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THE SIGNIFICANCE OF MITOCHONDRIAL RESPIRATORY FUNCTION IN REGULATING OXYGEN UPTAKE AND PERFORMANCE IN HUMANS

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“Science is a way of thinking much more than it is a body of knowledge”

Carl Sagan

THESIS FOR DOCTORAL DEGREE (Ph.D.)

The significance of mitochondrial respiratory function in regulating oxygen uptake and performance in humans

by
Daniele Cardinale

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ABSTRACT

The mitochondrion is one of the most fascinating organelles of our cells which has kept and keeps researchers busy in studying its origin, the complex morphology, the numerous functions, the rapid adaptations to a variety of stimuli and its role in health and disease. Exercise challenges cellular homeostasis and skeletal muscle mitochondria greatly adapt to repeated bouts of exercise by increasing mitochondrial respiratory function and content to match energy requirements and to better sustain future perturbations induced by muscle contractions. The oxidative capacity of mitochondria has been shown to exceed the capacity of the cardiorespiratory system to supply oxygen to active muscle at maximal exercise intensity. Despite this, exercise training further increases this overcapacity. Little is known about the role of this excess oxidative capacity of mitochondria in regulating oxygen consumption, the role of oxygen delivery in determining exercise-induced skeletal muscle adaptations, and whether any sex-related differences exist. The assessment of mitochondrial respiratory function in high resolution respirometer is largely used for clinical and scientific purposes. However, the reliability of this method has not been systematically investigated and warrant further investigation.

With this background, specific measures of reliability associated with repeated determination of maximal mitochondrial oxidative phosphorylation in saponin-permeabilized fibres, comparison of the right and left legs, variability with measurements at different time-points and over time, as well as influence of the local anesthetic and wet weight of the fiber bundle on determined maximal mitochondrial oxidative phosphorylation were investigated in **paper I**. The importance of having the same technicians in preparing the samples, and that the major source of variation in measuring mitochondrial oxidative capacity is the sample preparation per se were shown. Furthermore, other factors such as the possible difference between left and right limbs, two time points of sample collection, fibres bundle weight, time that elapsed after collection of the biopsy, and the use of an anesthetic have only a minor impact on the standard error of the measurement.

In **paper II** the physiological significance of having a mitochondrial oxidative capacity in excess of the capacity of the central circulation to deliver oxygen to the tissue was shown by integrating measures of *ex vivo* mitochondrial respiratory function with direct *in vivo* measure of oxygen consumption when performing two-legged cycling and one-legged knee extension exercise while inspiring atmospheric air and oxygen enriched air

in the same participants. Excess capacity of mitochondria allows submaximal mitochondrial activation at maximal oxygen delivery, thereby maintaining a high mitochondrial oxygen affinity and a high oxygen extraction peripherally. Considering the widespread and increasing sedentary behavior in a society plagued by diseases often linked to mitochondrial dysfunction, these results suggest the importance of preserving a high muscle oxidative capacity throughout life, which can be of significance in patients with heart, circulatory, and overall metabolic diseases.

Despite known sex-specific metabolic differences in human skeletal muscle and that animal models have consistently shown females having a superior mitochondrial function compare to males, data in humans are lacking. In **paper III** the first evidence that women possess higher mitochondrial quality compared to men with equal cardiorespiratory fitness and endurance performance was provided. Mitochondrial oxygen affinity varied with the degree of mitochondrial respiration rate and was lower in women compared to men. These results indicate that the higher mitochondrial quality in women may be an important physiological adaptation that compensates for the lower mitochondrial oxygen affinity allowing a higher oxygen extraction peripherally. Moreover, these results could possibly be linked to the difference in life expectancy, disease occurrence and aging between women and men.

Lastly, in **paper IV** it was shown that increasing oxygen delivery and exercise intensity by means of breathing hyperoxia during high-intensity exercise did not enhance cardiorespiratory fitness and exercise-induced skeletal muscle adaptations but still resulted in a small beneficial effect on performance in trained cyclists. This small positive effect on performance can be exploited in elite athletes; however, considering the cost/benefit, the unknown health-related problems, and ethical issues of performing hyperoxic-supplemented endurance training, it is arguable if the use of this strategy to maximize endurance performance is worthwhile.

Overall, this thesis provides useful information for future research on various factors influencing the error of the measurement when assessing mitochondrial respiratory function. Moreover, this thesis sheds light on novel factors that regulate oxygen consumption during exercise, highlighting the importance of maintaining a good mitochondrial function. This thesis also provides possible directions for future studies on mitochondrial function, metabolism and exercise-induced adaptations.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers referred to by their Roman numerals:

I. **Cardinale D.A.**, Gejl K.D., Ørtenblad N., Ekblom B., Blomstrand E., Larsen F.J. *Reliability of maximal mitochondrial oxidative phosphorylation in permeabilized fibres from the vastus lateralis employing high-resolution respirometry.* Physiol Rep. 2018 Feb; 6(4).

II. **Cardinale D.A.**, Larsen F.J., Jensen-Urstad M., Rullman E., Søndergaard H., Morales-Alamo D., Ekblom B., Calbet J.A.L., Boushel R. *Muscle mass and inspired oxygen influence oxygen extraction at maximal exercise: Role of mitochondrial oxygen affinity***.** Acta Physiol (Oxf). 2018 Jun 4; e13110.

III. **Cardinale D.A.*****, Larsen F.J.*****, Schiffer T.A., Morales Alamo D., Ekblom B., Calbet J.A.L., Holmberg H.C., Boushel R. *Superior intrinsic mitochondrial respiration in women than in men.* Front Physiol. 2018 Aug; 17; 1133

IV. **Cardinale D.A.**, Larsen F.J., Lännerström J., Manselin T., Södergård O., Mijwel S., Lindholm P., Ekblom B., Boushel R. *Influence of hyperoxic-supplemented high-intensity interval training on training adaptation in trained cyclists* (in manuscript)

***** equal contribution

In addition, some unpublished data are included. Study II is reprinted with permission from the publisher John Wiley and Sons. Paper I and III are licensed under CC BY 4.0.

Other papers not included in this thesis:

Mijwel S, **Cardinale D.A.**, Ekblom-Bak E, Sundberg CJ, Wengström Y, Rundqvist H. *Validation of 2 submaximal cardiorespiratory fitness tests in patients with breast cancer undergoing chemotherapy.* Rehabil Oncol. 2016 Oct; 34(4):137-143.

Cardinale D.A., Lilja M., Mandić M., Gustafsson T, Larsen F.J., Lundberg T.R. *Resistance training with co-ingestion of anti-inflammatory drugs attenuates mitochondrial function*. Front Physiol. 2017 Dec; 19;8:1074.

Gejl K.D., Thams L.B., Hansen M, Rokkedal-Lausch T., Plomgaard P., Nybo L., Larsen F.J., **Cardinale D.A.**, Jensen K., Holmberg H.C., Vissing K., Ørtenblad N. *No superior adaptations to carbohydrate periodization in elite endurance athletes.* Med Sci Sports Exerc. 2017 Dec; 49(12):2486-2497.

Mijwel S.*****, **Cardinale D.A.***, Norrbom J., Chapman M., Ivarsson N., Wengström Y., Sundberg C.J., Rundqvist H. *Exercise training during chemotherapy preserves skeletal muscle fiber area, capillarization, and mitochondrial content in patients with breast cancer.* FASEB J. 2018 May 11; fj201700968R.

Cardinale D.A., Ekblom B. *Hyperoxia for performance and training.* J Sports Sci. 2018 Jul; 36(13):1515-1522.

***** equal contribution

CONTENT

ABBREVIATIONS

O_2	Oxygen
OXPHOS, V_{MAX}	Mitochondrial maximal oxidative phosphorylation
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
$NADH_2$	Nicotinamide adenine dinucleotide
$FADH_2$	Flavin adenine dinucleotide
F_IO_2	Inspired oxygen fraction
ETS	Electron transfer system
complex I, CI	NADH dehydrogenase ubiquinone oxidoreductase
complex II, CII	Succinate ubiquinone oxidoreductase
complex III, CIII	Ubiquinol cytochrome c oxidoreductase
complex IV, CIV	Cytochrome c oxidase
complex V	ATP synthase
CoQ	Coenzyme Q
Cytc	Cytochrome c
CS	citrate synthase
$p50_{mito}$	The oxygen tension where mitochondrial respiration proceeds at 50% of its maximum in the presence of saturating ADP concentrations
HAD	β-hydroxyacyl-CoA dehydrogenase
HIIT	High-intensity interval training
SaO_2	Arterial oxygen saturation
RER	Respiratory exchange ratio
P/O ratio	Phosphate/Oxygen ratio
TMPD	N,N,N′,N′-tetramethyl-p-phenylenediamine

1. INTRODUCTION

It is well known that endurance training improves the aerobic capacity (VO_2max) and changes occur both in the cardiorespiratory system (Ekblom et al., 1968) and within the muscle machinery itself with profound metabolic (Varnauskas et al., 1970;Morgan et al., 1971;Gollnick et al., 1972) and morphological adaptations (Hoppeler et al., 1973) which result in a reduced utilization of glycogen and increased reliance in fat oxidation concomitant with a lower lactate production during exercise at a given intensity (Holloszy and Coyle, 1984).

The concept of symmorphosis proposes that the structural capacity of each step of the oxygen (O_2) cascade is tuned according to the functional demand (Weibel, 1987). Several lines of evidence have shown the importance of O_2 delivery in defining VO_2max (Åstrand, 1961;Andersen, 1985a;Saltin and Calbet, 2006b) and its improvements with training (Ekblom and Hermansen, 1968). At the bottom of the O_2 cascade, specific mitochondrial enzymes can increase up to 50% after a few weeks of endurance training (Henriksson, 1977) and mitochondria can occupy from 3% to 10% of the myocyte in untrained and endurance athletes, respectively (Hoppeler et al., 1973;Boushel et al., 2014b;Ørtenblad et al., 2018). This rapid and large adaptive response of mitochondria is not paralleled by an similar upregulation of other sites of the oxygen cascade (Gifford et al., 2015). This adaptive response seems redundant since skeletal muscle already possesses an apparent excess oxidative capacity compared to O_2 delivery (Boushel et al., 2011;Boushel and Saltin, 2013). Little is known about the role of this excess oxidative capacity of mitochondria in regulating oxygen consumption, if any related sex differences exist as well as the role of O_2 delivery relative to muscle mass in determining skeletal muscle metabolic adaptation.

Therefore, this thesis is centered on the study of distinct aspects of skeletal muscle metabolism when altering O_2 delivery to the working muscle acutely and chronically during exercise in healthy trained individuals. Mitochondrial respiratory function was assessed with special reference to mitochondrial O_2 affinity, sex difference, performance and training, as well as methodological considerations for permeabilized and isolated mitochondrial preparations.

2. BACKGROUND

2.1 THE OXYGEN CASCADE IN HUMANS

O_2 is vital for the formation of high-energy phosphate compounds within the mitochondria. Oxygen transport from the atmosphere to mitochondria involves multiple steps which are referred to as the oxygen cascade (figure 1). The oxygen cascade involves diffusive O_2 transport driven by large O_2 pressure gradients and convective O_2 transport by blood flow (Weibel, 1987). During the ventilation process, the inspired O_2 with a partial pressure (pO_2) in the atmospheric air of 159 mmHg reaches the alveoli with a pO_2 of 100 mmHg via the bronchial tree. The large pO_2 gradient (alveolar-capillary diffusion) allows O_2 to diffuse from the alveoli (100 mmHg) to the pulmonary capillaries (40 mmHg) where it largely binds to haemoglobin (Hb) within the circulating erythrocytes with a small O_2 fraction (~2%) freely dissolved in the blood plasma. O_2 is then transported from the pulmonary capillaries to the systemic capillaries (O_2 convective transport) and delivered to organs and muscles with different flow according to the metabolic energy demand. From a cardiac output (the product between the stroke volume and heart rate) of ~ 4-5 L blood per minute at rest, the heart can pump maximally ~20-25 L of blood per minute during maximal exercise in healthy women and men (Åstrand et al., 1964;Calbet et al., 2007) and up to 40 L of blood per minute in elite endurance athletes (Ekblom and Hermansen, 1968). The vast range of adjustment of the cardiac output together with the variable resistance of the arterioles through a highly regulated process of contraction and relaxation of the smooth muscle cells of the vessels regulates the blood flow according to the tissue demand. From the capillaries, due to the pO_2 gradient, O_2 diffuses into the myocyte (microvasculature to myocyte diffusion) crossing the carrier free region which includes capillary endothelial cell, the interstitium, the muscle sarcolemma and cytoplasm to finally reach the mitochondria (mitochondrial respiration).

Across multiple species, it is thought that the structural capacity of each step in the O_2 cascade is matched to the functional demand and this balance is consistent across diverse mammalian species of different size. This concept is termed symmorphosis (Weibel, 1987). Each of the above mentioned steps are important to sustain repeated muscle contractions as well as regulating cell metabolism and could represent a limiting factor during exercise in humans (Wagner, 1993). Pulmonary diffusion capacity, cardiac output and

oxygen carrying capacity of blood can be classified as central factors influencing the O_2 consumption, while oxygen extraction and factors within the skeletal muscle such as mitochondrial respiratory capacity are considered as peripheral factors (Bassett and Howley, 2000).

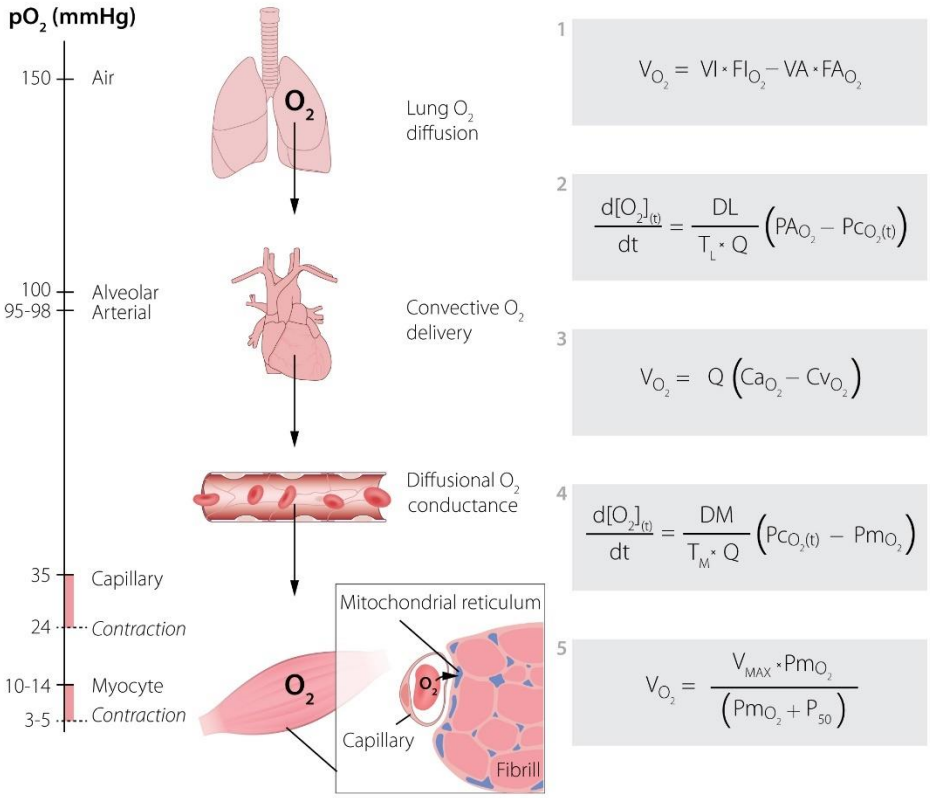

Figure 1. Schematic diagram of the oxygen cascade from the environment to the mitochondria and the five equations controlling the oxygen cascade at the different sites. Inspired O_2 fraction (FIO_2), ventilation inspired (VI); ventilation expired (VA), lung diffusing capacity (DL), cardiac output (Q), alveolar pO_2 (PAO_2), capillary pO_2 (PCO_2), transit time lungs (TL), arterial O_2 content (CaO_2), venous O_2 content (CvO_2), muscle diffusing capacity (DM), transit time muscle (TM), mitochondrial pO_2 (PmO_2), mitochondrial maximal oxidative phosphorylation (V_{MAX}) here referred to as OXPHOS, mitochondrial oxygen affinity (P_{50}).

2.2 THE MITOCHONDRION

Mitochondria are organelles delimited by a double bilayer phospholipidic membrane and are found in most cells. The outer membrane is in contact with the cytoplasm and is highly permeabilized allowing the passage of relatively large molecules. The inner membrane encloses the mitochondrial matrix and has a high cardiolipin content which makes this membrane impermeable to most of the small molecules and ions. This transport across the inner membrane is tightly regulated by specific multi-protein complexes controlled by energy demand and a proton gradient. The inner membrane folds back on itself, in a pattern called cristae (Mannella, 2006), forming a large surface-to-volume ratio which maximizes the energy production per mitochondrion (Demongeot et al., 2007). The mitochondrial matrix contains enzymes for the citric acid cycle, fatty acid β-oxidation, amino acid oxidation and the pyruvate dehydrogenase complex.

Embedded in the inner membrane are the four multi-protein complexes responsible for the electron transfer system together with the adenosine diphosphate (ADP) - adenosine triphosphate (ATP) translocase, ATP synthase and uncoupling proteins (figure 2).

Mitochondria play a variety of roles ranging from cell energy production to cell signal transduction e.g. reactive oxygen species essential for regulating cell adaptation, survival and homeostasis processes (Hepple, 2016). However, the production of ATP coupled to substrate oxidative phosphorylation and electrons/hydrogen transfer by the chemiosmosis mechanism (Mitchell, 1961) are the main functions of mitochondrial metabolism.

Improvement in mitochondrial respiratory function is associated with greater endurance performance and health (Jacobs et al., 2011;Jacobs and Lundby, 2013) whereas a decreased mitochondrial function is linked to aging (Tower, 2015;Hepple, 2016) and disease (Wallace, 2005). The key role of mitochondria in health and disease highlights the importance of further gaining knowledge about the mechanisms regulating mitochondrial function (Nunnari and Suomalainen, 2012).

2.2.1 The electron transfer system

Oxidative phosphorylation is the last step of the of energy-yielding aerobic metabolism where O_2 is reduced to H_2O with electrons donated from nicotinamide adenine dinucleotide ($NADH_2$) and flavin adenine dinucleotide ($FADH_2$), obtained from substrate metabolism. Several multi-protein complexes embedded in the inner mitochondrial membrane are involved in the electron transfer system (ETS) which lead to production of ATP while reducing O_2 to H_2O (figure 2). These include NADH dehydrogenase ubiquinone oxidoreductase (complex I, CI), succinate ubiquinone oxidoreductase (complex II, CII), the ubiquinol cytochrome c oxidoreductase (complex III, CIII), cytochrome c oxidase (complex

IV, CIV) and ATP synthase (complex V). Furthermore, the elaborated ADP-ATP turnover is possible due to two mobile electron carriers; the coenzyme Q (CoQ) and the cytochrome c (cytc).

The first four complexes facilitate the transfer of electrons to the O_2 molecule. $NADH_2$ and $FADH_2$ donate electrons to complexes I and II, respectively. Coenzyme Q (CoQ) shuttles these electrons to complex III. From here electrons are moved through cytc to complex IV which reduces O_2 to H_2O. This process is coupled to proton pumping from the matrix to the intermembrane space through complexes I, III and IV. Through this process, a proton gradient is created across the intermembrane, termed membrane potential, which is utilized by complex V to drive ATP synthesis (Reid et al., 1966).

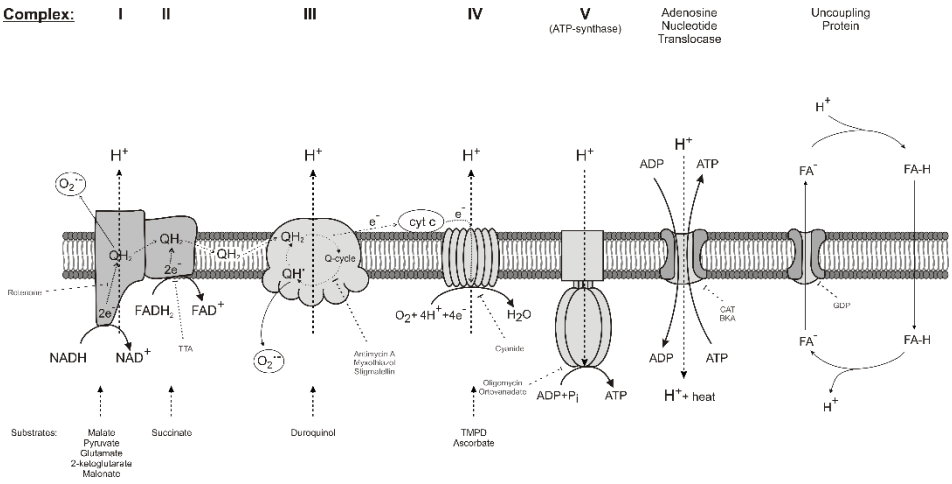

Figure 2. Illustration of the electron transport system together with substrates, inhibitors and other proteins that regulate membrane potential. Reprinted from Friederich et al. (2009) with permission from Bentham Science Publishers Ltd.

2.2.2 Supercomplexes

Knowledge regarding the enzyme complexes (Chance and Williams, 1955a) and their function (Chance and Williams, 1955b) was established about 60 years ago. However the understanding about the structural organization of these multi-protein complexes has evolved from the notion of being assembled within the inner membrane in a “rigid state” (Chance and Williams, 1955a) to being able to freely move in a diffusional and redox state named “fluid state” (Hochli and Hackenbrock, 1976), and later to be both present as individual complexes as well as in supramolecular protein aggregations (Hochman et al., 1982) called supercomplexes (Schagger and Pfeiffer, 2000). These supercomplexes are

sometimes constituted of all the required proteins to sustain the electron transfer from complex I to IV and they are termed respirasomes (Schagger and Pfeiffer, 2000). The structure of the supercomplexes and respirasomes is optimized to increase the mitochondrial catalytic efficiency (Bianchi et al., 2004) and their abundance can be upregulated by exercise training (Greggio et al., 2016).

