q. F., Ye, T. Z., Meng, F. Y., Zhou, Y. W., . . . Ji, K. M. (2018). Human erythrocyte lifespan measured by Levitt's CO breath test with newly developed automatic instrument. *J Breath Res*, *12*(3), 036003. https://doi.org/10.1088/1752-7163/aaacf1

Ziegler, A. K., Grand, J., Stangerup, I., Nielsen, H. J., Dela, F., Magnussen, K., & Helge, J. W. (2015). Time course for the recovery of physical performance, blood hemoglobin, and ferritin content after blood donation. *Transfusion*, *55*(4), 898-905. https://doi.org/10.1111/trf.12926

Appendix I: Supplementary materials

Supplemental Table 5.1. Pearson correlations between aerobic capacity and selected hematological measures disaggregated by sex.

		Females (n=34)						Males (n=45)						Total (n=79)					
		$\dot{V}O_2$max ($mL \cdot min^{-1}$)		$\dot{V}O_2$max ($mL \cdot kg\ BM^{-1} \cdot min^{-1}$)		$\dot{V}O_2$max ($mL \cdot kg\ FFM^{-1} \cdot min^{-1}$)		$\dot{V}O_2$max ($mL \cdot min^{-1}$)		$\dot{V}O_2$max ($mL \cdot kg\ BM^{-1} \cdot min^{-1}$)		$\dot{V}O_2$max ($mL \cdot kg\ FFM^{-1} \cdot min^{-1}$)		$\dot{V}O_2$max; ($mL \cdot min^{-1}$)		$\dot{V}O_2$max; ($mL \cdot kg\ BM^{-1} \cdot min^{-1}$)		$\dot{V}O_2$max; $mL \cdot kg\ FFM^{-1} \cdot min^{-1}$	
	Units	***r***	***P***	***r***	***P***	***r***	***P***	***r***	***r***	***r***	***P***	***r***	***P***	***r***	***P***	***r***	***P***	***r***	***P***
Hb_{mass}	g	0.707	<0.001	0.400	0.019	0.427	0.012	0.657	<0.001	0.076	0.620	0.030	0.847	0.840	<0.001	0.374	0.001	0.166	0.144
Hb_{mass}	$g \cdot kg\ BM^{-1}$	0.534	0.001	0.659	<0.001	0.541	0.001	0.560	<0.001	0.707	<0.001	0.568	<0.001	0.752	<0.001	0.718	<0.001	0.480	<0.001
Hb_{mass}	$g \cdot kg\ FFM^{-1}$	0.464	0.006	0.424	0.013	0.499	0.003	0.440	0.003	0.466	0.001	0.590	<0.001	0.585	<0.001	0.530	<0.001	0.537	<0.001
BV	mL	0.695	<0.001	0.401	0.019	0.425	0.012	0.690	<0.001	0.128	0.400	0.077	0.615	0.828	<0.001	0.376	0.001	0.197	0.082
BV	$mL \cdot kg\ BM^{-1}$	0.477	0.004	0.646	<0.001	0.520	0.002	0.601	<0.001	0.713	<0.001	0.572	<0.001	0.576	<0.001	0.714	<0.001	0.551	<0.001
BV	$mL \cdot kg\ FFM^{-1}$	0.397	0.020	0.410	0.016	0.479	0.004	0.520	<0.001	0.511	<0.001	0.603	<0.001	0.182	0.108	0.352	0.001	0.513	<0.001
RBCV	mL	0.705	<0.001	0.406	0.017	0.422	0.013	0.656	<0.001	0.079	0.606	0.046	0.762	0.839	<0.001	0.376	0.001	0.173	0.127
RBCV	$mL \cdot kg\ BM^{-1}$	0.529	0.001	0.654	<0.001	0.526	0.001	0.551	<0.001	0.706	<0.001	0.587	<0.001	0.741	<0.001	0.720	<0.001	0.492	<0.001
RBCV	$mL \cdot kg\ FFM^{-1}$	0.465	0.006	0.431	0.011	0.491	0.003	0.415	0.005	0.446	0.002	0.586	<0.001	0.550	<0.001	0.514	<0.001	0.539	<0.001
PV	mL	0.646	<0.001	0.375	0.029	0.401	0.019	0.674	<0.001	0.154	0.312	0.094	0.539	0.758	<0.001	0.349	0.002	0.204	0.071
PV	$mL \cdot kg\ BM^{-1}$	0.408	0.017	0.593	<0.001	0.477	0.004	0.577	<0.001	0.654	<0.001	0.534	<0.001	0.323	0.004	0.561	<0.001	0.484	<0.001
PV	$mL \cdot kg\ FFM^{-1}$	0.310	0.075	0.351	0.042	0.418	0.014	0.519	<0.001	0.485	0.001	0.536	<0.001	-0.080	0.485	0.167	0.141	0.366	0.001
[Hb]	$g \cdot dL^{-1}$	0.086	0.625	-0.115	0.519	-0.076	0.668	-0.142	0.354	-0.281	0.061	-0.247	0.102	0.395	<0.001	0.059	0.604	-0.083	0.467
Hct	%	0.071	0.687	-0.122	0.492	-0.107	0.547	-0.159	0.297	-0.307	0.040	-0.255	0.090	0.346	0.002	0.017	0.883	-0.108	0.345

$\dot{V}O_2$max = maximal oxygen uptake; BM = Body mass; FFM = Fat-free mass; Hb_{mass} = Hemoglobin mass; BV = Blood volume; RBCV = Red blood cell volume; PV =Plasma volume. n=79.