2.2.3 Mitochondrial morphology

Typically, mitochondria in skeletal muscle are classified by their location in the subsarcolemmal and intermyofibrillar regions and possess a spherical or elongated shape (Elander et al., 1985) organized in a mitochondrial reticulum (Kirkwood et al., 1986;Ogata and Yamasaki, 1997). However, recent advances in electron microscopy together with immunostaining has facilitated the detection of five types of mitochondria according to their localization and morphology (figure 3) (paravascular mitochondria, I-band mitochondria, fiber parallel mitochondria, cross-fiber connection mitochondria and intra-fibrillar mitochondrial) interconnected to each other forming a mitochondrial reticulum where membrane potential conduction permits quick intracellular energy distribution (Glancy et al., 2015).

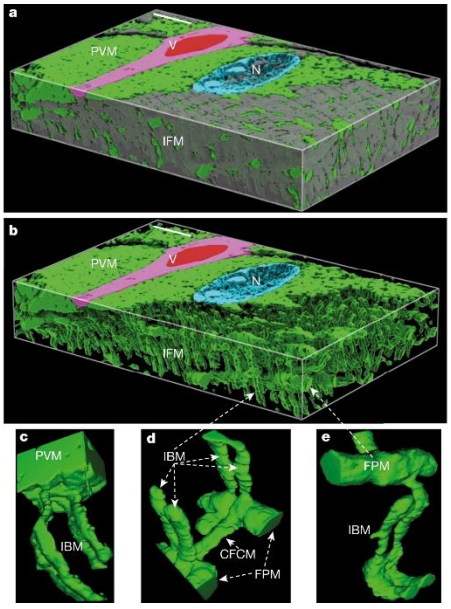

Figure 3. a, 3D imaging of mitochondria (green) and other structures (nucleus (N), cyan; capillary (V), magenta; red blood cell, red; myofibrils, grey). b, Removing myofibrils highlights different morphologies within intrafibrillar mitochondrial (IFM) network. c–e, Zooming in reveals projections from paravascular mitochondria (PVM) into I-band mitochondria (IBM) (c), and numerous interactions between IBM and cross-fiber connection mitochondria (CFCM) (d) and fiber parallel

mitochondria (FPM) (d, e). Reprinted from (Glancy et al., 2015) with permission from Nature Publishing Group.

2.2.4 Mitochondrial DNA

A unique feature of the mitochondrion is that it possesses its own genome i.e. mitochondrial DNA (mtDNA) which is used to encode 22 tRNA, 2 rRNA and 13 of the ~90 proteins involved in energy production (e.g. complex IV subunits) (Falkenberg et al., 2007).

According to the widely accepted endosymbiotic theory the mitochondrion developed from an α-proteobacterium which during the course of evolution transferred most genes to the nuclear genome (Gray et al., 1999). The functional advantage of keeping the small mtDNA in control of the expression of some proteins (such as cytochrome c oxidase subunit 1 and cytochrome b) which are central in the electron transport system is unclear. A simple explanation could be that it is difficult to translocate these highly hydrophobic proteins across the mitochondrial membranes and consequently these proteins are produced within the mitochondrion. Alternatively, these proteins are encoded within the mitochondrion for metabolic regulation purposes since evidence points towards a feedback loop being present between mitochondrial redox state and molecular signaling which regulate mitochondrial gene expression termed retrograde signaling (Falkenberg et al., 2007).

Another unique feature of the mtDNA is that it is almost exclusively maternally inherited (Tower, 2015). Findings from an animal model showed that male more than female mitochondria present a higher number of mutations due to maternal transmission of mitochondrial genes which have a direct impact on mitochondrial function (Innocenti et al., 2011). Maternal inheritance could be a strategy to bypass paternal mtDNA mutations which occur due to radical oxygen species exposure (Allen and de Paula, 2013). It appears that the part of the genome regulating mitochondrial function is optimized in women since mtDNA is maternally inherited (Tower, 2015). Indeed, strong sex differences have been shown in rodent models with females exhibiting superior structural and functional mitochondria in different organs compared to males (Ventura-Clapier et al., 2017). However, human data on mitochondrial function in relation to sex are lacking.

2.2.5 Mitochondrial respiration

In humans the maximal oxidative metabolic rate when exercising with large muscle mass can be 20 times higher than the basal metabolic rate (Weibel and Hoppeler, 2005) and up to ~100 times higher in skeletal muscle engaged in isolated exercise (Andersen, 1985b). Therefore, energy production is a crucial function of mitochondria. About 90% of total O_2 utilization in the mitochondria is coupled to ATP synthesis (Rolfe and Brown, 1997). Therefore, neglecting the uncoupled respiration caused by the proton leakage over the

inner membrane (Rolfe and Brown, 1997), maximal mitochondrial oxidative phosphorylation (i.e. OXPHOS) is indirectly a measurement of ATP synthesis and overall mitochondrial function.

Ex vivo (or *in vitro*) assessment of mitochondrial respiratory function of biological samples, such as a specimen of skeletal muscle obtained via biopsy (Bergström, 1962;Ekblom, 2017), is assessed in a closed-chamber respirometer (Gnaiger et al., 1995b). This device permits monitoring of O_2 concentration in the incubation medium measured amperometrically by Clark-type polarographic oxygen sensors. O_2 concentration is continuously measured and plotted together with O_2 consumption by the biological sample (Pesta and Gnaiger, 2012) which vary according to substrates titrated and mitochondrial activation state (Chance and Williams, 1955b). Mitochondrial respiratory function from samples of e.g. skeletal muscle can be either assessed in the isolated mitochondrial preparation (Chance and Williams, 1955b;Tonkonogi et al., 1997), cell cultures or in permeabilized fibres (e.g., saponin-or digitonin) (Kuznetsov et al., 2008;Pesta and Gnaiger, 2012). Considering the morphology-function relationship of the mitochondrial reticulum (Kirkwood et al., 1986;Glancy et al., 2015) the assessment of mitochondrial respiratory function *in situ* using the permeabilized myofiber preparation is advantageous compared to the use of isolated mitochondrial preparation (Picard et al., 2011) since mitochondrial reticulum is preserved during the experiment. Moreover, ten times as much tissue is needed to assess mitochondrial respiratory function in isolated mitochondria than in permeabilized fibres. However, mitochondrial ATP production (Wibom and Hultman, 1990), O_2 affinity ($p50_{mito}$) (Gnaiger et al., 1998) and the P/O ratio can only be determined by using isolated mitochondria. Furthermore, the small amount of tissue needed for permeabilized fibres is not solely an advantage since the small amount of fibres increases the risk of getting a fibre bundle from a very homogenous and unrepresentative part of the muscle.

In vivo assessment of mitochondrial respiratory function in the isolated mitochondria preparation (Larson-Meyer et al., 2001;Lanza et al., 2011) and *ex vivo* in the permeabilized fiber preparation (Layec et al., 2016) has been used as the gold standard for comparison with more advanced techniques such as high-resolution phosphorus-31 nuclear magnetic resonance spectroscopy (^{31}P MRS) where coils and magnet bores large enough to accommodate human limbs permit the noninvasive study of high-energy phosphate metabolism *in vivo* during recovery after exercise in human skeletal muscle (Gadian, 1982).

Since phosphocreatine resynthesis recovery time is a qualitative measure of mitochondrial oxidative capacity (Hultman et al., 1981), ^{31}P MRS is used for assessment of in vivo mitochondrial function (Arnold et al., 1984) and is capable of detecting differences in mitochondrial function between trained and untrained individuals (Larsen et al., 2009).

2.2.5.1 Mitochondrial respiratory flux states

Seminal work by Chance and Williams (1955b) described five mitochondrial activation states. State 1 is characterized by only endogenous substrates and inorganic phosphate in the mitochondrial respiration medium. No adenylates or substrates are added into the chamber; therefore, substrates and ADP levels are low, and respiration is slow. The titration of ADP leads to exhausting endogenous substrates and transition to state 2. Addition of pyruvate and succinate feed electrons to complex I and II of the ETS raising mitochondrial respiration to state 3. In state 3 O_2 flux rate is maximal and termed OXPHOS capacity. Gradually the ADP is phosphorylated to ATP and when ADP is exhausted, despite substrate availability, respiration falls to state 4. State 4 respiration is low but relatively higher than in state 1 and 2 due to ATP synthase activity and proton leak which occurs due to high membrane potential induced by the proton pumping coupled to the reduction of $NADH_2$ and $FADH_2$. State 5 occurs during anoxic state. An alternative to the described conventional protocol is to titrate substrates in the absence of ADP to determine state 2 where leak respiration occurs. For transition to state 3, ADP is added and O_2 flux rises reaching OXPHOS capacity if both complex I and II are maximally activated.

2.2.5.2 Normalization of the mitochondrial respiratory flux

The absolute mitochondrial respiration is usually expressed per mg initial tissue and is termed mass-specific mitochondrial respiration.

Intrinsic mitochondrial respiration refers to mitochondrial respiration normalized by mitochondrial content. Transmission electron microscopy is widely considered the gold standard technique for assessment of mitochondrial content. However, this technique is expensive, not always available and time consuming. For these reasons more easily assessed proxies of mitochondrial content such as citrate synthase (CS) activity, complex IV protein levels (Larsen et al., 2012), or mitochondrial protein levels of the isolated mitochondria preparation are more often described in the literature.

While a change in the respiratory capacity of each individual mitochondria is revealed by the intrinsic mitochondrial respiration, a change in mass-specific mitochondrial respiration can be due to 1) a higher number of mitochondria, 2) an enlargement of the already existing mitochondria and/or 3) an increase intrinsic mitochondrial respiratory function. The higher number of mitochondria and an enlargement of the already existing mitochondria lead to an increased mitochondrial content whereas an increased intrinsic mitochondrial function is dependent on specific protein upregulation such as upregulation of supercomplexes and/or increased mitochondrial cristae density without the necessity of concomitant change in mitochondrial volume. The assessment of mass-specific and intrinsic mitochondrial respiration allows the characterization of specific exercise-induced mitochondrial respiratory functions.

The importance of studying mitochondrial physiology in healthy and diseased populations (Nunnari and Suomalainen, 2012), together with the relative simplicity in the specimen preparation necessary for *ex vivo* mitochondrial respiratory function assessment and the accessibility of human skeletal muscle biopsy donors are some of the factors that contributed to the extensive use of high-resolution respirometry in clinical and scientific studies. However, the reliability and validity of this method has received less attentions and warrants further investigation.

2.2.6 Mitochondrial respiratory excess capacity

Despite the previously described concept of symmorphosis (Weibel, 1987), an apparent excess mitochondrial oxidative capacity compared to O_2 delivery (Boushel et al., 2011;Boushel and Saltin, 2013) is found when comparing direct measures of leg peak O_2 consumption during maximal cycling exercise to peak muscle O_2 consumption estimated from *ex vivo* OXPHOS from permeabilized fibres normalized per recruited lean muscle mass. In other terms, at maximal exercise intensity during cycling mitochondrial respiration of the recruited skeletal muscle (i.e. vastus lateralis muscle) proceeds submaximally at about 60% of its maximum (Boushel et al., 2011;Boushel et al., 2015a;Gifford et al., 2015). This is in contrast with the larger per unit muscle O_2 consumption observed during one-legged knee extension (Andersen and Saltin, 1985), suggesting that mitochondrial respiratory capacity is more closely matched to O_2 delivery with local or small muscle mass exercise. Thus, it can be debated how the 50% increase in mitochondrial content after only a few weeks of endurance training (Henriksson, 1977) contributes to the improvement of VO_2max and its overall physiological function.

2.2.7 Mitochondrial O_2 affinity ($p50_{mito}$)

Mitochondrial O_2 consumption is a function of OXPHOS, pO_2 in the tissue, and the apparent mitochondrial O_2 affinity ($p50_{mito}$), as described by equation 5 in figure 1. $p50_{mito}$ is the oxygen tension at which mitochondrial respiration proceeds at 50% of its O_2 saturated maximum.

Experimentally, $p50_{mito}$ is calculated by hyperbolic fitting of the data following smoothing of the exponential O_2 flux decay corrected for time-constant, zero drifting, background correction, and zero oxygen calibration of the high-resolution respirometer polarographic oxygen sensor. Equation 5 which is used to compute O_2 consumption at the muscle level demonstrates the possible influence exerted by the $p50_{mito}$ on the *in vivo* mitochondrial respiration. However, when considering the different factors influencing O_2 uptake, the tissue pO_2 of ~0.3 kPa at work rates corresponding to VO_2max has usually been considered sufficiently high to maintain mitochondrial respiration (Wagner, 1993)

and the $p50_{mito}$ has usually been considered to be low *in vivo* (high mitochondrial O_2 affinity) and has therefore been neglected. However, a wide range of *ex vivo* $p50_{mito}$ values can be found in the literature, from as low as 0.01 kPa to 0.3 kPa (de Groot et al., 1985;Gnaiger et al., 1995b). According to equation 5 in figure 1 a $p50_{mito}$ of about 0.01 kPa would have a minimal influence on O_2 flux rate whereas a $p50_{mito}$ of 0.3 kPa would have an impact of ~50% on the O_2 flux rate at physiological pO_2. Gnaiger and colleagues demonstrated that $p50_{mito}$ varies with the degree of coupling (Gnaiger et al., 1995b) and that the higher excess capacity of complex IV of the ETS leads to low $p50_{mito}$ (Gnaiger et al., 1998). More recently the interdependence between $p50_{mito}$ and efficiency was explained by the presence of different isoforms of complex IV subunit where isoform IV-1 is associated with high affinity for oxygen but a low efficiency, whereas isoform IV-2 has a low affinity for oxygen but high efficiency (Schiffer et al., 2016). Data from isolated mitochondria of human skeletal muscle have reported that a low O_2 affinity (high $p50_{mito}$) is associated with a low basal metabolic rate (Schiffer et al., 2016) and high aerobic efficiency during exercise (Larsen et al., 2011a). Furthermore, in human skeletal muscle, maximum ADP-stimulated $p50_{mito}$ seems to vary considerably between individuals (0.023-0.068 kPa) (Larsen et al., 2011b).

Although only few studies have reported on $p50_{mito}$ in humans (Larsen et al., 2011b;Boushel et al., 2015b;Schiffer et al., 2016), the $p50_{mito}$ variability and its possible impact on VO_2max in humans remains to be elucidated.

2.3 LIMITATIONS TO VO_2max

VO_2max is determined by the integrated capacity of the O_2 transport and muscular systems to deliver and utilize O_2 when performing maximal work with large muscle groups. VO_2max is a measurement of cardiorespiratory fitness and the best indicator associated to all-cause mortality (Kodama et al., 2009). Therefore, the study of the regulation of oxygen consumption and the possible limiting factors which impose the upper limit for VO_2max has great relevance not only in exercise physiology for sport applications (Bassett and Howley, 2000) but also in clinical populations (Booth et al., 2012).

2.3.1 Alveolar-capillary diffusion

In healthy individuals the pulmonary system generally poses a no, or only a small limitation on VO_2max, since even at exercise intensities near to maximal effort alveolar-capillary diffusion is not impaired and results in an arterial oxygen saturation (SaO_2) above 95% (Powers et al., 1989). However, ~50% of highly trained endurance athletes show an SaO_2 below 95% during exercise approaching $\dot{V}O_2$max at sea level (Powers et al., 1988).

This pattern is linked to the alveolar-capillary diffusion limitation resulting from a decreased Hb mean-transit time in the lung (Dempsey and Wagner, 1999) caused by mechanical ventilatory constraint during exercise (Dominelli et al., 2013). This condition, named exercise induced arterial hypoxemia (EIAH) (Dempsey et al., 1984) occurs both in running and cycling (Powers et al., 1989;Rice et al., 2000) and is more pronounced in women than in men (Harms et al., 1998;Richards et al., 2004).

Inspiring a mild hyperoxic gas ($F_IO_2 = 0.26$) completely prevents EIAH (Powers et al., 1989). This indicates that improving O_2 delivery by increasing O_2 carrying capacity affects VO_2max and that the demand imposed on the pulmonary system in endurance trained individuals may exceed the structural capacity of the system (Dempsey, 1986;Nielsen et al., 1999b).

Apart from breathing hyperoxia, there are other means for manipulating the O_2 carrying capacity of the blood and all have shown the dependence on O_2 delivery for VO_2max. It has been shown that the levels of carboxyhemoglobin in blood, a gas with a far higher Hb affinity than O_2 which competitively blocks Hb binding O_2, linearly relates to a reduction in VO_2max (Ekblom and Huot, 1972). Another way to alter O_2 carrying capacity is by manipulating Hb mass by blood loss and autologous blood reinfusion (Ekblom et al., 1972;Ekblom et al., 1976) or by increasing Hb mass resulting from recombinant erythropoietin treatment (Ekblom and Berglund, 1991;Thomsen et al., 2007). These studies have clearly shown that VO_2max increases in proportion to the elevated O_2 carrying capacity.

2.3.2 Convective oxygen transport

According to the Fick Principle shown in equation 3 (figure 1), VO_2max is determined by the product of the cardiac output and the arterial-venous difference in O_2 content (i.e. O_2 extraction). It has long been debated whether central or peripheral factors represent the dominant factor defining VO_2max (Saltin and Calbet, 2006a).

There are several lines of evidence that indicate the key role of the convective component for defining VO_2max and its improvements related to changes in VO_2max observed with training. It is well documented that cardiac output is a significant limiting factor for VO_{2max} (Andersen, 1985a) and that a change in cardiac output linearly relates to changes in VO_2max (Åstrand et al., 1964;Ekblom and Hermansen, 1968). Additionally, differences in maximal cardiac output, specifically differences in stroke volume, account for much of the variation in VO_2max among sedentary and trained individuals (Saltin et al., 1968) and that changes in VO_2max after 20 days bed rest followed by 50 days training are predominantly due to changes in cardiac output. A less precisely defined factor involved in the convective component of increases in VO_2max is the 'distribution' of the cardiac output to the active muscle bed that is regulated by the interaction between the

sympathetic nervous system and local vasodilatory mechanisms (Calbet et al., 2007;Boushel et al., 2014a).

Further evidence on the dependence of aerobic capacity on cardiac output and muscle blood flow has been provided in studies using the one-legged knee extension and two-legged cycling models (Saltin et al., 1976;Andersen, 1985b). If exercise is performed with only the quadriceps muscles of the leg (~2.5 kg muscle mass) such as during one-legged knee extension, the peak blood flow to the muscle is ~2-3 times higher and muscle VO_2 is ~1.5-2 times higher than when the same muscle performs two-legged cycling (~20 kg muscle mass) (Andersen and Saltin, 1985). It has been shown that locomotor muscle blood flow and VO_2max are reduced when respiratory muscles are fully loaded compared to when breathing heliox, a gas with less resistance than atmospheric air when passing through the airways of the lungs, and thus reducing the work of breathing. These results suggest that there is a competition for blood distribution as maximal exercise effort is approached (Harms et al., 1997;Calbet et al., 2007;Sheel et al., 2018).

Further evidence of the role of circulation for defining VO_2max comes from studies comparing maximal exercise performed with legs alone versus combined arms and legs, where O_2 delivery per unit muscle mass differs markedly. In a classical study by Secher and coworkers (1977), the portion of blood flow perfusing the leg muscles was reduced when upper body work was added to leg cycling. A later study reported a similar blood flow redistribution between arms and legs in leg in elite cross-country skiers (Calbet et al., 2004). However, according to O_2 delivery limitation, adding arm work to cycling should not result in a higher VO_2max as has been reported in some studies (Gleser et al., 1974;Secher et al., 1974;Holmberg et al., 2007) since O_2 delivery per unit muscle mass is not elevated under these conditions. This suggests that VO_2max is mainly dictated by the convective component of O_2, although other factors in the O_2 cascade may additionally regulate oxygen consumption.

2.3.3 Microvasculature to myocyte diffusion

Another often debated factor which may limit VO_2max is the possible O_2 diffusion limitation between the microvasculature and myocyte (Lundby and Montero, 2015;Wagner, 2015). Diffusive capacity is thought to play a contributory role in limiting O_2 transport and muscle VO_2 (Wagner, 1992;Richardson et al., 1995a). This argument is supported by the finding that the arterial-venous O_2 difference is in the range of 80-90 % in well-trained athletes at exercise intensities near to maximum (Ekblom et al., 1968), and there is therefore a theoretical possibility to enhance VO_2max if O_2 extraction is increased. Furthermore, the recently developed phosphorescence quenching technique has shown that the pO_2 gradient between microvasculature and myocyte is not altered when going from the resting condition to submaximal work. Therefore, according to the Fick Law of diffusion

[$VO_2 = DO_2 \cdot (pO_2cap\text{-}pO_2mit)$], since VO_2 increases from rest to contraction, diffusion must be the major determinant of muscle VO_2 observed from rest to contraction (Hirai et al., 2018). In contrast, a large functional reserve in muscle O_2 diffusing capacity exists and remains available at exercise to exhaustion in normoxia (Calbet et al., 2015).

2.3.4 O_2 utilization at the mitochondrial level

Further down in the O_2 cascade the mitochondria itself could theoretically limit VO_2max. As shown in equation 5 in figure 1, OXPHOS, pO_2 in the tissue and the $p50_{mito}$ all directly influence VO_2max. Mitochondrial respiratory rate, based on conservation of mass, is proportional to VO_2max since O_2 is consumed mainly by mitochondria. Indeed, a strong linear relationship exists between muscle mitochondrial volume and VO_2max across a wide range of species including humans (Weibel and Hoppeler, 2005). However, the possible role of mitochondria in limiting VO_2max is usually ruled out since mitochondria possess an apparent excess oxidative capacity compared to O_2 delivery when healthy individuals exercise with large muscle groups e.g. in cycling (Boushel et al., 2011).

In the case of exercise with smaller muscle mass there is a closer matching between the amount of oxygen delivered to the muscle and the maximal activity of the enzymes of the muscle mitochondria (Blomstrand et al., 2011;Boushel and Saltin, 2013). In other terms the mitochondrial metabolic capacity is nearly completely utilized during one-legged knee extension exercise in healthy individuals (Gifford et al., 2015). Interestingly, despite the close matching between O_2 delivery and metabolic capacity exists during one-legged knee extension exercise, breathing hyperoxia further increases muscle VO_2 in trained individuals which emphasizes the reliance of O_2 delivery for VO_2max (Richardson et al., 1999b). Nevertheless, divergent results have also been shown (Pedersen et al., 1999;Mourtzakis et al., 2004) which may be explained by the training status of the participants recruited in these studies (Gifford et al., 2015).

Despite a possible role played by the excess of mitochondrial capacity in regulating substrate utilization especially at a submaximal level (Gollnick and Saltin, 1982), there is no compelling evidence that mitochondria limit VO_2max during exercise with large muscle groups in healthy individuals. However, higher mitochondrial ADP sensitivity was associated with higher O_2 extraction in hypoxia in trained individuals (Ponsot et al., 2010) indicating that mitochondrial properties can play a role in regulating VO_2. Given that the decrement in VO_2max with age is mainly due to changes in O_2 extraction and that structural O_2 diffusion is unaffected with age (McGuire et al., 2001), these data suggest that mitochondrial oxidative capacity or other mitochondrial properties can finely regulate O_2 extraction. The finding that $p50_{mito}$ can influence mitochondrial respiration (Gnaiger et al., 1998) indicates a possible role of mitochondria in regulating VO_2 and this appears to vary between individuals (Larsen et al., 2011b) as mentioned above.

Thus, mitochondria can have a possible role in determining VO_2 by regulation of O_2 extraction. However, the role of mitochondria in regulating VO_2 is poorly investigated.

2.3.5 Endurance performance and training

Endurance performance in broad biological terms is determined by the integration of muscular, cardiovascular and neuromechanical and endocrine factors combined with motivational and environmental factors. From a physiologic standpoint endurance performance in humans is mainly determined by the individual VO_2max, percentage of VO_2max that can be sustained over time, and movement economy (Bassett and Howley, 2000). The importance of a high VO_2max as a prerequisite for high level endurance performance has been known for decades (Hill et al., 1924). VO_2max values of up to ~6 $L \cdot min^{-1}$ or ~80-90 $mL \cdot min^{-1} \cdot kg^{-1}$ have been found in elite endurance athletes (Ekblom and Hermansen, 1968;Holmberg et al., 2007;Burtscher et al., 2011;Haugen et al., 2018). These VO_2max values are 2 to 3 times higher than in untrained individuals and are mainly explained by differences in cardiac output (Ekblom et al., 1968;Saltin et al., 1968) which can reach values of 40 $L \cdot min^{-1}$ in endurance athletes (Ekblom and Hermansen, 1968) and an arterial-venous difference of ~95% (Calbet et al., 2005). These high cardiac output values in endurance athletes are obtained with a large left ventricular mass and the left ventricular end-diastolic volume (Levine et al., 1991;Levine, 2008), a feature which is not affected by aging *per se* but is a characteristic of the endurance athletes' heart (Steding-Ehrenborg et al., 2015a). Other equally important determinants of endurance performance are those influencing the O_2 carrying capacity such as the large blood volume resulting from a large Hb mass and plasma volume (Kjellberg et al., 1949;Sawka et al., 2000;Jacobs et al., 2011) found in endurance athletes (Lundby and Robach, 2015).

The percentage of VO_2max that can be sustained over time drastically differentiates endurance trained from untrained individuals. Athletes can sustain 87% and 83% of VO_2max for 1 and 2 h, respectively whereas untrained individuals can exercise for the same amount of time at 50% and 35% of VO_2max (Åstrand PO, 2003). Another performance determinant is the movement economy often quantified as gross efficiency (GE) which is the percentage of energy consumption that can be converted to actual work. GE in cycling ranges between 18% to 23% whereas a larger individual variation of up to 40% is found in running (Joyner and Coyle, 2008). Superior GE has been linked to a higher proportion of slow-twitch fibres (Coyle et al., 1992;Mogensen et al., 2006); however, other studies have reported that only the individual body mass rather than cardiorespiratory, skeletal muscle morphology, mitochondrial respiratory function or biochemical factors explains the variation in GE in individuals with a VO_2max range of 45.5-72.1 $mL \cdot min^{-1} \cdot kg^{-1}$ (Lundby et al., 2017). Interestingly cyclists with higher VO_2max seem to possess lower GE and vice versa (Lucía et al., 2002) indicating that a

relative optimum rather than an absolute maximum is found in endurance trained individuals. Despite the mitochondrial oxidative excess capacity over the O_2 delivery shown in endurance trained individuals (Gifford et al., 2015) OXPHOS is positively related to the individual fitness level of people ranging from sedentary to elite athletes (Bishop et al., 2014) and is a determinant of time trial performance in endurance trained athletes (Jacobs et al., 2011).

2.3.6 Adaptation to endurance training

Repeated exercise bouts generate systemic and tissue specific activation and/or repression of specific signaling pathways that regulate gene expression through transcription and translation, and which lead to a gradual functional adaptation and remodeling due to the alteration in protein content and enzyme activity changes (Perry et al., 2010;Egan and Zierath, 2013). Endurance training has been shown to increase cardiac output and arterial-venous difference and up to double the VO_2max in previously untrained individuals after a few weeks of endurance training (Ekblom et al., 1968;Saltin et al., 1968). The rapid blood volume expansion obtained after a few days of training explains the greater cardiac output at the beginning of an endurance training intervention in untrained individuals (Bonne et al., 2014). Repeated bouts of endurance exercise will consequently induce cardiac morphological remodeling that increase left ventricular end diastolic volume which in turn increases the cardiac output and VO_2max (Arbab-Zadeh et al., 2014). Despite this profound cardiac morphological remodeling observed in previously untrained individuals, the cardiac morphological remodeling that appears to plateau after ~9 months of endurance training hinders a previously untrained individual from reaching similar levels of cardiac compliance and performance to those found in athletes (Arbab-Zadeh et al., 2014;Steding-Ehrenborg et al., 2015b). Indeed, a strong genetic component appears to dictate the training-induced response (Karavirta et al., 2011;Sarzynski et al., 2016), however recent findings have shown that training-adaptation can be elicited in people not possessing the best genetic endowment by increasing the training volume (Montero and Lundby, 2017). The endurance training-induced increment in cardiac output paralleled by a higher muscle blood flow and muscle capillary density which enhance the capacity to extract O_2 from the blood (Ekblom et al., 1968;Andersen and Henriksson, 1977;Henriksson, 1977;Boushel et al., 2014a) while the increase in mitochondrial respiratory capacity found following endurance training enhances the capacity to utilize oxygen, reduces the magnitude of anaerobic metabolism at a given work rate and increases the skeletal muscle metabolic flexibility (Holloszy, 1975;Gollnick and Saltin, 1982;Tonkonogi and Sahlin, 2002;Storlien et al., 2004).

High-volume training with continuous exercise at moderate intensity (MICT) has been shown to be a fundamental training component for endurance athletes (Seiler and

Kjerland, 2006;Laursen, 2010). This form of training evokes increases in muscle blood flow as well as expansion of capillary and mitochondrial volume in untrained individuals (Andersen and Henriksson, 1977;Henriksson, 1977;Orlander et al., 1977). On the other end of the training intensity spectrum, low-volume high-intensity interval training (HIIT) and sprint interval training (SIT), the most intense form of HIIT, have been widely shown to time efficiently enhance cardiorespiratory fitness, skeletal muscle adaptation and endurance performance (Burgomaster et al., 2005;Daussin et al., 2007;Helgerud et al., 2007;Daussin et al., 2008) in healthy and diseased populations (Sloth et al., 2013;Weston et al., 2014;Milanović et al., 2015). Several lines of evidence indicate the importance of exercise intensity for exercise-induced adaptations (MacInnis and Gibala, 2017). As little as two minutes of total sprint exercise time per week (10s sprint exercise repeated four times for three times a week) can induce change in cardiorespiratory fitness and performance in young adults active people (Hazell et al., 2010). Recent findings showed that skeletal muscle adaptation such as mitochondrial content and respiratory function are enhanced more following HIIT than after moderate continuous exercise (MacInnis et al., 2016;Robinson et al., 2017) and after SIT the intrinsic mitochondrial respiratory function is improved (Granata et al., 2015). Interestingly, the higher change in mitochondrial respiratory function induced by SIT compared to MICT or HIIT was not related to a superior improvement in endurance performance (Granata et al., 2015). Conversely, too intense SIT training has been shown to inhibit mitochondrial respiration in skeletal muscle of arms and legs through inhibition of the citric acid cycle enzyme aconitase due to high ROS production with evidence of protein carbonylation (Larsen et al., 2016). Furthermore, the cross-sectional study by Jacobs et al. (2011) indicated mitochondrial respiratory function as a determinant of endurance performance. Despite the importance of training intensity for improving mitochondrial respiratory function, mitochondrial content adaptation appears to be related to training volume and not training intensity (Bishop et al., 2014;Granata et al., 2018).

2.4 SEXUAL DIMORPHISM

Differential gene expression between men and women (Rigby and Kulathinal, 2015) combined with social and cultural factors result in sexual dimorphism of anatomical, physiological, hormonal and behavioral characteristics. Sexual dimorphism in humans has important consequences for life expectancy (Seifarth et al., 2012), disease occurrence and aging (Popkov et al., 2015;Tower, 2017).

Women are generally shorter, lighter and have more fat mass (FM) than men (Cureton and Sparling, 1980). There are distinct differences mainly attributed to hormonal levels such as a ten-fold higher testosterone level in men than in women and a four-fold higher

estrogen level in women than in men (Khosla et al., 2005a;Khosla et al., 2005b;Turpeinen et al., 2008). These hormones affect numerous systems including musculoskeletal tissue, erythropoietin production, immune system function, and behavioral patterns (Mooradian et al., 1987;Rickenlund et al., 2003).

Furthermore, women possess smaller airways compared to men which theoretically compromises the O_2 diffusion at the lung level (Dominelli et al., 2013), however a study direct comparing men and women did not find any difference in EIAH (Guenette et al., 2004). Interestingly, exercise-induced cardiac remodeling appears to be sex dependent and hormonally influenced with women showing a plateau after 3 months of training in contrast to between 9 and 12 months in men (Howden et al., 2015). Consequently, women have a smaller heart size compared to men and subsequently a lower stroke volume which is compensated by a higher heart rate resulting in equal cardiac output in women compared to men when exercising at the same absolute exercise intensity (Salton et al., 2002).

The lower hemoglobin concentration (Murphy, 2014) and the lower hemoglobin oxygen affinity (Humpeler et al., 1989) is partially compensated by a higher microvasculature-myocyte O_2 extraction and a higher blood flow in women at a given exercise intensity. Therefore, even though O_2 delivery per a given amount of blood is lower in women than in men compensatory mechanisms equalize the O_2 available at the skeletal muscle level between women and men (Lewis et al., 1986). In fact equal oxygen consumption per lean muscle mass is observed in women and men (Freedson et al., 1979). Women oxidize more fatty acids and less carbohydrates than men at the same relative exercise workload (Tarnopolsky et al., 1990;Horton et al., 1998) possibly due to a higher mitochondrial content (Montero et al., 2018) and higher baseline lipoprotein lipase levels (Skelly et al., 2017) compared to men with similar cardiorespiratory fitness. The proteins involved in the regulation of muscle lipid metabolism have been reported to be sex dependent with a higher number of intramyocellular lipid droplets found in skeletal muscle of women compared to men (Tarnopolsky et al., 2007). The acute response of a continuous endurance exercise bout has shown to increase mRNA content of citrate synthase (CS) and β-hydroxyacyl-CoA dehydrogenase (HAD) to a larger extent in women compared to men (Roepstorff et al., 2005). During SIT gene expression (Scalzo et al., 2014;Skelly et al., 2017) and cell signaling responses (Fuentes et al., 2012) are similar in men and women with a few exceptions. The mRNA content of the glucose transporter 4 (GLUT-4) has been reported to be higher in men compared to women at baseline but increased 3 hours post sprint bout in women only. Similarly, lipoprotein lipase was only increased in women at the end of the sprint bout and 3 hours post exercise. In contrast, Atrogin-1 was similar pre and directly post sprint bouts but was significantly higher in men at 3 hours post exercise (Skelly et al., 2017). 5´AMP-activated protein kinase (AMPK) which is implicated in the regulation of fatty acid uptake, handling, and oxidation (Thomson and Winder, 2009;O'Neill et al., 2013) as well as mitochondrial biogenesis

via phosphorylation of peroxisome proliferator activated receptor c co-activator-1a (Norrbom et al., 2011), is acutely upregulated more in men than women following a bout of endurance exercise, suggesting that women better preserve muscle cellular homeostasis compared to men following an exercise bout (Roepstorff et al., 2006). Other factors involved in mitochondrial function such as 3-beta-Hydroxyacyl CoA dehydrogenase, complex II-III, complex IV, and CS activity have been reported to be similarly improved by endurance training in men and women (McKenzie et al., 2000;Carter et al., 2001;Skelly et al., 2017) while muscle protein synthesis and mitochondrial biogenesis may be greater in men compared to women following sprint interval training (Scalzo et al., 2014).

Despite the available literature on sexual dimorphism showing several important differences between women and men (Lewis et al., 1986) most of the studies in exercise physiology have been conducted on men, without attention to potential physiological differences between sexes (Della Torre and Maggi, 2017). The fact that women are significantly less studied than men in sports and exercise medicine research (Costello et al., 2014) highlights the need for further research on sex-based differences in exercise and physiological function (Clayton and Collins, 2014).

3. AIMS AND HYPOTHESES

The general aim of this thesis was to examine on the significance of mitochondrial respiratory function in regulating oxygen uptake and performance.

The specific aims were to assess:

- The influence of various methodological factors on the error in maximal mitochondrial oxidative phosphorylation measurements.
- The possible role exerted by mitochondrial excess capacity and mitochondrial oxygen affinity in regulating VO_2.
- The individual differences in mitochondrial respiratory function and mitochondrial oxygen affinity in relation to biological sex.
- The exercise-induced adaptations following hyperoxic-supplemented high-intensity interval training in trained cyclists.

It was hypothesized that mitochondrial respiratory function regulates oxygen uptake and would be different between women and men. Moreover, it was hypothesized that increasing oxygen delivery would enhance exercise-induced adaptations.

4. RESULTS AND DISCUSSION

4.1 Paper I: Reliability of maximal mitochondrial oxidative phosphorylation in permeabilized fibres from the vastus lateralis employing high-resolution respirometry.

The keystones of any scientific method are validity and reproducibility. Despite the widespread assessment of mitochondrial respiratory function in clinical and scientific laboratories, no systematic study has been conducted on the reliability of OXPHOS measurement in both permeabilized myofibres and isolated mitochondrial preparations. In paper I OXPHOS values using permeabilized myofibres preparation obtained from biopsies from human *vastus lateralis* muscle were compared in four different ways, 1) the values for two bundles of fibres in the same biopsy; 2) the values for the left and right thighs of the same subject; 3) the values obtained at two time-points 27 ± 6 days apart; and 4) measurements by two different technicians. Furthermore, the potential implication of fiber bundle weight, time after collection of the biopsy, and the use of an anaesthetic on OXPHOS measurements was evaluated. In addition, the reliability of OXPHOS and $p50_{mito}$ measurements from isolated mitochondria are reported here (unpublished data).

4.1.1 Repeated determination of OXPHOS is associated with a relatively large variability between fiber bundles and this variability remains similar when comparing OXPHOS between right and left thigh.

To determine the OXPHOS reliability in repeated measurements, OXPHOS was measured in 50 samples using two fiber bundles per biopsy obtained from 25 participants. The standard error of the mean (SEM) and the coefficient of variation (CV), two measurements of reliability, were 10.5 pmol $\cdot$ s^{-1} $\cdot$ mg^{-1} and 15.2% respectively (figure 4). These results indicate a relatively large OXPHOS variation in repeated measurements involving the same biopsy. In simple terms, the "noise" in the repeated determination of OXPHOS

measurements is about ~10 pmol · s^{-1} · mg^{-1} and this error implies that the smallest beneficial/harmful change in OXPHOS detectible following an intervention cannot be smaller than the measured SEM. The analysis of OXPHOS from specimens collected from the left and right *vastus lateralis* muscle of the same subject revealed no significant difference in OXPHOS ($p > 0.05$). This is a particularly important consideration when one leg acts as treatment and the contralateral leg of the same subject is used as the control (Andersen, 1985b). Interestingly, the calculated SEM from OXPHOS measurements in left and right *vastus lateralis* muscle (i.e. 9.4 pmol · s^{-1} · mg-1) was similar to the one calculated from repeated measurements.

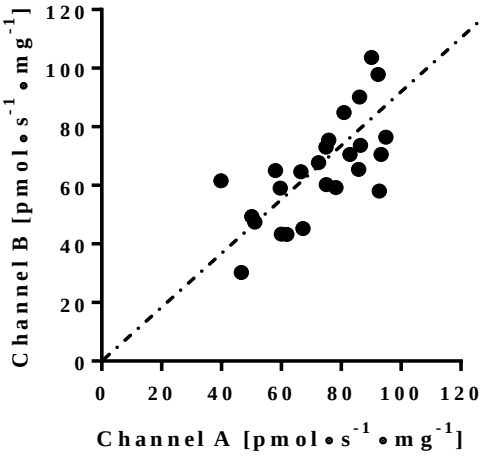

Figure 4: Maximal mitochondrial oxidative phosphorylation (OXPHOS) (pmol · s^{-1} · mg^{-1}) measured simultaneously on two different fibre bundles in two different channels (A and B) of a high-resolution respirometer (n = 25). Each circle represents one individual and the dotted line shows the line of identity.

The calculated SEM is the reflection of the technical error arising from the high-resolution respirometry apparatus, the human error introduced by the technician in preparing the fibre bundles and the possible biological variation between the analyzed fiber bundles. Assuming the pure machine error playing a marginal role and considering that the same technician conducted the experiments, the SEM would indicate either a role of biological variation between the analyzed fiber bundles or that the sample preparation *per se* is the major cause of variability in measurements of OXPHOS on isolated bundles of muscle fibres. In the case of biological variation, it is possible that mitochondrial content differed between fiber bundles due to variation in fiber composition thus accounting at least in part to the found variability. However, if heterogeneity of the fibre bundles were a major source of the observed methodological error, bundles composed of a larger number of fibres (i.e., heavier) should exhibit a smaller variation in OXPHOS than bundles with

fewer fibres (i.e. lighter), which was not the case (figure 5A). Moreover, the variation in OXPHOS between fiber bundles was not dependent on the O_2 flux rate per se as shown in figure 5B.

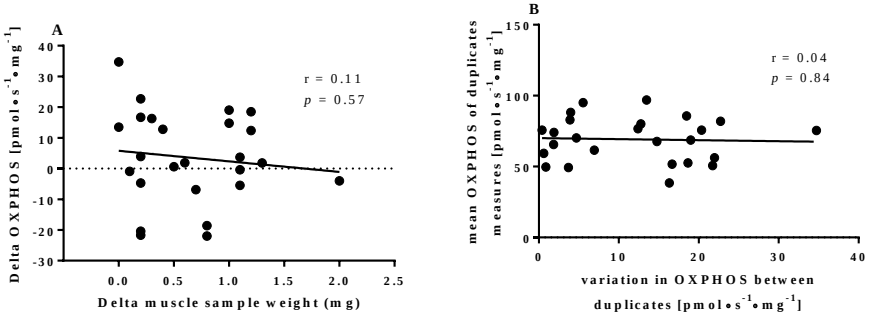

Figure 5. **A**: No significant relationship between the difference in OXPHOS (Y axis) and two fiber bundles wet weight (X axis) from the same subject (n = 25) indicating no effect of fiber bundle weight on OXPHOS variation. **B**: No significant relationship between the mean OXPHOS between fiber bundles (Y axis) and its variation (X axis) from the same subject (n = 25) indicating the variation in OXPHOS between two bundles is not dependent on the absolute O_2 flux. Each measurement is represented by a circle and the black continuous line depicts the trend, with the Pearson's product moment correlation coefficient (r) and the relative *p* value also indicated.

In line with these results, CS activity and myosin heavy chain type distribution has been reported to not differ between two biopsies collected from the same leg or contralateral leg of the same individual (Sahl et al., 2018) and that fiber type distribution in the *vastus lateralis* muscle of the right and left legs is similar (Blomstrand and Ekblom, 1982)(Blomstrand and Ekblom, 1982). Thus, ruling out the biological variability as a major contributor of methodological variability, sample preparation remains the primary cause of measurement error.

4.1.2 The technician sample preparation but not fiber bundle weight, the use of anaesthetic or time elapsed from biopsy is the source of variation of OXPHOS measurement

The use of the permeabilized fiber technique requires the technician to consistently and carefully dissect out, permeabilize and precisely weigh the different fiber bundles prior to the assessment of mitochondrial respiratory function in the high-resolution respirometer. Since sample preparation appears to be a key factor in determining OXPHOS variability, it was evaluated whether the sample preparation by the technician would have a

significant impact on the reliability of the OXPHOS measurements. The comparison between OXPHOS measurements obtained by two experienced technicians who prepared 12 fiber bundles each from the same biopsy revealed consistent differences in OXPHOS measurements which did not reach the level of significance ($p = 0.12$, figure 6). However, this observation indicates the importance of having the same technician prepare, weigh and permeabilize the samples in any given study.

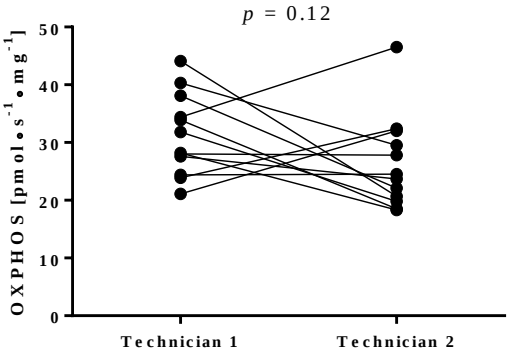

Figure 6. The variance in maximal mitochondrial oxidative phosphorylation (OXPHOS) by bundles of fibres from the same biopsy as measured by two experienced technicians. Each measurement is represented by a circle. Two measurements performed in the same respirometer at the same time of the day, but permeabilized by the different technicians are connected with a line. *p* indicates the level of significance (no significant difference was observed).

Another possible source of methodological error is the fiber bundle weight which could affect OXPHOS in two distinct ways. Firstly, O_2 and substrate diffusion may be limited in large fiber bundles since mitochondria could be located distantly from the permeabilized pores and the more internal fibres in a bundle may not be sufficiently permeabilized. However, no support was found for this notion as no significant correlation was found between the wet weight of the fiber bundle and OXPHOS (figure 7).

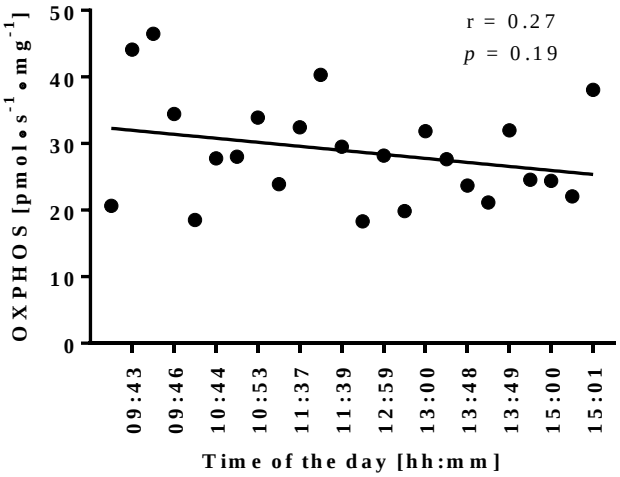

Figure 7. Correlation between maximal mitochondrial oxidative phosphorylation (OXPHOS) and the wet weight of the fiber bundle in 50 permeabilized samples. Each measurement is represented by a circle and the black continuous line shows the trend, with the Pearson's product moment correlation coefficient (r) and the relative *p* value also being indicated.

Secondly, since O_2 flux rate is usually normalized by the fiber bundle wet weight this procedure could be a source of methodological error. The wet weight is assessed by positioning the fiber bundle on a filter paper for ~5 seconds without applying any external pressure before weighing it on a precision scale. Longer blotting periods or squeezing the fiber bundle would remove more liquid but also intracellular water and is not recommended. To avoid this possible issue, fiber bundles could be recollected at the end of an experiment and O_2 flux rate could be expressed per dry weight; thus avoiding variability due to the moisture content and intracellular liquid weight. However, to recollect the fiber bundle is not always possible since the stir bar rotation usually causes fragmentation of the bundle. Additionally, a fiber bundle with a wet weight of 2 mg would weigh ~0.4 mg when dried, making the weighing procedure even more difficult.

Alternatively, O_2 flux rate could be normalized by the CS activity of the assessed fiber bundle to eliminate the fiber bundle weighing as a possible source of OXPHOS variation. Despite the positive relationship between OXPHOS and CS activity, it has been reported that CS activity does not differ between specimens collected from the same leg or contralaterally (Sahl et al., 2018). Therefore, the normalization of O_2 flux rate per CS activity would not only be redundant to reduce the variability observed in repeated OXPHOS measurements involving the same biopsy or different biopsies from the same individual but would even introduce another methodological error to the OXPHOS measurements.

Local anesthetics are commonly injected subcutaneously in the area were the incision through the skin and fascia is made prior to biopsy collection. Long lasting exposure to anaesthetics has been reported to raise the production of reactive oxygen species and alter mitochondrial physiology including impairment of mitochondrial oxidative phosphorylation in animal models (Nouette-Gaulain et al., 2012). In the observations from paper I, OXPHOS was not influenced by direct exposure of the fibres to carbocaine ($p > 0.05$), although, this does not definitely exclude that OXPHOS was impaired by carbocaine since the biopsy may already be influenced by the local anaesthetic given to the subject before the biopsy collection.

Another factor that could be a source of OXPHOS variation between samples is the time elapsed between biopsy collection and OXPHOS assessment. It can be challenging for the investigator to standardize the length of this period and the fresh muscle sample is kept in ice-cold BIOPS buffer (specifically designed to preserve mitochondrial function) for a variable time until the technician is ready to start the experiment. Here it was indicated that OXPHOS remained unchanged for at least 5 hours after collection of the biopsy (figure 8). It should be stressed that the OXPHOS measurements started as soon as the fiber permeabilization procedure was completed.

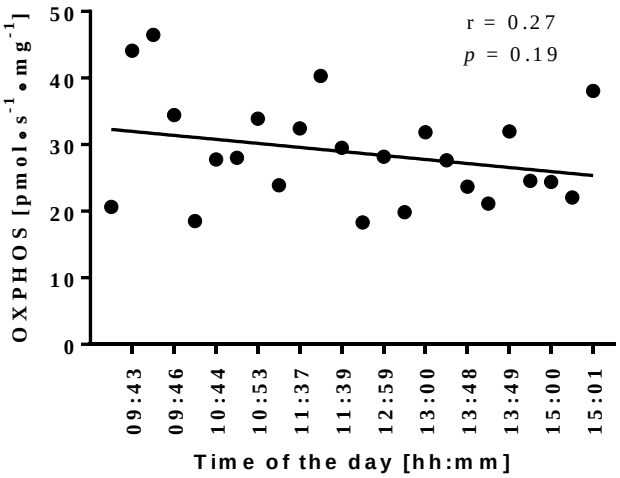

Figure 8. Values obtained from 24 repeated measurements of maximal mitochondrial oxidative phosphorylation (OXPHOS) from fibre bundles from the same sample at various times after collection of the biopsy. Each measurement is represented by a circle and the black continuous line depicts the trend, with the Pearson's product moment correlation coefficient (r) and the relative *p* value also being indicated also being indicated.

4.1.3 Determination of OXPHOS in permeabilized fibres is similar at two different time-points

Another important aspect influencing the OXPHOS reproducibility is the magnitude of the daily OXPHOS variability. Therefore, the variance in OXPHOS in two biopsies per leg obtained from participants who were instructed not to change their physical activity level between two laboratory visits separated by ~4 weeks were assessed. The analysis of variance indicated no main effect of time ($p > 0.05$) or interaction between time and leg ($p > 0.05$). The SEM between the two time points was ~15 pmol · s^{-1} · mg^{-1} for both left and right legs which is slightly higher than the SEM found in repeated OXPHOS measurements from the same biopsy probably due to the effect of subjects' circadian variability and other factors such as nutrition (van Moorsel et al., 2016).

4.1.4 Reproducibility of repeated OXPHOS and $p50_{mito}$ measurements in isolated mitochondria

Contrary to OXPHOS measurements with permeabilized fiber bundles which only require a few milligrams of fresh skeletal muscle tissue, mitochondrial isolation requires about ~30-50 mg of fresh skeletal muscle tissue which is homogenized and centrifuged in a series of defined steps leading to a final mitochondrial pellet. The pellet is then resuspended and diluted in a few microliters buffer specifically designed to preserve mitochondrial function. About 5 µl of the mitochondrial suspension volume is then transferred into the high-resolution respirometer for assessment of the mitochondrial respiratory function. Contrary to the permeabilized fiber technique, the mitochondrial isolation procedure *per se* ensures homogeneity of the sample used for OXPHOS measurements. Therefore, repeated OXPHOS measurements of portions of the same mitochondrial suspension allow assessment of the technical error arising from the high-resolution respirometry apparatus, under the assumption of a negligible influence exerted by the technician in titrating the substrates and the mitochondrial suspension. It was observed an SEM of 15.8 pmol · s^{-1} and a CV of 7.6%. In the same experiments the SEM and CV for repeated $p50_{mito}$ measurements were 0.01 kPa and 12.5%, respectively. Similarly, when OXPHOS was determined in duplicates from the same mitochondrial suspension using a total of 21 participants, it was observed an SEM of 33.0 pmol · s^{-1} and a CV of 10% (figure 9). These results are in line with recent findings investigating the reliability of OXPHOS measurement using yeast cell suspension and isolated mitochondria from mouse soleus muscle (Sahl et al., 2018).

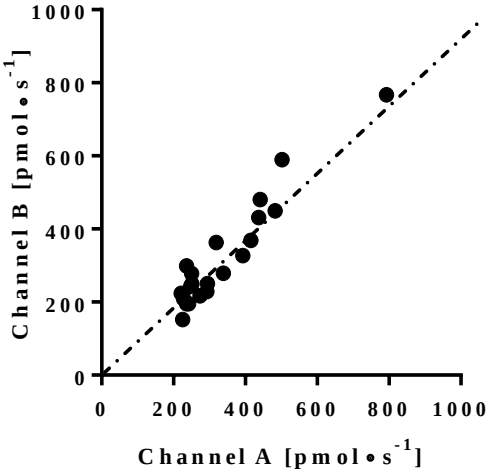

Figure 9: Maximal mitochondrial oxidative phosphorylation (OXPHOS) (pmol · s^{-1}) in isolated mitochondria measured simultaneously in two different chambers (A and B) of a high-resolution respirometer (n = 21). Each circle represents one individual and the dotted line shows the line of identity.

4.2 Paper II: Muscle mass and inspired oxygen influence oxygen extraction at maximal exercise: Role of mitochondrial oxygen affinity

Despite decades of research indicating that O_2 delivery is a key factor determining VO_2max, the regulation of VO_2 and the functional role of mitochondrial properties such as the OXPHOS excess capacity over O_2 delivery (Boushel et al., 2011) and mitochondrial oxygen affinity ($p50_{mito}$) (Gnaiger et al., 1995b) are not well understood. VO_2 at the muscle level can be calculated as $VO_2 = [OXPHOS \cdot pO_2)/(pO_2 + p50_{mito})]$. Consequently, VO_2 can be influenced by mitochondrial oxygen affinity ($p50_{mito}$) even when other variables in the equation are unchanged. I.e. $p50_{mito}$ may play a role in regulating VO_2max.

When applying the Fick law of diffusion [$VO_2 = DO_2 \cdot (Pcap-Pmito)$], previous studies have considered mitochondrial oxygen affinity so high (low $p50_{mito}$) that the role of $p50_{mito}$ could be neglected and the muscle pO_2 sufficiently high (~0.3 kPa) to maintain maximal mitochondrial respiration (Gayeski and Honig, 1988;Wagner, 1993;Richardson et al., 1995b). However the relationship between pO_2 and mitochondrial respiration in isolated mitochondria indicates that at a cellular pO_2 of ~0.3kPa which has been measured in muscle, respiration would be reduced by about ~15% of its maximum (Gnaiger, 2003).

Therefore, the functional role of the OXPHOS excess capacity could be to preserve high rates of mitochondrial respiration *in vivo*. However *in vivo* data proving the validity of this role is not existent. In addition, contrary to a $p50_{mito}$ set to zero, members of our research group have discovered that $p50_{mito}$ in human skeletal muscle varies between individuals (Larsen et al., 2011b) and that $p50_{mito}$ is influenced by the relative activation of mitochondria by ADP (Boushel et al., 2015b). Therefore, in paper II, the possible role exerted by mitochondrial properties in regulating VO_2 was evaluated by examining the Fick components together with mitochondrial O_2 affinity ($p50_{mito}$) in relation to O_2 extraction and O_2 uptake in trained individuals during two-legged cycling (BIKE) and one-legged knee extension (KE) exercise while breathing normoxia (NORM) or hyperoxia (HYPER). The exercise mode was chosen to recreate two distinct levels of *in vivo* mitochondrial activation by altering the active muscle mass during exercise. Additionally, two minor changes in mitochondrial activation were obtained by altering inspired O_2 fraction to influence oxygen limitation. Skeletal muscle samples were collected from the same individuals to assess mitochondrial respiratory properties such as OXPHOS and *ex vivo* $p50_{mito}$.

4.2.1 *Ex vivo* mitochondrial activation is linked to $p50_{mito}$

In line with previous findings (Gnaiger et al., 1995a;Gnaiger et al., 1998) $p50_{mito}$ measured *ex vivo* (i.e. in vitro) was related to the rate of mitochondrial respiration (figure 10). By varying substrate titration feeding complex I only or both complex I and II (i.e. OXPHOS) it was possible to stimulate ATP coupled mitochondria respiration at ~50 and 100% of its maximum. This increment in mitochondrial respiration resulted in a significantly higher $p50_{mito}$ (from 0.06±0.02 to 0.17±0.04 kPa; $p<0.05$). This clearly demonstrates that $p50_{mito}$ increases (i.e. O_2 affinity is reduced) as mitochondrial respiration approaches O_2 flux rates close to their maximum capacity.

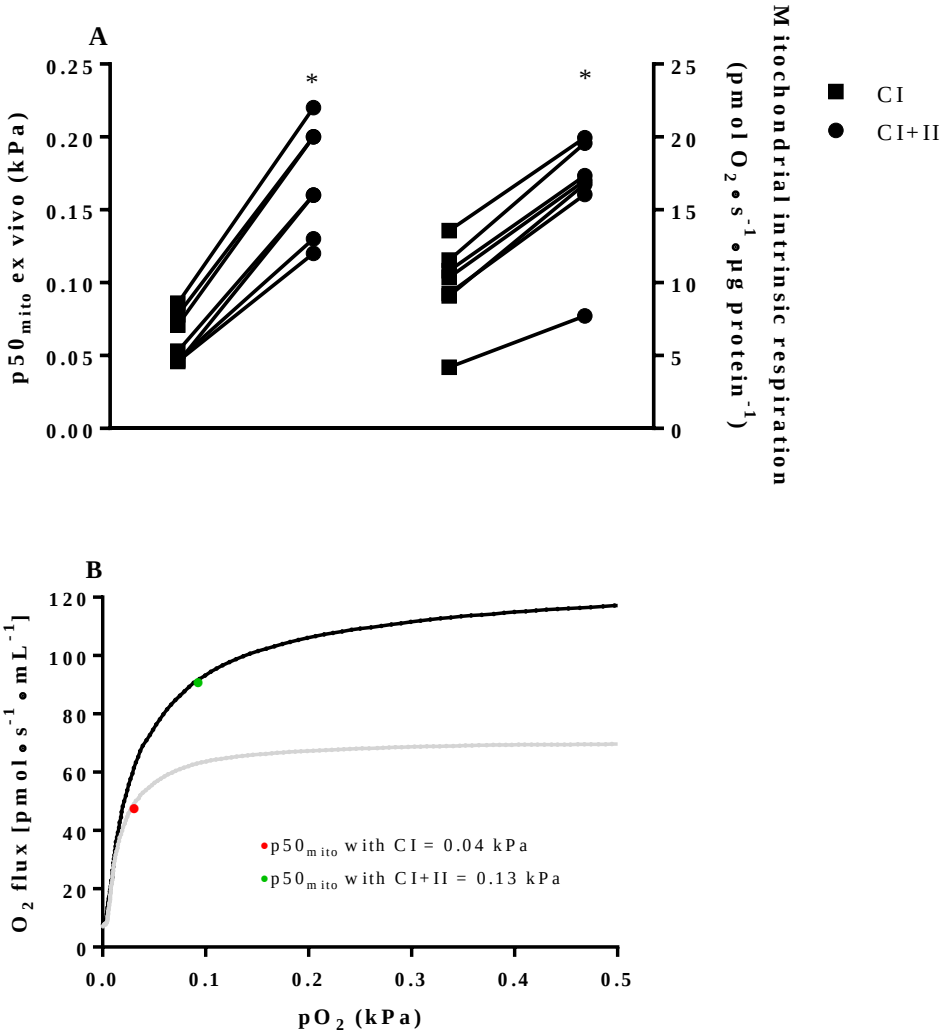

Figure 10. **A**: Mean±SD of *ex vivo* $p50_{mito}$ (kPa) and mitochondrial intrinsic respiration (pmol O_2 · s^{-1} · µg $protein^{-1}$) at maximal ADP-induced activation measured in isolated mitochondria when supplying complex I (CI) substrates (titrating pyruvate and malate) and complex I and II (CI+II) substrates (pyruvate, malate and succinate). Individual points are represented by filled circles and squares. * $p< 0.05$ between CI and CI+II. **B**: Relationship between pO_2 (kPa) and O_2 flux [pmol · s^{-1} · mL^{-1}] for a single subject with two levels of mitochondrial O_2 flux rate. In grey the data obtained when mitochondrial respiration is sustained by complex I (i.e. titrating pyruvate and malate), and in black when activating complex I and II (i.e. titrating pyruvate, malate and succinate). The curves are obtained by plotting the raw data exported from DatLab software. These figures indicate that $p50_{mito}$ is increased (i.e. O_2 affinity is reduced) as the mitochondria approaches O_2 flux rates close to their maximum capacity. Differences in the shape of the curves were observed corresponding to relative activation of mitochondria. Of note is that when mitochondria respire submaximally

and $p50_{mito}$ is lower, mitochondrial respiration is less sensitive to changes in PO_2 in the low oxygen range.

4.2.2 Increasing mass specific O_2 delivery decreases O_2 extraction and muscle diffusion capacity

The reproducibility of previous findings showing the interdependence of $p50_{mito}$ and mitochondrial O_2 flux rate in an *ex vivo* model (Gnaiger et al., 1998) does not prove the validity of these findings *in vivo*. Therefore, trained individuals performed BIKE and KE exercise to recreate two distinct levels of *in vivo* mitochondrial activation by altering the active muscle mass during exercise. The leg and thigh muscle mass was determined by magnetic resonance imaging. In line with previous findings (Andersen, 1985a), hemodynamic data showed that mass-specific VO_2 in KE was 1.7-fold higher than in BIKE due to a higher mass-specific blood flow (~2-fold) which was accompanied with an increase in capillary pO_2 (+22%), a reduction in O_2 diffusion (~2-fold) and a lower O_2 extraction (-23%) than during BIKE (figure 11 and table 1). The significantly higher mass-specific *in vivo* VO_2 in KE *vs*. BIKE indicates that mitochondria respire sub-maximally (~60% of its maximum) at exercise intensities approaching VO_2max during BIKE. Assuming that the mitochondrial respiratory rate *in vivo* follows the same hyperbolic decline with decreasing *ex vivo* pO_2 (figure 10), these results imply that *in vivo* $p50_{mito}$ would be substantially lower (higher affinity) at VO_2max during BIKE than during KE, resulting in higher muscle diffusion capacity, and in turn a higher O_2 extraction. These results provide the first evidence for an underlying link between a higher mitochondrial activation, a higher $p50_{mito}$ and lower O_2 extraction.

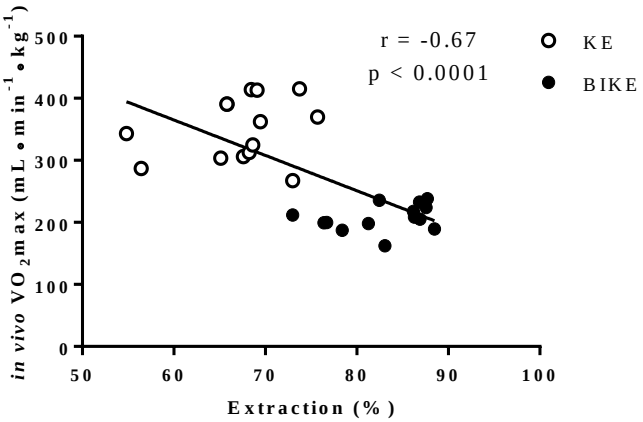

Figure 11. Relationship between oxygen extraction (X axis) at VO_2 peak exercise (Y axis) for two-legged cycling (BIKE; filled dots) and one-legged knee extension (KE; empty dots). The Pearson's product moment correlation coefficient (r) and the *p* value are also indicated.

Table 1. Hemodynamic data

	BIKE NORM	BIKE HYPER	KE NORM	KE HYPER
Leg O_2 delivery ($ml \cdot min^{-1} \cdot kg^{-1}$)	238.8±21.3†	263.2±25.4*†	497.0±82.8	547.4±64.9*
Leg blood flow ($ml \cdot min^{-1} \cdot kg^{-1}$)	1234.9±137.9†	1311.2±157.1*†	2746.7±626.5	2868.7±539.4*
Leg VO_2 ($ml \cdot min^{-1} \cdot kg^{-1}$)	197.7±19.3†	217.9±18.8*†	333.9±56.7	365.8±41.5*
v pO_2 (mmHg)	13.6±2.4†	13.9±4.1†	20.7±2.9	21.6±2.9
Extraction (%)	82.9±4.4†	83.0±6.0†	67.4±6.2	67.1±5.9
PaO_2 (mmHg)	101.9±9.1†	140.5±17.4*	112.6±17.5	139.3±26.9
CaO_2 ($ml \cdot min^{-1}$)	19.4±1.2†	20.2±1.7*	18.4±1.8	19.4±2.7
MDO_2 ($ml \cdot min^{-1} \cdot mmHg^{-1}$)	31.8±8.6†	29.5±9.2†	16.5±2.6	16.0±4.0
pO_2cap (mmHg)	44.4±4.9†	54.5±8.5*	54.1±5.1	62.9±7.8
pO_2tissue (kPa)	0.30±0.03†	0.37±0.06*	0.37±0.03	0.43±0.05

Values are means±SD during normoxia (NORM) and hyperoxia (HYPER) in two-legged cycling (BIKE) and one-legged knee extension exercise (KE); VO_2, O_2 consumption; v pO_2, venous PO_2; CaO_2, arterial O_2 content; MDO_2, 1-leg muscle O_2 diffusion capacity; PO_2cap, mean muscle capillary PO_2; PO_2tissue, mean muscle PO_2; * $p < 0.05$ between conditions i.e. NORM and HYPER in BIKE and KE; † $p < 0.05$ between work modality i.e. BIKE and KE in NORM and HYPER.

4.2.3 The decreased O_2 extraction when increasing O_2 delivery is determined by the relationship between mitochondrial capacity and O_2 delivery, not by higher blood flow and reduced transit time

Opponents of a cause/effect relationship between mitochondrial activation, $p50_{mito}$ and O_2 extraction may argue that the reduction in the erythrocyte mean transit-time caused by the higher blood flow in KE is the basis for the 23% lower O_2 extraction in KE compared to BIKE. In contrast to this notion, no correlation (r=0.08, p=0.86) between the increase in blood flow and the decrease in O_2 extraction was found in the same participant when moving from BIKE to KE (figure 12 panel a). Instead, we found a strong, significant correlation (r=0.74, p<0.0001) between the O_2 extraction in BIKE and KE and the mitochondria excess capacity over O_2 delivery (figure 12 panel b). These results reinforce the importance of having a high OXPHOS capacity relative to O_2 delivery by maintaining high O_2 extraction by a reduction in $p50_{mito}$.

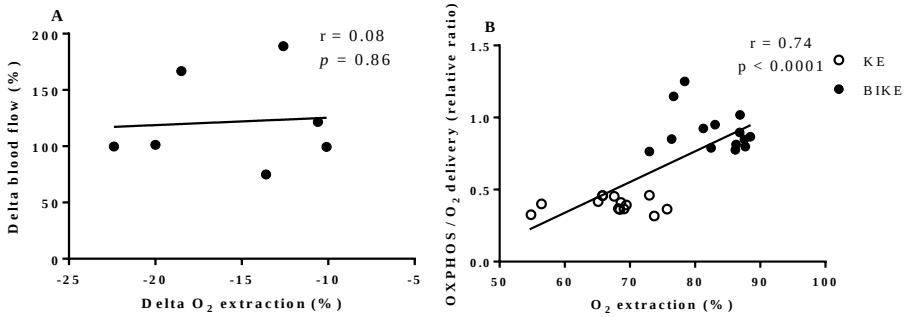

Figure 12. **A**: Relationship between delta O_2 extraction (X axis) and delta blood flow (Y axis) at peak exercise for two-legged cycling (BIKE) and one-legged knee extension (KE). Delta was calculated between BIKE and KE in NORM. **B**: Relationship between oxygen extraction (X axis) and OXPHOS excess capacity (Y axis) in BIKE (filled dots) and KE (empty dots), showing the importance of a high OXPHOS excess capacity compared to O_2 delivery for maintaining a high O_2 extraction. OXPHOS excess capacity was calculated as: [(saponin-permeabilized maximal mitochondrial respiration · lower limb muscle mass) / (2 · peak oxygen delivery measured over the leg during BIKE)]. The whole leg muscle mass including gluteus muscles mass was used to calculate the OXPHOS excess capacity. The Pearson's product moment correlation coefficient (r) and p value are also indicated. These figures indicate that the lower O_2 extraction seen in KE compared to BIKE is not a function of blood flow but that the magnitude of mitochondrial excess capacity over maximal O_2 delivery controls O_2 extraction.

4.2.4 Comparing $p50_{mito}$ *ex vivo* with $p50_{mito}$ *in vivo* reveals full mitochondrial activation in the KE HYPER exercise model and the effect of *in vivo* $p50_{mito}$ on O_2 extraction

It could be argued that the proposed synergetic effect of OXPHOS excess capacity and $p50_{mito}$ in regulating O_2 extraction is based only on indirect evidence and not on experimentally measured $p50_{mito}$ *in vivo*. Therefore, we estimated the *in vivo* $p50_{mito}$ values at peak exercise during BIKE and KE in NORM and HYPER. These calculations are possible by the integration of Fick data, previous findings and some reasonable assumptions by modifying equation 5 in figure 1 i.e. $p50_{mito}$= (O_2 delivery · pO_2)/(Leg VO_2)-pO_2.

The underlying assumption for the calculation of *in vivo* $p50_{mito}$ are that 1) leg VO_2 represents the sum of mitochondrial VO_2, 2) when mitochondrial OXPHOS is in excess of the leg VO_2, OXPHOS can be substituted for O_2 delivery i.e. leg VO_2 = (O_2 delivery · pO_2)/(pO_2+$p50_{mito}$), and 3) the group mean tissue pO_2 in BIKE NORM is equal to 0.3 kPa (Richardson et al., 1995b) at maximal exercise intensity and 4) the change in mean capillary pO_2 was reflected by a corresponding proportional change in the muscle pO_2 when going from NORM to HYPER and from BIKE to KE (Richardson et al., 1995a). In other words, it was assumed that an increase in mean capillary O_2 pressure by, for example 30%, was directly reflected by an increased tissue pO_2 by 30%.

It was shown that the calculated values of *in-vivo* $p50_{mito}$ were lowest in BIKE and significantly increased in KE reaching similar values to the ones observed *ex vivo* or above in the case of KE exercise in HYPER (figure 13). Consistently, HYPER increased the *in-vivo* $p50_{mito}$ in both BIKE and KE partially limiting the potential increase in VO_2 produced by the increased O_2 delivery. Furthermore, the *in-vivo* $p50_{mito}$ was inversely related to mitochondrial excess capacity (figure 14), and the change in *in-vivo* $p50_{mito}$ when shifting from BIKE to KE was related to the change observed in O_2 extraction (figure 15).

All together, these results indicated that in BIKE, where OXPHOS capacity is in excess of O_2 delivery, *in-vivo* $p50_{mito}$ is kept low to maximize O_2 extraction. On the other end of the mitochondrial activation spectrum, OXPHOS capacity is nearly fully utilized during KE thus increasing *in-vivo* $p50_{mito}$ and compromising O_2 extraction. Thus, these results show the importance of having a high mitochondrial OXPHOS which keeps $p50_{mito}$ low, to increase VO_2 by improving O_2 extraction. These conclusions are supported by Rud et al. (2012) who elegantly showed that trained individuals following one-legged knee extension training improved muscle VO_2 only in the exercised leg as a result of improved O_2 extraction, higher blood flow, and a 30% higher mitochondrial content. Furthermore, in the study by Rud and co-authors the increased blood flow in the exercised

leg did not compromise O_2 extraction which further supports the importance of mitochondrial capacity and $p50_{mito}$ for a high O_2 extraction at a reduced mean transit time (Andersen and Saltin, 1985).

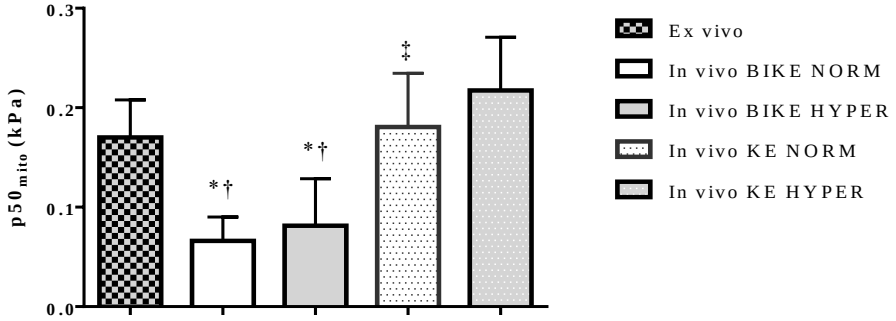

Figure 13. Mean ± SD of *ex-vivo* and *in-vivo* $p50_{mito}$ (kPa) at peak exercise for two-legged cycling (BIKE) and one-legged knee extension (KE) in normoxia (NORM) and hyperoxia (HYPER). * $p<$ 0.05 between *ex-vivo* and *in-vivo*; † $p<0.05$ between BIKE and KE; ‡ $p<0.05$ between NORM and HYPER. *In vivo* $p50_{mito}$ is lowest in BIKE NORM and increased with increasing O_2 delivery until it reaches similar values to those values measured *ex vivo*. This reveals how mitochondrial activation approaches its upper limit in the KE modality, especially under HYPER. Note that the calculated *in vivo* $p50_{mito}$ in KE HYPER is higher than the *ex vivo* $p50_{mito}$ obtained with fully saturated ADP concentration. This would indicate a) less than full activation of mitochondrial capacity in KE NORM, b) less than full activation of mitochondrial capacity when measured *ex vivo*.

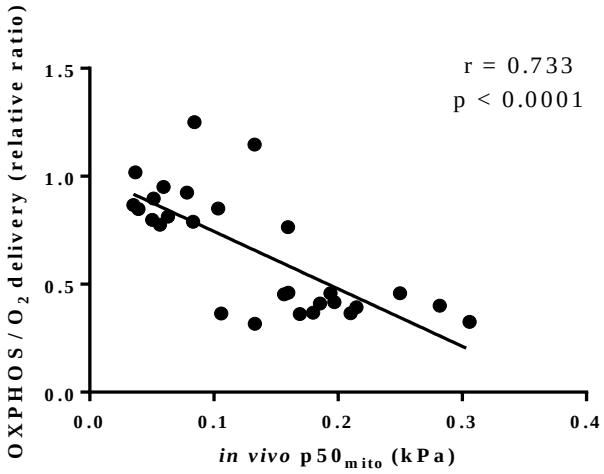

Figure 14. Relationship between *in-vivo* $p50_{mito}$ and OXPHOS capacity showing the importance of a high OXPHOS excess capacity relative to O_2 delivery for maintaining a low in-vivo $p50_{mito}$. OXPHOS excess capacity was calculated as: [(saponin-permeabilized maximal mitochondrial respiration · lower limb muscle mass) / (2 · peak oxygen delivery measured over the leg during BIKE)].

In vivo $p50_{mito}$ was calculated with the equation *in-vivo* $p50_{mito} = O_2$ delivery $\cdot$ pO_2 / (VO_2-pO_2). The Pearson's product moment correlation coefficient (r) and the relative *p* value is also indicated.

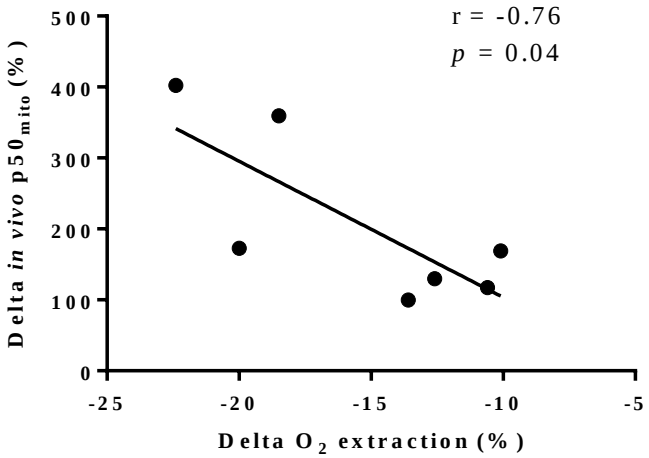

Figure 15. Relationship between delta oxygen extraction (X axis) and delta *in vivo* $p50_{mito}$ (Y axis) when shifting from BIKE and KE in NORM. *In vivo* $p50_{mito}$ was calculated with the equation $p50_{mito} = O_2$ delivery $\cdot$ pO_2 / (VO_2-pO_2). The Pearson's product moment correlation coefficient (r) and the relative *p* value is also indicated.

4.2.5 Regulation of $p50_{mito}$ *in vivo* and *ex vivo*

The concordance of findings showing $p50_{mito}$ being related to mitochondrial respiration rate *ex vivo* and *in vivo* does not imply that mechanisms regulating $p50_{mito}$ are similar in the two conditions. *Ex vivo* $p50_{mito}$ was assessed resembling physiological tissue pO_2 in high-resolution respirometry using isolated mitochondria in the presence of both substrates and ADP saturated concentrations during an aerobic-anoxic transition. In this setup, the relative activation of complex IV, the enzyme where O_2 is bound and reduced to water (Belevich et al., 2006) determines *ex vivo* $p50_{mito}$. The lower the activation of complex IV, the lower is the *ex vivo* $p50_{mito}$ and vice versa. Importantly, saturated concentrations of substrates and ADP as well as optimal pO_2 in the high-resolution respirometer chamber do not drive complex IV activity to its kinetic limit. Therefore, complex IV capacity was measured by titrating the electron donor N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD) to complex IV, held constant by concomitant titration of ascorbate in a redox reaction. The measured O_2 flux was then adjusted for chemical auto-oxidation by measuring the O_2 flux obtained in the presence of sodium azide which inhibits the activity of complex IV. In this study complex I mitochondrial respiration led to a ~40% activity of complex IV capacity whereas complex I and II linked respiration utilized ~60% of complex IV capacity. The complex IV excess capacity relative to the capacity

of the integrated respiratory system keeps mitochondrial oxygen affinity high and permits the preservation of mitochondrial respiration even in case of competitive inhibition of complex IV by cyanide or nitric oxide (Gnaiger et al., 1998;Larsen et al., 2011a). Linking results from *in vitro* to the whole-body level, recent findings have shown that complex IV has important thermodynamic properties. High protein levels of complex IV isoform IV-2 have been positively associated with high *ex vivo* $p50_{mito}$, lowered resting metabolic rate and high mitochondrial efficiency (Schiffer et al., 2016). Furthermore, high *ex vivo* $p50_{mito}$ is associated with better exercise efficiency (Larsen et al., 2011b).

In contrast to the factors influencing *ex vivo*, in the *in vivo* model $p50_{mito}$, mitochondria may be exposed to less than saturating concentrations of both substrates, ADP and O_2. At a submaximal exercise level, mitochondrial activation is determined primarily by the ADP/ATP ratio and substrate availability but at exercise intensities near to maximum, the blood flow and O_2 delivery to the working muscles become the limiting factors of mitochondrial respiration e.g. exercise during which a large muscle mass is engaged. I.e. during maximal cycling the observed mitochondrial activation is dependent on O_2 delivery since mitochondrial respiration possesses an excess capacity over O_2 delivery when exercising with large muscle mass. In this study the *in vivo* kinetic limit of complex IV was not measured. However, the higher *in vivo* $p50_{mito}$ found during KE compared to BIKE (figure 13), indicated that complex IV activation was higher in KE than in BIKE (at maximum exercise intensity). Thus, the lower the activation of mitochondria in BIKE, the lower the $p50_{mito}$ is and the less sensitive mitochondria are to changes in pO_2 in the low O_2 range (figure 10) since low $p50_{mito}$ results in less decline in mitochondrial VO_2 per unit change in pO_2. In line with this concept it was shown that OXPHOS excess capacity was related to *in vivo* $p50_{mito}$ (figure 14) and that both OXPHOS excess capacity and *in vivo* $p50_{mito}$ were related to O_2 extraction (figure 12 and 15).

4.2.6 Breathing hyperoxic gas increases O_2 delivery to a similar extent regardless of the muscle mass involved with no effect on muscle diffusion capacity and O_2 extraction

The maximum VO_2 achieved in BIKE and KE was increased by ~10% when breathing hyperoxia. These results are in line with previous findings on endurance-trained subjects where VO_2 at peak KE exercise was increased by ~17% (Richardson et al., 1999a), and in cycling by ~8% (Knight et al., 1996;Cardús et al., 1998). The increased VO_2 found in HYPER was explained by the ~10% increase in O_2 delivery, which in turn was the result of a similar increase in O_2 arterial content and quadriceps blood flow with HYPER compared to NORM regardless of exercise modality. Moreover, HYPER did not change O_2 extraction and O_2 diffusion. The increased VO_2 and *in vivo* $p50_{mito}$ in KE induced by HYPER revealed that mitochondria excess capacity was not yet fully utilized in KE

NORM in these trained participants despite the small muscle mass involved. These results are in line with findings showing that mitochondrial excess capacity was already fully utilized in untrained individuals exercising with small muscle mass such as in KE (Pedersen et al., 1999) in contrast to submaximal activation of mitochondria in trained individuals (Gifford et al., 2015). Another observation was that HYPER increased the capillary-to-myocyte pO_2 gradient mainly by increasing mean capillary O_2 pressure with an unchanged muscle O_2 diffusion. Interestingly, the high muscle mass-specific VO_2 in KE was achieved at ~half of the muscle O_2 diffusion capacity found in BIKE (muscle O_2 diffusion BIKE 31.8±8.6 ml and KE 16.5±2.6 ml $\cdot$ min^{-1} $\cdot$ mmHg^{-1}) indicating that muscle O_2 diffusion capacity did not restrain KE VO_2 to increase.

4.3 Paper III: Superior intrinsic mitochondrial respiration in women than in men

Despite the clear sexual dimorphism present in humans resulting from a differential genetic expression (Rigby and Kulathinal, 2015), with direct impact on life expectancy (Seifarth et al., 2012), disease occurrence and aging (Popkov et al., 2015;Tower, 2017), women are significantly less studied in medical research (Costello et al., 2014). Moreover, most of the findings proven in men are usually assumed to be transferable to women despite women presumably having been exposed to higher evolutionary pressure than men (Della Torre and Maggi, 2017). To date, several sex differences in skeletal muscle metabolism (Roepstorff et al., 2002;Kiens et al., 2004;Roepstorff et al., 2005;Roepstorff et al., 2006) and differential adaptations to exercise training (Scalzo et al., 2014;Skelly et al., 2017) have been found. Despite animal studies having consistently shown superior mitochondrial function in females compared to males (e.g. higher oxidative capacity, lower ROS production, higher fatty acid utilization, lower ADP-stimulated respiration in females compared to males) (Ventura-Clapier et al., 2017), data from humans are limited to few studies (Thompson et al., 2013;Miotto et al., 2018;Montero et al., 2018) with mixed outcomes. In paper III of this thesis, women's' mass-specific and intrinsic mitochondrial respiratory function (i.e. mitochondrial quality) as well as $p50_{mito}$ were compared to men with similar (i.e. VO_2max ~50 mL O_2 min^{-1} $\cdot$ kg^{-1}) and higher fitness levels (i.e. VO_2max ~67 mL O_2 min^{-1} $\cdot$ kg^{-1}).

4.3.1 Intrinsic but not mass-specific mitochondrial respiration differs between men and women with similar VO_2max

Using isolated mitochondria preparation from *vastus lateralis* muscle biopsies, it was found that women's intrinsic mitochondrial respiration (i.e. O_2 flux per mitochondrial protein), a measurement of mitochondria quality, with complex I alone or linked complex I and II respiration, was higher in women than in men with a similar fitness level ($p<0.05$, fig. 16C, 16D). Of note, women's mitochondrial quality was similar to trained men with ~32% higher VO_2max. Furthermore, when complex II was activated by the addition of succinate, respiration increased more in women than in men when respiration was only supported by complex I (figure 16J). This would indicate a functional difference of specific proteins of the electron transfer system between women and men. This is the first data showing a superior intrinsic mitochondrial respiration in relatively fit women (i.e. VO_2max ~50 mL O_2 $min^{-1} \cdot kg^{-1}$) compared to men of similar fitness. It can be speculated that the higher intrinsic mitochondrial respiration shown in women is a strategy that compensates the blunted cardiovascular adaptations to one year of endurance training reported in women compared to men (Howden et al., 2015). Alternatively, the metabolic and mechanic stress caused by endurance training is more favorable in inducing peripheral than central adaptations in women compared to men. The higher intrinsic mitochondrial respiration shown in women contrasts with previous findings showing no difference between men and women in intrinsic mitochondrial respiratory function assessed in permeabilized fibre preparations from gastrocnemius muscle (Thompson et al., 2013). However, in Thompson´s study men and women were not matched for fitness level and all subjects had endothelial dysfunction; two major limitations that presumably influenced the study outcomes.

The total skeletal muscle mitochondrial oxidative capacity coupled to ATP resynthesis is mostly dependent on the total mitochondrial content, its quality and the coupling control ratio when substrate and O_2 availability is not limited. Therefore, the higher intrinsic mitochondrial respiration found in women compared to men with similar fitness could have potentially been balanced out by these other factors. To test this idea, mitochondrial respiration rate was first normalized per initial wet weight of the sample from mitochondria which were isolated. This allowed for the comparison of O_2 flux per unit muscle instead of per mitochondrial protein as in the case of intrinsic mitochondrial respiration.

It was found that mass-specific mitochondrial respiration did not differ between women and men with similar VO_2max and as expected O_2 flux per unit muscle was lower in women compared to trained men ($p<0.05$, fig. 16H, 16I). Thus, these findings indicate that the women had lower mitochondrial content than men of similar fitness level and than the trained men.

These results are in line with two recent studies using the permeabilized fiber technique showing that women possess similar maximal oxidative phosphorylation capacity per unit muscle mass (Miotto et al., 2018;Montero et al., 2018), but lower ADP sensitivity and different substrate sensitivity independent of maximal oxidative phosphorylation and mitochondrial content or protein level compared to men. The other important factor influencing total mitochondrial oxidative capacity is how coupled the mitochondrial respiration is to the ATP synthesis. In muscle tissue, this is mainly regulated by uncoupling protein-3 (Gong et al., 1997) and adenine nucleotide translocator (ANT) protein activity (Andreyev et al., 1989;Azzu et al., 2008) which, by increasing proton leak, dissipates energy as heat and affects ATP production. Leak respiration accounts for a substantial part of the differences in basal metabolic rate between species (Porter and Brand, 1993;Rolfe and Brown, 1997). Considering the lower basal metabolic rate per unit muscle found in women compared to men (Benedict and Emmes, 1915) it can be expected that women would have lower leak respiration. OXPHOS control ratio was determined as the ratio between OXPHOS and leak respiration (mitochondrial respiration in absence of adenylates). Despite the leak respiration and state 4 respiration, a proxy for passive proton leak over the inner mitochondrial membrane, being significantly higher in women compared to men ($p<0.05$, figure 16A, 16B), the OXPHOS control ratio was similar between women and men of similar fitness level and was higher in trained men ($p<0.05$, figure 16E).

Altogether, these results would indicate that women and men with similar VO_2max possess similar skeletal muscle mitochondrial oxidative capacity per unit muscle which is achieved differentially. In women this may have been achieved by a higher mitochondrial quality, lower mitochondrial content and higher proton leak whereas in men a similar O_2 flux rate may have been obtained by a higher mitochondrial content, lower mitochondrial quality and lower proton leak. An equally plausible interpretation of these data is that women do not need to upregulate mitochondrial content due to the inherently higher intrinsic mitochondrial respiration compared to men with similar fitness level.

The higher mass-specific mitochondrial respiration in trained men compared to women indicates that trained men had a higher mitochondrial content than women and men with lower fitness level.

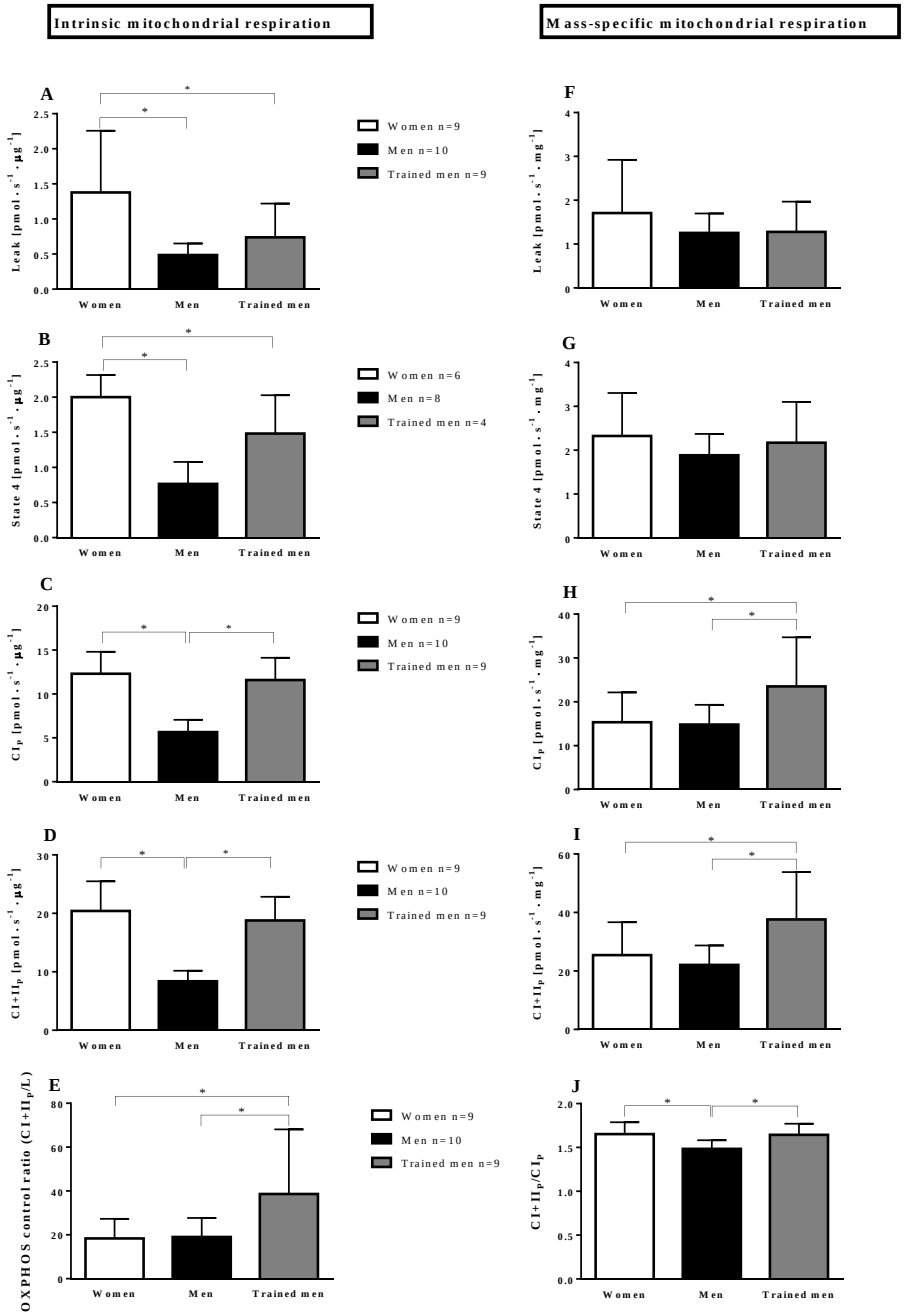

Figure 16. Mean ± SD of mitochondrial respiration rates normalized by protein levels of mitochondrial suspension (pmol · s^{-1}· µg $protein^{-1}$; intrinsic mitochondrial oxidative respiration) and by the initial muscle wet weight (pmol · s^{-1}· mg^{-1}; mass-specific mitochondrial respiration) in women, men and trained men groups. **A** and **F**, leak respiration is the respiratory rate in the presence of substrates without addition of adenylates; **B** and **G**, state 4 is the respiratory rate when ADP is

phosphorylated maximally to ATP; **C** and **H**, complex I_P is the maximum ADP stimulated respiration rate in the presence of complex I substrates; **D** and **I**, complex $I+II_P$ is similar to **C**, **H** but with added convergent electron flux through complex II by adding succinate ($CI+II_P$); **E**, maximal oxidative phosphorylation capacity (OXPHOS) control ratio ($CI+II_P$/Leak) indicates the limitation of OXPHOS by the phosphorylation system; **J**, $CI+II_P/CI_P$ indicates the contribution of CII respiration to maximal respiration. Note: * = $p<0.05$ between groups.

4.3.2 Mitochondrial quality in women: possible implication in muscle metabolism and performance

The higher intrinsic mitochondrial respiration in women compared to men with similar fitness level could be an important strategy to permit a higher O_2 extraction peripherally by a reduced mitochondrial activation (Cardinale et al., 2018) to counteract the more centrally-mediated limitations of a higher work of breathing (Dominelli et al., 2013;Dominelli et al., 2017) and lower O_2 carrying capacity of blood (Murphy, 2014). The higher intrinsic mitochondrial respiration would also improve fat oxidation which is often (Tarnopolsky et al., 1990;Roepstorff et al., 2006) but not always (Zehnder et al., 2005) reported to be higher in women compared to men during exercise at a given work rate. In line with this notion, it has been reported that transcription and translation of proteins involved in muscle lipid metabolism is sex dependent (Kiens et al., 2004) and women possess a higher number of intramyocellular lipid droplets compared to men (Tarnopolsky et al., 2007). The higher intrinsic mitochondrial respiration would potentially allow a higher fat oxidation presumably by the same mechanisms observed when mitochondrial content is increased (Gollnick and Saltin, 1982). The concept of the higher mitochondrial quality for a higher fat oxidation in women could be mediated by an upregulation AMP-activated protein kinase (AMPK). AMPK has been implicated in regulation of fatty acid uptake, handling, and oxidation (Thomson and Winder, 2009;O'Neill et al., 2013) and has been shown to activate mitochondrial biogenesis via direct phosphorylation of peroxisome proliferator activated receptor c co-activator-1a (Norrbom et al., 2011) (the transcriptional regulator of genes involved in oxidative metabolism). However, no sex differences have been found in resting AMPK, while a lower AMPK activation following exercise has been reported in women compared to men (Roepstorff et al., 2006), indicating that AMPK signaling may not the be key regulator of fat oxidation during prolonged exercise in women and not differentially regulating intrinsic mitochondrial respiration in women and men. An argument that goes against the potential superior skeletal muscle metabolic capacity of women attained by the higher mitochondrial quality is that it would be expected that women would perform better in endurance and in ultra-endurance events compared to men (Gollnick and Saltin, 1982). On the contrary, the performance gap between women and men in endurance events ranging between 1500 meters

to marathon is ~11% (Sparling et al., 1998) and rises to ~20% in ultra-events (mainly running and cycling events) (Knechtle et al., 2014;Zingg et al., 2014) with the only exception of swimming events where women perform equally or better compared to men (Knechtle et al., 2015), presumably due to the increased buoyancy in women attributed to the caudally located higher percentage of fat mass (McLean and Hinrichs, 1998). Therefore, these compelling data indicate that the superior intrinsic mitochondrial respiration in women compared to men does not lead to better endurance performance. However, when pulling together the three groups, the maximal power output pedaled during an incremental cycling test was significantly correlated to intrinsic and mass-specific mitochondrial respiratory function and even better correlated to VO_2max (figure 17).

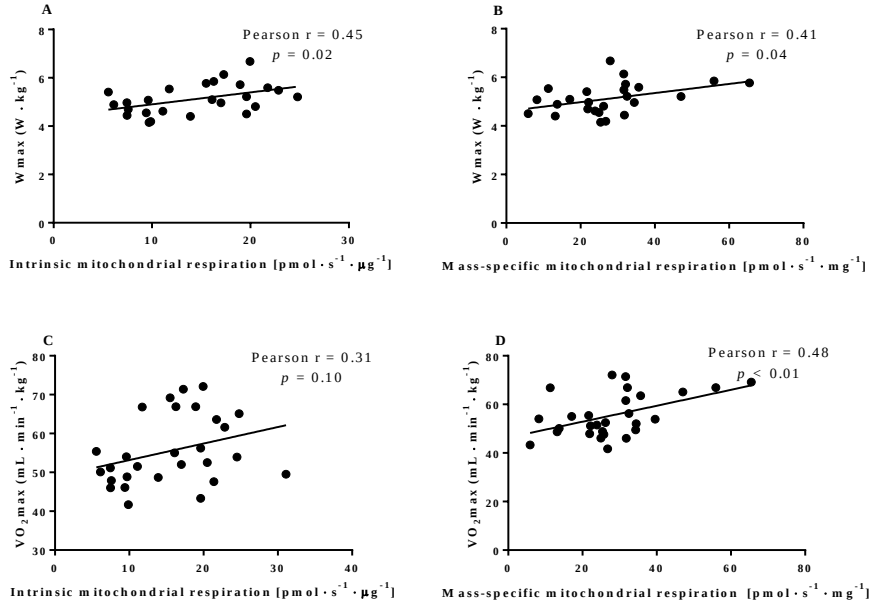

Figure 17. Correlations between the maximal mitochondrial oxidative phosphorylation (State 3 complex I+II$_P$), maximal cycling power output (Wmax) achieved on a gradual incremental exercise test and maximal oxygen consumption (VO_2max) for the combined group of subjects. Maximal mitochondrial oxidative phosphorylation was normalized for mitochondrial protein content (intrinsic mitochondrial respiration; panel A and B) and for initial wet weight (mass-specific mitochondrial respiration; figure panel C and D).

4.3.3 The role of the high skeletal mitochondrial oxidative capacity and high $p50_{mito}$ in regulating oxygen consumption in women compared to men

In paper II, it was demonstrated that both OXPHOS excess capacity and $p50_{mito}$ synergistically regulate O_2 extraction thus affecting muscle oxygen consumption since a)

higher excess capacity would reduce mitochondrial activation and b) at a physiological intracellular pO_2, a lower $p50_{mito}$ (high mitochondrial O_2 affinity) would result in a higher tissue O_2 consumption [$VO_2 = OXPHOS \cdot pO_2 \cdot (pO_2 + p50_{mito})^{-1}$] (Cardinale et al., 2018). Considering that women have a lower basal metabolic rate per unit muscle (Benedict and Emmes, 1915) and lower exercise energy expenditure (Åstrand PO, 2003;Fares et al., 2017), and in light of recent findings showing that high $p50_{mito}$ is associated with a low basal metabolic rate (Schiffer et al., 2016) and high aerobic efficiency during exercise (Larsen et al., 2011a), a higher $p50_{mito}$ in women compared to men of similar fitness level was expected. As hypothesized, the $p50_{mito}$ with complex I substrates (0.10±0.05 kPa) and with complex I+II substrates (0.22±0.07 kPa) was significantly higher ($p< 0.05$) in women than in men with similar VO_2max ($p50_{mito}$ with CI_P = 0.04±0.01 kPa, $p50_{mito}$ with $CI+II_P$ = 0.07±0.02 kPa), and also higher compared to trained men with the higher VO_2max ($p50_{mito}$ with CI_P = 0.05±0.02 kPa, $p50_{mito}$ with $CI+II_P$ = 0.12±0.03 kPa) (figure 18A and figure 18B). The physiological significance of the higher $p50_{mito}$ found in women compared to men needs to be carefully interpreted since it could be misinterpreted as indicative of a higher mitochondrial activation which consequently could reduce O_2 extraction *in vivo* as previously shown with an impact of VO_2 (Cardinale et al., 2018). The higher intrinsic mitochondrial respiration could possibly increase VO_2, however the higher $p50_{mito}$ could negatively influence extraction of O_2. Thus, the impact of this sex difference on whole body VO_2 is unknown.

4.3.4 Factors influencing mitochondrial quality

Given the key role played by mitochondria in healthy and diseased populations (Nunnari and Suomalainen, 2012) the identification of factors able to upregulate mitochondrial quality is important. A denser mitochondrial cristae density (Hoppeler et al., 1973), as shown in rodent muscle undergoing several weeks of exercise training (Gollnick and King, 1969) (Gollnick and King, 1969), may be a factor determining mitochondrial quality. Data on mitochondrial density in human are limited to few studies with small sample sizes (Hoppeler et al., 1973;Hoppeler and Lindstedt, 1985;Hoppeler, 1986;Hoppeler and Fluck, 2003;Larsen et al., 2012) that have supported the notion that mitochondrial cristae density is constant in humans (Hoppeler and Fluck, 2003). However, recent findings indicated that mitochondrial cristae density became denser following long-term exercise training and can discriminate active individuals from elite athletes (Nielsen et al., 2016).

It is proposed that endurance exercise induces mitochondrial biogenesis and leads to the development of new mitochondria which at the initial stage become enlarged, followed by an increase in length (Glancy et al., 2015;Lundby and Jacobs, 2015), and lastly an increase in mitochondrial cristae density, since further increases in mitochondrial content would impair muscle contractile function (Nielsen et al., 2016).

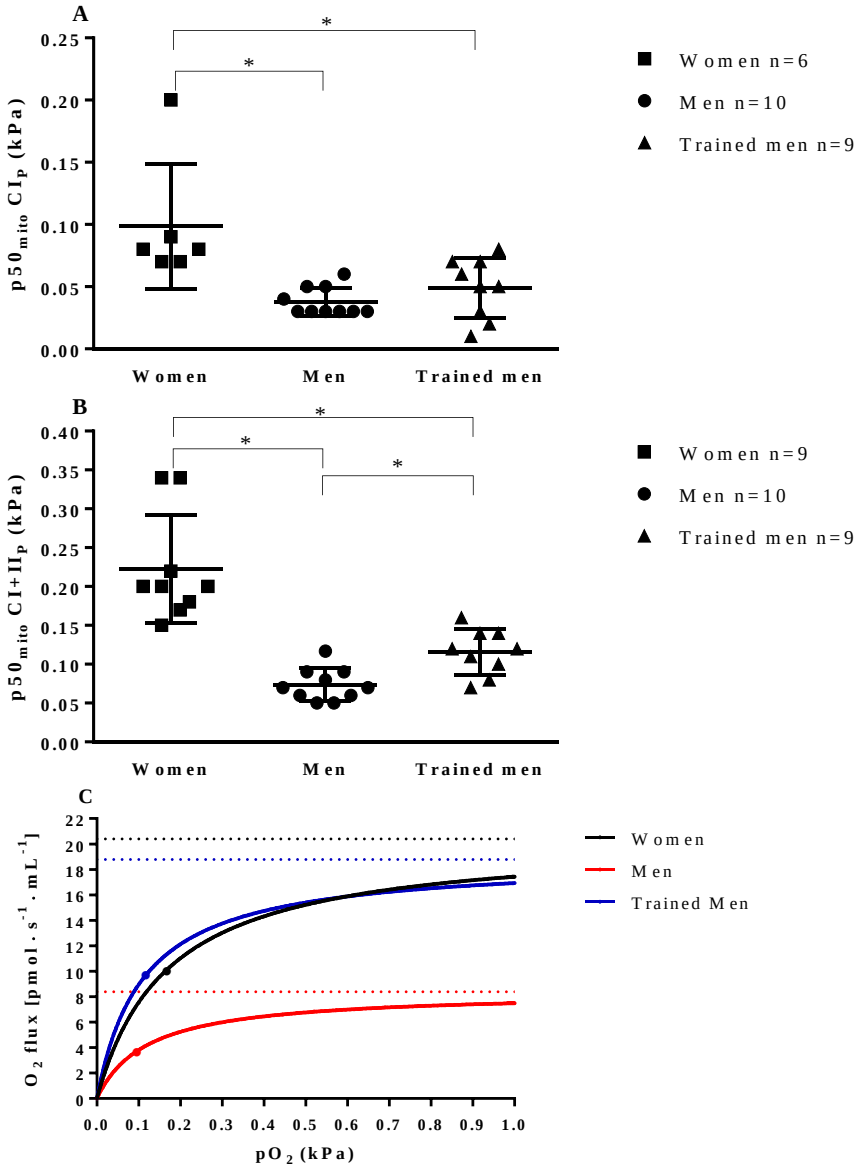

Figure 18 A: Individual and mean ± SD values of *ex vivo* mitochondrial p50 ($p50_{mito}$; kPa) at maximal ADP-induced activation measured in isolated mitochondria when activating complex I (CI_P) for women, men and trained men groups **B**: Individual and mean ± SD values of *ex vivo* $p50_{mito}$ as shown in A, but with complex I+II-linked substrate state ($CI+II_P$) for women, men and trained men groups. Note: * = $p<0.05$ between groups. **C**: Hyperbolic curves showing the relation between pO_2 (kPa) and mitochondrial O_2 flux [pmol · s^{-1} · mL^{-1}]. The curves were obtained by the following equation [$VO_2 = (CI+II_P \cdot pO_2) \cdot (pO_2 + p50_{mito})^{-1}$] using continuous pO_2, the *ex vivo* $p50_{mito}$ (graphically indicated with a dot) and $CI+II_P$ (graphically indicated with a dashed lines) mean values

measured in this study in women, men and trained men groups. This figure shows the role of mitochondrial $p50_{mito}$ for muscle oxygen consumption since for a given intracellular pO_2, a lower $p50_{mito}$ would result in a higher tissue O_2 consumption [$VO_2 = CI+II_P \cdot pO_2 \cdot (pO_2 + p50_{mito})^{-1}$] and vice versa. The superior intrinsic mitochondrial respiration in women compared to men with similar VO_2max shown in this study may be an important physiological adaptation that compensates for the higher mitochondrial p50 allowing a higher O_2 extraction peripherally.

Interestingly in Nielsen´s study cross country-skiers with the highest VO_2max had similar mitochondrial cristae density and higher mitochondrial volume than soccer players with lower VO_2max. These results would indicate that soccer players could theoretically further increase in mitochondrial volume without any impairment of the muscle contractile properties. To picture this concept, one could parallel this physiologic adaptation to modern urbanization and the increasing population density in an urban area. For a city (mitochondria) to grow, it is initially easier to urbanize previous rural areas (mitochondrial biogenesis and elongation of previous existing mitochondria). However, when the urbanization process is reaching the upper limit, the population density of a city grows (increased mitochondrial cristae density). Importantly, it is rarely seen that a city has a high-density population (high mitochondrial cristae density) and a great unexploited urbanization potential (low mitochondrial volume). In this view it is unlikely that mitochondrial cristae density become denser in fibres where mitochondrial reticulum is not yet maximized. With this background, it is tempting to speculate that the superior intrinsic mitochondrial respiration observed in women compared to men of a similar fitness level is explained by a higher mitochondrial cristae density in women compared to men.

A second plausible factor that could explain at least in part the higher mitochondrial quality found in women is a higher abundance of supercomplexes and respirasomes (Schagger and Pfeiffer, 2000;Lobo-Jarne and Ugalde, 2018) in women than in men. Growing evidence has shown that the structure of these multi-protein aggregates is optimized to increase the mitochondrial catalytic efficiency (Bianchi et al., 2004) and that supercomplex abundance is upregulated by exercise training in young and old individuals (Greggio et al., 2016). However, since no differences in supercomplex abundance between women and men have been reported, this remains speculative.

4.3.5 Mitochondrial quality: born or made?

The superior intrinsic mitochondrial respiration in women compared to men raises the important question of whether this difference is a true sex difference as a consequence of evolutionary pressure to which the two sexes have been subjected to (Della Torre and Maggi, 2017) or if it is the result of environmental factors such as the response to exercise training or other factors such as nutrition and lifestyle.

Despite the ~50-year accumulated knowledge in studying the extraordinary plasticity shown by skeletal muscle mitochondria in response to repeated endurance-based exercise stimuli (Holloszy, 1967;Morgan et al., 1971), not much is known about mitochondrial quality in relation to specific exercise-induced responses (Bartlett et al., 2017). The common finding following endurance-based exercise training is an increase in mass-specific mitochondrial respiratory capacity which is paralleled by an increased mitochondrial content (Holloszy, 1967). Thus, mitochondrial respiration per content has traditionally been conceptualized to remain unchanged following exercise training (Granata et al., 2016;MacInnis et al., 2016).

Both high-volume training at moderate intensity continuous exercise (MICT) and low-volume high-intensity interval training (HIIT) or sprint interval training (SIT) (Orlander et al., 1977;Daussin et al., 2008;Granata et al., 2015;Vincent et al., 2015) are strategies capable of improving mitochondrial content and oxidative capacity. However, cross-sectional data indicate that exercise intensity is a key factor to stimulate higher mitochondrial respiration whereas training volume is linked to change in mitochondrial content (i.e. higher CS activity) (Bishop et al., 2014;Vigelsø et al., 2014;Granata et al., 2018). Along these lines, SIT has a lower impact on mitochondrial content, produces the largest increase in mass-specific mitochondrial respiration per exercise unit time (both including and excluding rest period in a training session) and has so far been able to increase intrinsic mitochondrial respiratory function compared to HIIT and MICT (Granata et al., 2015).

It appears that the extreme metabolic demand of SIT training induces adaptations within the mitochondria itself as opposed to an increase in mitochondrial content. The SIT stimuli on skeletal muscle mitochondria is so powerful that impaired mitochondrial respiratory function has been reported, as a consequence of aconitase inhibition, when SIT volume is presumably overdosed in untrained individuals (Larsen et al., 2015). With this background it cannot be excluded that the intrinsic mitochondrial respiration in women compared to men with similar VO_2max is the result of different exercise-induced training adaptations.

Another interesting topic of discussion is the possible effect of elevating O_2 delivery to the working muscle on mitochondrial quality. In the above discussed effect of SIT on mitochondrial quality, it must be noted that mitochondrial respiratory capacity outstrips the capacity of cardiac output for delivering O_2 to the working muscle. The ~100-fold rise of skeletal muscle metabolic demand at the initiation of a sprint bout forces mitochondria to adapt to this enormous challenge. Another possible means of inducing a higher mitochondrial quality is to elevate O_2 delivery to the working muscle performing exercises engaging small mass. In support for this argument, it has been shown that mitochondria respire close to their maximal capacity when the exercised muscle is highly perfused such as in the case of one-legged knee exercise (Blomstrand et al., 2011;Cardinale et al., 2018).

Furthermore, peripheral adaptations of skeletal muscle following one-legged cycling are greatly enhanced compared to double-legged cycling where lower O_2 delivery per active muscle mass occurs (Abbiss et al., 2011). Generally, women possess higher percentual body fat than men and therefore it can be speculated that the superior intrinsic mitochondrial respiration in women compared to men with similar VO_2max reported here was the result of a chronic exposure of higher O_2 delivery per lean muscle mass in women than in men. Unfortunately, body composition was not systematically measured in this study and it was therefore not possible to scale VO_2max to lean muscle mass.

If we assume body fat values of 20%, 18% and 12% in women, VO_2max matched men and trained men respectively, women with a VO_2max of 51.0±4.1 mL O_2 $min^{-1} \cdot kg^{-1}$ had a slightly higher oxidative capacity per kg lean mass than men with similar VO_2max when accounting for their higher percentage of body fat. This hypothesis would favor the idea of the higher mass-specific O_2 delivery to increase mitochondrial quality. However, comparing women to trained men would presumably lead to a rejection of this hypothesis. Trained men would have ~76 ml O_2 per kg lean muscle mass (~12% fat mass and a VO_2max of ~67 mL O_2 $min^{-1} \cdot kg^{-1}$) versus ~62 ml O_2 per kg lean muscle mass in women (~18% fat mass and a VO_2max of ~51 mL O_2 $min^{-1} \cdot kg^{-1}$).

Therefore, since women and trained men exhibited similar mitochondrial quality, the women would need to possess ~33% body fat in order to be matched for O_2 delivery per lean muscle mass of trained men (~76 ml O_2 per kg lean muscle mass) which is inconsistent with the lean and active group of women recruited in this study. This indicates that O_2 delivery per unit muscle mass does not explain the difference in mitochondrial quality observed in women and men recruited in this study. This is consistent with the differences in Wmax between groups (figure 19).

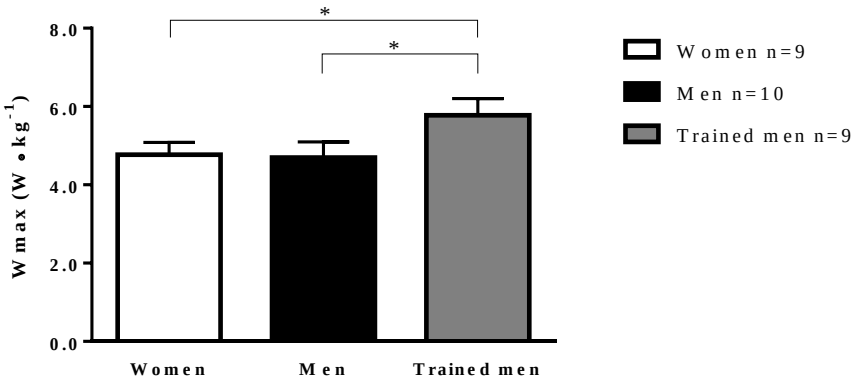

Figure 19. Mean ± SD values of maximal cycling power output (Wmax; $W \cdot kg^{-1}$) achieved on a gradual incremental exercise test for women, men and trained men groups. Note: * = p<0.05 between groups.

4.4 Paper IV: Influence of hyperoxic-supplemented high-intensity interval training on training adaptations in trained cyclists

It is debatable whether greater exercise-induced adaptations are achieved if a system is challenged by the shortage of for example a needed compound or by pushing a system over its maximum by facilitating or ergogenically supplying what the system lacks. Subsequently, it is intriguing to compare hyperoxic versus normoxic exercise-induced adaptations since breathing hyperoxia would partially release the constraint imposed by a limited O_2 delivery at exercise intensities near to maximum (Saltin and Calbet, 2006b), increase mitochondrial activation, as shown in paper II, (Cardinale et al., 2018) and enable higher power output and endurance compared to normoxia (Powers et al., 1989;Nielsen et al., 1999b).

Previous studies on hyperoxic-supplemented endurance training (Ploutz-Snyder et al., 1996;Hamalainen et al., 2000;Morris et al., 2000;Perry et al., 2005;Perry et al., 2007;Kilding et al., 2012) have shown an overall 'likely positive' effect on performance compared to normoxic training (Cardinale and Ekblom, 2017;Mallette et al., 2017). However the few studies that have attempted to identify the possible mechanisms of exercise adaptations are inconclusive (Ploutz-Snyder et al., 1996;Perry et al., 2007;Przyklenk et al., 2017) and too few studies have been conducted on trained individuals (Hamalainen et al., 2000;Morris et al., 2000;Kilding et al., 2012) to demonstrate the clear efficacy of this training method in already trained individuals.

Therefore, to investigate the influence of hyperoxic-supplemented HIIT on training adaptations, trained cyclists (i.e. n= 23, VO_2max ~4.5 L·min^{-1} and 5.3 W·kg^{-1}) performed 6 weeks of polarized and periodized endurance training on a cycle ergometer consisting of supervised HIIT sessions 3 days/week and additional low-intensity training 2 days/week. Participants were randomly assigned to either HYPER (F_IO_2 0.30, n=12) or NORM (F_IO_2 0.21, n=11) breathing conditions during HIIT. Mitochondrial respiration in permeabilized fibres and isolated mitochondria together with maximal and submaximal VO_2, hematological parameters and endurance cycle performance were tested pre and post training intervention. It was expected that this group of athletes would show only a small magnitude of change in performance, cardiorespiratory fitness and metabolic adaptation even when intensifying their training, thus this setup would isolate the effect induced by breathing hyperoxia during HIIT.

4.4.1 Exercise Intensity, Training Load

It was found that HYPER consistently trained at a 3.3±1.2% numerically but significantly higher relative intensity than NORM despite reporting a similar rate of perceived exertion (i.e. NORM RPE 8.3±1.0 and HYPER RPE 8.2±0.7) during the HIIT sessions. However, this low percentual difference in exercise intensity did not lead to a significantly higher training load over the intervention in HYPER compared to NORM ($p = 0.37$) (figure 20). The higher relative training intensity enabled by breathing hyperoxia is a common characteristic described in the literature (Welch, 1982;Cardinale and Ekblom, 2017), although a larger change in exercise intensity between hyperoxic and control group has been reported in other studies (Perry et al., 2005;Perry et al., 2007;Kilding et al., 2012). This could be explained by the lower F_IO_2 (i.e. ~0.30) during exercise used in this study since previous reports have shown a linear relationship between time to exhaustion and F_IO_2 (F_IO_2 between 0.40 and 0.1) (Wilson and Welch, 1975).

There are several possible underlining mechanisms for the lower perceived exertion and the higher relative power output during HIIT observed in the HYPER group compared to NORM. It has been shown that breathing hyperoxia prevents the fall in arterial oxygen saturation often found in endurance athletes when approaching maximal exercise intensity (Dempsey et al., 1984;Nielsen, 2003). This condition, as described in the background section, termed exercise induced arterial hypoxemia (EIAH) is linked to the alveolar-capillary diffusion limitation due to decreased Hb mean-transit time in the lung (Dempsey and Wagner, 1999) caused by mechanical ventilatory limitation during exercise (Dominelli et al., 2013) and its incidence is about 50% in endurance trained athletes (Powers et al., 1988). In line with previous findings, 57% of the cyclists exhibited EIAH at intensities near to maximum; 7 of the 11 cyclists in NORM, and 6 of the 12 cyclists in HYPER. Thus breathing hyperoxia increased arterial O_2 content and O_2 delivery to the working muscle independently of blood flow (Ekblom et al., 1975) and could consequently explain the higher exercise intensity observed at lower relative effort in the HYPER group compared to NORM (Nielsen et al., 1999a).

Hyperoxia could also be involved in the modulation of mechanisms involved in fatigue (Amann et al., 2006). It has been shown that transient local hypoxia in conjunction with muscle metabolite accumulation during high intensity exercise inhibits α-motor unit activation via discharge of group 3 and group 4 muscle afferents, which are transmitted by metaboreceptor and nociceptor signaling (Kaufman et al., 1984), causing a reduction in motor cortex activation. In this context, hyperoxia attenuates muscle free ADP and AMP, reduces PCr utilization and decreases arterial epinephrine concentrations with no changes in pyruvate accumulation and oxidation (Stellingwerff et al., 2006). In addition, muscle and blood lactate production/efflux is reduced (Ekblom et al., 1975;Hogan et al., 1983;Plet et al., 1992); consequently the reduction in peripheral fatigue keeps α-motor

units activated during high intensity exercise (Amann et al., 2006) as shown by the enhanced muscle activation (Tucker et al., 2007), which contradicts what has been shown in hypoxia conditions (Kayser et al., 1994) during high intensity exercise. Another plausible explanation for the lower perceived exertion and higher relative power output during HIIT observed in the HYPER group compared to NORM is that cerebral oxygenation is reduced during high intensity exercise (Santos-Concejero et al., 2017) despite having higher priority than O_2 supply to the working muscle (Curtelin et al., 2017), and is found to be preserved by hyperoxia at maximal exercise (Nielsen et al., 1999a;Oussaidene et al., 2013). In summary, the increased O_2 delivery by preventing EIAH, the reduced stress within the muscle itself during high intensity exercise together with a maintained cerebral oxygenation are factors that could have contributed independently or acted synergistically to improve performance acutely in the hyperoxia condition.

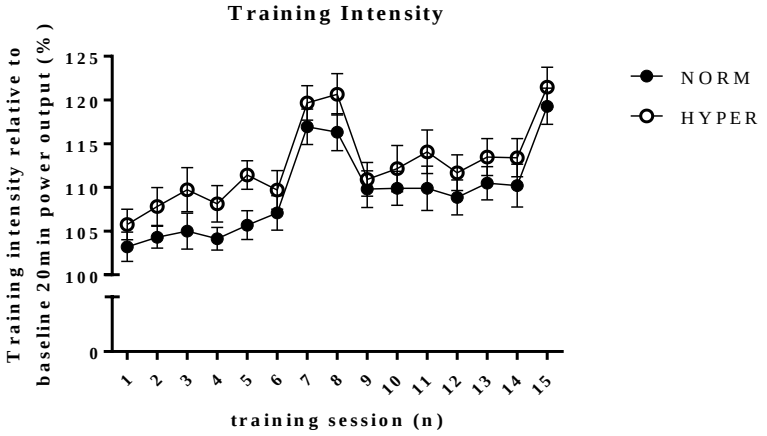

Figure 20. Overview of the training intensity and progression of the normoxic (NORM) and hyperoxic (HYPER) group during the 6-week training intervention. Training intensity of each high-intensity interval session is expressed in percentage of the mean power output obtained during the baseline self-paced 20-minute cycling trial. Note that the higher training intensity during training session number 7, 8 and 15 is attributed to the higher exercise intensity obtained when performing 4x4 minutes intervals compared to the 3x8 minute intervals performed in the other sessions. This figure shows a) the numerically but not significantly higher training intensity performed by HYPER compared to NORM throughout the training intervention which was independent of the interval duration, b) the increasingly higher training intensity performed by both groups during the intervention period which indicated that cycle performance was enhanced in both groups independent of hyperoxia.

4.4.2 Skeletal Muscle Mitochondrial Adaptations

Considering that mitochondrial oxidative capacity outstrips the O_2 delivery capacity (Boushel et al., 2011) and since hyperoxia is expected to increased O_2 delivery to the working muscle (Ekblom et al., 1975), especially at exercise near to maximum preventing EIAH (Dempsey et al., 1984), it was hypothesized that the acute hyperoxic-induced increase in O_2 delivery would augment mitochondrial activation of the working muscle as discussed in paper II (Cardinale et al., 2018); thus, leading to a superior skeletal muscle mitochondrial adaptation compared to normoxia. To test this hypothesis mitochondrial respiratory function was assessed in permeabilized fibres and isolated mitochondria preparation. Contrary to what was expected, no difference was seen between the groups in the effect of the overall training intervention on mass-specific mitochondrial leak respiration, fatty acid oxidation, maximal oxidative phosphorylation respiratory, or electron transfer system capacity when assessed in permeabilized fibres from the *vastus lateralis* muscle with (figure 21A). Overall, OXPHOS numerically increased (22.6±46.1%) following the intervention but did not reach the level of significance (p = 0.20), and was not different between (p = 0.37, ES= -0.06) or within groups (NORM 16.5±49.1% p = 0.86; HYPER 27.3±46.0%, p = 0.15). In line with these findings intrinsic mitochondrial function assessed in isolated mitochondria showed only an increase of 21.0±75.1% (p = 0.90) over the intervention with no change between (p = 0.66, ES= -0.57) or within groups (NORM 15.9±73.3% p = 0.99; HYPER 26.1±80.1%, p = 0.54) (figure 21B).

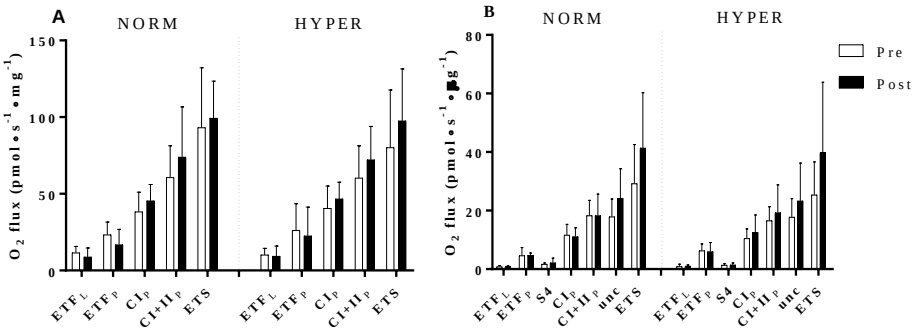

Figure 21. Mitochondrial respiration pre and post 6-wks polarized and periodized high-intensity interval training either in normoxia (NORM) or hyperoxic-supplemented (HYPER). All values are expressed as means ± SD. In figure **A** O_2 flux rates obtained from measurement of mitochondrial respiration using permeabilized fibres technique are expressed in pmol · s^{-1} · mg^{-1} fibres initial wet weight, whereas in figure **B** O_2 flux obtained from measurement of isolated mitochondria the O_2 flux rates are presented in pmol · s^{-1} · μg^{-1} protein. Electron-transferring flavoprotein complex (ETF), state 4 (S4), complex I (CI), complex I+II-linked substrate state (CI+II), uncoupling state (Unc), electron transport system capacity (ETS). Leak respiration and oxidative phosphorylation are indicated by subscripts L and P respectively.

The total oxidative capacity of the skeletal muscle is determined by the oxidative capacity of each mitochondrion (intrinsic mitochondrial respiratory function) and the total mitochondrial content. The unchanged intrinsic and mass-specific mitochondrial respiration would indicate that no effect of mitochondrial content occurred over the intervention. Accordingly, CS activity, a validated biomarker of mitochondrial content (Larsen et al., 2012), was found to be unchanged over the intervention (p = 0.42) and did not differ between groups (p = 0.41) or within groups. Previous studies have reported an increased CS activity with no difference between hyperoxia and normoxia treatment following cycling exercise (Ploutz-Snyder et al., 1996;Perry et al., 2007). However, 4-weeks of one-legged exercise was not able to induce in an increase in CS activity with no difference between hyperoxia, normoxia or hypoxia conditions (Przyklenk et al., 2017).

The unchanged CS activity could simply indicate that the training intervention induced a training volume which was not sufficiently high to stimulate mitochondrial adaptation in this group of trained cyclists. This assertion is supported by findings by Granata et al. (2018) showing a positive correlation between training volume and CS activity with no plateau in CS activity expected to occur if training volume is constantly increased (Granata et al., 2018). Since the cyclists aimed to achieve maximal effort at each HIIT session which increased exercise intensity as clearly shown in figure 20, two other variables could have been manipulated: the length of the HIIT session and the weekly training frequency. However, it would have been logistically problematic to extend the training session by adding one or two extra intervals, since cyclists performed the training intervention in groups, cycling in the afternoon and on a limited number of cycle ergometers. Furthermore, an increment in training load by adding a fourth HIIT session per week would possibly have caused a high dropout rate. Unquestionably, participants with a lower fitness level could have been recruited and the training intervention would possibly have induced larger skeletal muscle training adaptations. However, such a group would also have exhibited large cardiorespiratory adaptations over the intervention which would have blunted the possible effect induced by hyperoxia.

Another possible explanation for the unchanged mitochondrial content observed pre to post intervention was that this group of trained cyclists already possessed a high mitochondrial content, as indicated by the high CS activity [increase in NORM from 238.7±53.2 to 239.3±39.5 nM $\cdot$ min$^{-1}\cdot$ mg^{-1} (p = 0.97) and in HYPER from 239.4±37.3 to 221.9±48.6 nM $\cdot$ min$^{-1}\cdot$ mg^{-1} (p = 0.28)] prior to the intervention and that mitochondrial content could not be further augmented. This idea is supported by evidence indicating that mitochondrial content cannot have an unlimited upregulation since it would impair myocyte contractile function (Nielsen et al., 2016), and that a plateau in mitochondrial volume could have already occurred after a few weeks endurance training (Montero and Lundby, 2017). In line with the recent study by Przyklenk et al. (2017), immunoblotting

analysis of complex IV protein levels indicated no change pre to post intervention (p = 0.93) or between groups (p = 0.95). However, by titrating artificial electron donors to complex IV, i.e. titrating TMPD, complex IV activity was found to be increased pre to post intervention (p = 0.03) with no difference between HYPER and NORM groups when measured *ex vivo* in isolated mitochondria preparation. The overall upregulation of complex IV activity pre to post intervention would indicate an even larger complex IV capacity over the integrated respiratory system which is expected to lower $p50_{mito}$ and positively affect submaximal and maximal O_2 consumption as shown in paper II. Therefore, it is tempting to speculate that the lower mitochondrial activation at a submaximal level could improve fat oxidation (Gollnick and Saltin, 1982). Previous studies have suggested an increased fat oxidation during hyperoxia indicated by the lower respiratory exchange ratio (RER) (Welch, 1982). However, gas exchange analysis while breathing hyperoxia is technically difficult to measure and can overestimate VO_2 uptake causing an erroneously lower RER (Welch and Pedersen, 1981;Prieur et al., 1998). Data obtained using phosphorus-31 nuclear magnetic resonance spectroscopy showed that hyperoxia reduces the ATP cost of a dynamic low intensity contraction by a reduction of energy demand during contraction indicated by lower EMG amplitude per Watt and lower oxidative phosphorylation (Layec et al., 2015). Furthermore, there is no clear evidence supporting the increased fat oxidation induced by breathing hyperoxia when assessing metabolites in skeletal muscle biopsies. Breathing hyperoxia (F_IO_2 0.60) during continuous cycling at 70% of VO_2max has been reported to reduce glycogenolysis with no effect on pyruvate production and oxidation (Stellingwerff et al., 2005). This would indicate that the lower blood lactate found when breathing hyperoxia compared to normoxia is not a proof of a shift towards higher fat oxidation but simply that less pyruvate is available for conversion to lactate. Indeed, breathing hyperoxia has been shown to reduce lactate efflux with no effect on lactate clearance (Stellingwerff et al., 2006). Many factors may influence the net fat oxidation by regulation of fatty acid availability, transport and oxidation into the mitochondria (Purdom et al., 2018). In this study, fatty acid oxidation capacity measured in permeabilized fibres and isolated mitochondria *ex vivo* was found unchanged pre to post intervention. However, due to methodological limitations, factors such as fatty acid availability, transport into mitochondria, substrate sensitivity and free creatine levels may have changed *in vivo* and influenced this outcome. Nevertheless, if breathing hyperoxia during exercise increases fat oxidation compared to normoxia, it is expected that hyperoxic training lead to enhanced 3-hydroxyacyl-CoA dehydrogenase (HAD) enzyme activity, which is both directly involved in the fat oxidation and transport of fatty acid into the mitochondria. On the contrary, continuous cycling has been shown to increase HAD in normoxia but not in hyperoxia (Ploutz-Snyder et al., 1996), whereas HIIT has been shown to have a similar effect on HAD activity independent of the breathing conditions (Perry et al., 2007).

In conclusion this data does not support the hypothesis that hyperoxia leads to superior skeletal muscle adaptations despite the higher relative exercise intensity and the higher O_2 delivery.

4.4.3 Endurance cycling performance

The 6-week training intervention improved the endurance cycling performance measured with a self-paced 20-minute cycling trial. Mean power output increased pre to post intervention both when expressed as absolute power change (3.7±4.6%; $p < 0.001$) and power change relative to the cyclists' body mass (4.3±4.7%; $p < 0.001$) (figure 22). The magnitude of change in mean power output during the 20-minute cycling trial was larger in HYPER compared to NORM but this difference was not significant (for mean power output $p = 0.07$, ES= 0.22; for mean power output relative to body mass $p = 0.06$, ES= 0.32).

However, the calculated effect size indicated a small beneficial effect on performance in HYPER compared to NORM group. The subsequent within-group analysis showed that NORM only numerically increased absolute mean power output during the 20-minute cycling trial by 1.6±4.3% ($p = 0.23$), whereas HYPER significantly enhanced power output by 5.6±4.2% ($p < 0.001$) (figure 22). Similar differences were observed when mean power during the 20-minute test was normalized per body mass: 2.4±6.0% ($p = 0.15$) in NORM and 6.0±3.7% ($p < 0.001$) in HYPER.

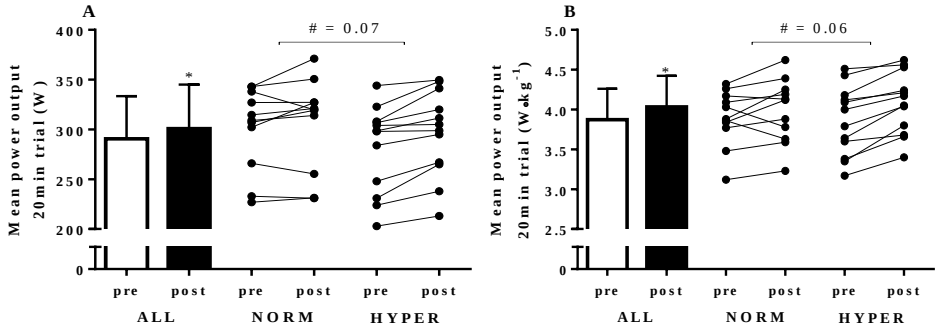

Figure 22. Mean ± SD of the mean power output during the self-paced 20-minute cycling trial pre and post intervention expressed in absolute Watts (a) and Watts relative to body mass (b) for all the participants as well as the individual response of the participants in normoxia (NORM) and hyperoxia (HYPER) groups. Note: * = p value < 0.05, # = p value between groups.

In a separate set of experiments using a subgroup of the same participants recruited in this study, the reliability of the mean power measurement in the 20-minute test was assessed. Despite several difficulties in performing a relatively long self-paced maximal

effort trial, the calculated standard error of the measurement was 8.7 watts which corresponded to 2.9% of the mean group value (figure 23). Therefore, since the change in performance observed in HYPER over the intervention was ~2 fold larger than the standard error of the measurement, these results would indicate that the change in performance in HYPER was detectable and indicates a true improvement in performance, whereas the change in performance in NORM was within the error of the measurement. It is important to not confuse statistical significance and practical significance when interpreting these results. The significance level in this study was arbitrary and conventionally set to an alpha level of 0.05, thus the found *p* values indicates that there is a ~6% probability that these results are by chance and that the alternative hypothesis (hyperoxia is superior in improving performance than normoxia) should be rejected. However, from a practical point of view the observed change in performance is meaningful in sport.

Importantly, findings from paper IV have an ecological validity in sport (Seiler, 2010) since low to moderate continuous training was supplemented during the high intensity exercise sessions in a polarized fashion. This training periodization strategy is a common practice in endurance athletes (Seiler, 2010) and it has been shown to be superior than the traditional block periodization in improving performance in endurance elite athletes (Stöggl and Sperlich, 2014). Furthermore, the 6th week of the training study was designed to gradually reduce the training volume still maintaining the exercise intensity to reduce possible residual fatigue from the HIIT and as a tapering strategy to improve performance during the post-test assessments.

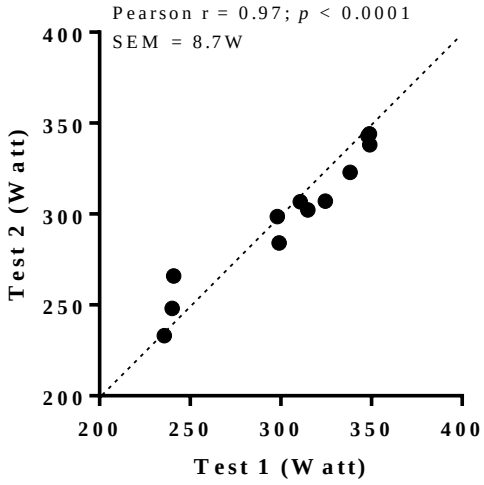

Figure 23. Reliability measurement of the mean power output during the 20-minute self-paced maximal effort cycling trial obtained from two trials performed a week apart by a subgroup of 12 cyclists.

Considering that the lower the SaO_2 during exercise intensity near to maximum is, the larger is the effect of breathing hyperoxia on exercise tolerance (Ohya et al., 2016) and considering that ~50% of all the trained cyclists in both groups showed EIAH during the 20-minute trial test, it can be expected that the performance gain following the training intervention would be larger in those individuals that could exercise with fully saturated Hb and smaller in cyclists exhibiting the largest level of EIAH. In contrast, as depicted in figure 24, no clear relationship was found in any group between SaO_2 and change in power output or when the two groups were pooled together.

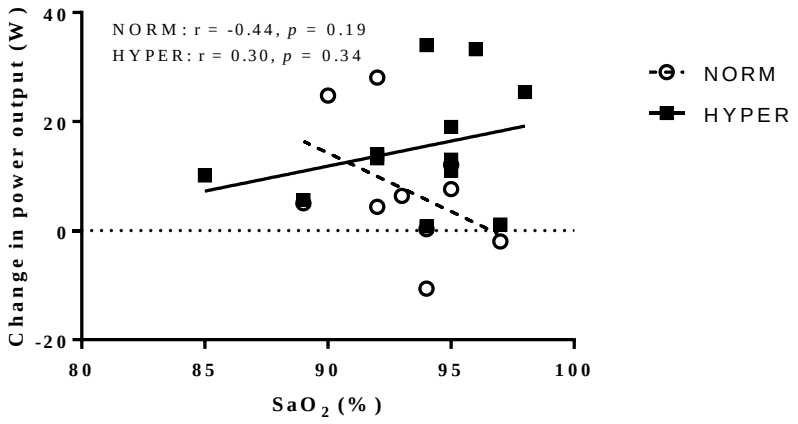

Figure 24. Relationship between the change in power output during the 20-minute self-paced cycling test over the training intervention and the mean arterial oxygen saturation (SaO_2) measured during the same test in participants in normoxia (NORM) and hyperoxia (HYPER) groups. In the HYPER group, it appears that cyclists manifesting the lowest SaO_2 had a lower magnitude of change in performance following the intervention.

Previous studies have shown that hyperoxic-supplemented endurance training is not a superior exercise training strategy in improving physical performance versus breathing normoxia in a randomized controlled trial with parallel groups (Ploutz-Snyder et al., 1996), a single-blinded randomized control trial with parallel groups (Kilding et al., 2012), a single-blind crossover design (Perry et al., 2007) or a non-blinded crossover design (Przyklenk et al., 2017). However, others have shown higher performance enhancement from hyperoxic-supplemented training using a single-blind crossover design (Perry et al., 2005) or a single-blinded randomized controlled trial with parallel groups (Hamalainen et al., 2000) and at high altitude (Morris et al., 2000) when compared to normoxic condition. The differences in study designs, training intervention lengths (3 to 6 weeks), exercise modalities (continuous or high-intensity interval training), fractions of O_2 inspired (F_IO_2 range: 0.26 to 0.70), training status of the participants (i.e. untrained or

trained cyclists) and different measurements of endurance performance (i.e. time to exhaustion vs. time trial) make it difficult to draw parallels between changes in performance observed in paper IV and previous published work.

When designing the current study, a priority was to construct an exercise session that would challenge the participants' cardiorespiratory capacity. Thus, breathing hyperoxia would maintain or improve O_2 delivery and prevent the occurrence of EIAH. Furthermore, with the intent to isolate the experimental factor hyperoxia, only trained cyclists were included in the study since it is expected that this group of athletes would show only a small magnitude of change in performance, cardiorespiratory fitness and metabolic adaptation even when intensifying their training. In contrast, most of the studies conducted to elucidate the effect of breathing hyperoxia were done in untrained participants, using poor performance measurements if any. For instance the untrained participants in the study conducted by Ploutz-Snyder et al. (1996) trained 5 days a week for 5 weeks, cycling for 40 min at 70% of maximum heart rate, breathing an F_IO_2 of either 0.70 or 0.21. At such low exercise intensity, it is doubtful that participants showed any sign of EIAH and that arterial O_2 content differed between groups even using a F_IO_2 of 0.70 in the hyperoxic group. Despite the low exercise intensity, these untrained participants improved their VO_2max by as much as 20%, which is a clear sign of the low fitness level possessed by the participants at baseline. It can be speculated that the training adaptations achievable within a few weeks training period are already maximized in individuals with low-fitness level since they respond so robustly, independent of the type of training intervention. Therefore, hyperoxia cannot further enhance physiological adaptations and performance or the small effect induced by hyperoxia can be blunted and difficult to detect. Another issue is the use of time to exhaustion as an indicator of performance in individuals who are not accustomed to sustaining high relative efforts for an indefinite time. For instance, motivational factors could have influenced the contradictory results reported by Perry et al. (2005);(2007) who found a 2-fold change in time to exhaustion pre to post intervention.

When excluding the studies conducted on untrained individuals, only three studies remain that have reported on the effect of hyperoxic training on performance in well-trained individuals (Hamalainen et al., 2000;Morris et al., 2000;Kilding et al., 2012). The study on runners by Hamalainen et al. (2000) found a significant reduction in 3000 meters running time and maximal anaerobic running velocity after hyperoxic-supplemented training compared to normoxia despite no difference in VO_2max. However, the study of Hamalainen et al. (2000) is only available as an abstract and therefore the results need to be interpreted with caution. The study conducted by Morris et al. (2000) tested the effect of hyperoxic-supplemented HIIT in trained jonior cyclists during an altitude training camp. Therefore, the control group did not perform in normoxia but in a natural high-altitude enviroment. Morris et al. (2000) found that submaximal performance and time

trial performance improved in the hyperoxic group only (4.5%); however, the lack of a normoxic comparison group, precludes a direct comparison with this study. Of note, Morris et al. (2000) did not report statistical differences between groups but only the within group change was stated in the paper. Therefore, it can be assumed that no difference between groups was detected despite a larger magnitude of change in performance exhibited by the hyperoxic group. These results would therefore resemble the results from paper IV.

The most suitable comparison with the literature can presumably be made with the study conducted by Kilding et al. (2012). Trained cyclists alternated between 12 · 2 minutes maximal effort cycling with 2 minutes complete recovery and 5 · 5 minutes cycling with 3 minutes recovery breathing hyperoxic ($F_IO_2 = 0.6$) or room air 2 days · week^{-1} for 4 weeks. Hyperoxia was not found to be a superior training strategy in improving power output at submaximal intensity and time trial performance compared to normoxia in a group of cyclists with similar fitness level to those recruited in paper IV. In agreement with the study by Kilding et al. (2012), the overall training intervention induced only minimal changes in lactate threshold, VO_2max, time trial performance in this group of trained cyclists.

Based on the results reported here, hyperoxic-supplemented HIIT could possibly induce a small positive effect in cycling performance compared to normoxic training, which is not explained by changes in mitochondrial respiratory function, VO_2max, lactate threshold, blood O_2 carrying capacity, and cycling efficiency. Although this small positive effect in performance may be advantageous in elite competition, considering the cost/benefit, unknown health-related effects and ethical issues of performing hyperoxic-supplemented HIIT, it is arguable if the use of this strategy to maximize endurance performance in already trained cyclists is worthwhile.

5. STUDY LIMITATIONS

In paper II, the importance of having a high OXPHOS capacity relative to O_2 delivery for maintaining a high O_2 extraction by a reduction in $p50_{mito}$ in well-trained individuals was shown. These results were obtained by combining *ex vivo* measures of mitochondrial O_2 affinity in isolated mitochondria with direct measures of leg blood flow and VO_2 which were determined by thermodilution and the Fick method. However, the inclusion of only well-trained healthy individuals may have impacted the generalization of the findings to other populations. The possible influence of heterogeneity of muscle recruitment, metabolism and blood flow during maximal exercise was not considered; however, this has been shown to be relatively low. Moreover, contrary to the precise measure of the quadriceps muscle mass by MRI, a good estimation of the muscle mass recruited during cycling is difficult and may have influenced the mass-specific normalization but not the conclusion drawn.

In paper III, when comparing women and men with equal VO_2max it would have been more appropriate to scale VO_2max to lean muscle mass than per kg of body mass. Moreover, the research design did not control for diet between participants and menstrual cycle phase when testing women; thus, a certain variability in the measured outcomes may have been introduced by these factors.

In paper IV, neuromuscular measurements were not included as part of the test battery. Therefore, it cannot be excluded that the higher training intensity induced by hyperoxia increased muscle contractile properties which may in turn have resulted in small improvements in performance compared to normoxia.

6. CONCLUSIONS

In this thesis, the first systematic report on the influence of several methodological factors on the measurements of maximal mitochondrial oxidative phosphorylation in permeabilized fibres from the *vastus lateralis* muscle employing high-resolution respirometry were described. The importance of having the same technician preparing the samples was shown, as was that the major source of variation in OXPHOS measurements is the sample preparation *per se*. Furthermore, other factors such as the possible difference between left and right limbs, two time points of sample collection, fibre bundle weight, time that elapsed after collection of the biopsy and the use of anesthetics all only had a minor impact on the standard error of the measurement. These methodological considerations are central for the design of future studies and for calculating appropriate sample sizes.

The significance of having a mitochondrial OXPHOS capacity that is in excess of the capacity of the central circulation to deliver O_2 to the tissue was shown. Excess capacity of mitochondria allows submaximal mitochondrial activation at maximal O_2 delivery, thereby maintaining a low $p50_{mito}$ and a high peripheral extraction of O_2. Mitochondrial oxygen affinity varied with the degree of mitochondrial respiration rate and was lower in women compared to men. Considering the widespread and increasing sedentary behavior in a society plagued by diseases often linked to mitochondrial dysfunction, these results suggest the importance of preserving a high muscle oxidative capacity throughout life, which can be of significance in patients with heart, circulatory, and overall metabolic diseases.

This thesis also provides the first evidence that women possess higher mitochondrial quality compared to men with equal cardiorespiratory fitness and endurance performance. Moreover, it was shown that increasing oxygen delivery and exercise intensity by means of breathing hyperoxia during high-intensity exercise does not enhance exercise-induced skeletal muscle adaptations although it resulted in a small beneficial effect on performance in trained cyclists.

Altogether, this thesis highlights the importance of maintaining high mitochondrial function and provides further insights on the regulation of O_2 supply and O_2 uptake that create an updated platform for future studies centered on mitochondrial function, metabolism and exercise-induced adaptations.

7. FUTURE PERSPECTIVES

Having shown that both skeletal muscle mitochondrial OXPHOS and $p50_{mito}$ act in concert with O_2 delivery to determine the upper limit of VO_2max, future studies should focus on validating these findings in *in vivo* models possibly using direct measurements of mitochondrial $p50_{mito}$ using e.g. phosphorus-31 nuclear magnetic spectroscopy. The effect of $p50_{mito}$ on O_2 uptake could be tested altering O_2 delivery by means of venesection and reinfusion of red blood cells without altering blood volume. This experimental setup can be a better model instead of comparing hemodynamic responses between two- and one-legged exercise where skeletal muscle mass greatly differs. Since the quadriceps muscle is recruited differently in one-legged knee extension compared to cycling exercise, the hemodynamic responses of one-legged knee extension could be compared to two-legged knee extension or one-legged cycling could be compared to two-legged cycling if two levels of muscle mass activation are desirable in the experimental setup.

From a clinical perspective, it is of importance to transfer these findings to clinical populations since the physiological importance of a high muscle oxidative capacity to exploit a given O_2 delivery by the circulation is of significance in patients with heart and circulatory disease and overall metabolic diseases. The study of skeletal muscle mitochondrial morphology could also elucidate the structural properties determining $p50_{mito}$ and possibly explain the reported variability in $p50_{mito}$ between individuals shown in paper II and III. The study of mitochondrial function in relation to biological sex is also of interest considering the longer life expectancy, the lower disease occurrence and the rate of aging in women compared to men. Studies on twins assessing function and adaptation of skeletal muscle mitochondria could elucidate how the magnitude of change is induced by the genetic component only.

In this thesis it was shown that $p50_{mito}$ was highly dynamic within the same individual. However, it is unknown if $p50_{mito}$ can be altered over time pharmacologically or by means of exercise training and consequently optimize O_2 extraction. Since it appears, at least in *ex vivo* models, that the capacity of complex IV determines $p50_{mito}$, it would be interesting to enhance complex IV capacity/activity without a major increase in mitochondrial content to study the effect of $p50_{mito}$ on O_2 uptake. This specific adaptation could be tested in animal models overexpressing complex IV capacity but also in humans performing sprint

interval training which has been shown to increase intrinsic mitochondrial respiratory function with minimal change in mitochondrial content.

Hyperoxic-supplemented HIIT induced small improvements in performance which were not explained by superior mitochondrial adaptations in trained individuals. This would indicate that normoxic training is probably the optimal stimuli for skeletal muscle adaptation. However, studying mitochondrial biogenesis and mitophagy in relation to increased or decreased O_2 delivery could be of interest to further elucidate possible mechanisms involved in mitochondrial adaptation during training.

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9. SAMMANFATTNING

Mitokondrier är fascinerande organeller som finns i de flesta celltyper. De har en komplex och dynamisk morfologi och mitokondriell funktion har dessutom kopplats till hälsa och sjukdom. Fysisk träning leder till att mitokondrierna blir fler och större för att öka den mitokondriella oxidativa förmågan vilket är viktigt för att matcha det ökade energibehovet och för att bättre kunna hantera framtida träningsinducerad stress.

Mitokondrierna i skelettmuskulaturen hos friska individer har en respiratorisk förmåga som överträffar mängden syre som det kardiorespiratoriska systemet kan leverera till muskeln. Trots detta ökar uthållighetsträning denna så kallade överkapacitet av mitokondrierna ytterligare. Med tanke på att evolutionen har sett till att varje steg i alla metabola processer är matchade med det metabola kravet och att naturen sällan slösar med resurser, är det anmärkningsvärt att mitokondrierna har en sådan överkapacitet. Rollen som denna överkapacitet spelar är fortfarande oklar i regleringen av syreförbrukning, liksom rollen av extra syretillförsel för skelettmuskulens anpassning. Det är även oklart om könsrelaterade skillnader existerar kring mitokondriell funktion hos människor. Mätningar av den mitokondriella oxidativa förmågan i en respirometer används numera regelbundet för kliniska och vetenskapliga ändamål, dock är metodens reproducerbarhet inte välstuderat.

I **studie I** studerades olika faktorer som kan påverka reproducerbarheten av mätningen av den mitokondriella oxidativa förmågan. Resultaten visade vikten att av att samma tekniker utförde fiberseparationen och att provberedningen i sig var den huvudsakliga källan till mätningsvariationen. Dessutom har andra faktorer såsom eventuella skillnader mellan vänster och höger ben, tid efter insamling av biopsi och användning av bedövningsmedel endast en liten inverkan på mätningen.

I **studie II** visades den fysiologiska betydelsen av att ha en mitokondriell oxidativ kapacitet som överstiger kapaciteten av det kardiorespiratoriska systemet att tillföra syre till skelettmuskulaturen vid maximalt arbete. Detta visades genom att kombinera mätningar av den mitokondriella oxidativa funktionen med direkta mätningar av syreförbrukning under cykel och bensparksarbete med och utan extra syretillförsel. Mitokondriernas överkapacitet tillåter en hög syreextraktion i muskeln vid ett lågt syretryck.

Studie III visade att kvinnor har en högre mitokondriell kvalitet jämfört med män med jämförbar kondition. Denna högre mitokondriella kvalitet hos kvinnor kan vara resultatet av en viktig fysiologisk anpassning som möjligtvis är en kompensation för kvinnors lägre Hb-värden. . Dessutom kan dessa resultat eventuellt kopplas till skillnaden i förväntad livslängd, sjukdomstillstånd och åldrande mellan kvinnor och män.

I **studie IV** visades att extra syretillförsel under högintensiv uthållighetsträning inte förbättrade konditionen eller anpassningar i skelettmuskulaturen men resulterade i en liten fördelaktig effekt på prestation hos vältränade cyklister. Denna lilla positiva effekt på prestation skulle kunna vara betydelsefull hos elitidrottare. Dock, med tanke på kostnaden/nyttan, de okända hälsorelaterade effekterna och etiska problem med att utföra uthållighetsträning med extra syretillförsel, är det tvivelaktigt om användningen av denna träningsmetod bör användas för att maximera uthållighetsprestandan hos idrottare.

Sammantaget ger denna avhandling användbar information för framtida forskning om olika faktorer som kan påverka mätningen av mitokondriell oxidativ funktion, visar nya faktorer som reglerar syreförbrukningen under träning och framhäver vikten av att upprätthålla en god mitokondriell funktion.

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