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## Adjustment of pulmonary O<sub>2</sub> uptake, muscle deoxygenation and metabolism during moderate-intensity exercise transitions initiated from low and elevated baseline metabolic rates

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Graduate Program in Kinesiology  
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science  
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Adjustment of pulmonary O<sub>2</sub> uptake, muscle deoxygenation and metabolism during moderate-intensity exercise transitions initiated from low and elevated baseline metabolic rates

(Thesis format: Integrated Article)

by

Joshua Peter Nederveen

Graduate Program in Physiology

A thesis submitted in partial fulfillment  
of the requirements for the degree of  
Master of Science

The School of Graduate and Postdoctoral Studies  
The University of Western Ontario  
London, Ontario, Canada  
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## ABSTRACT

When instantaneous step-wise transitions within the moderate intensity domain are initiated from elevated metabolic rates, the rate of pulmonary oxygen uptake ( $\dot{V}O_{2p}$ ) adjustment is slowed, and the  $\dot{V}O_{2p}$  gain ( $\Delta \dot{V}O_{2p} / \Delta WR$ ) is greater. This study sought to determine the relationship between  $\dot{V}O_{2p}$  kinetics and energy status during step transitions from low and elevated metabolic rates within the moderate intensity domain. Ten young men completed six double-step constant load cycling bouts, consisting of step-wise transitions from 20 W to 45%  $\theta_L$  and 45%  $\theta_L$  [lower step (LS)] to 90%  $\theta_L$  [upper step (US)], one double-step bout included needle biopsies at baseline, steady-state values and during transitions. Gas exchange was analyzed breath-by-breath and muscle de-oxygenation status ([HHb]) was measured with near infrared spectroscopy. The  $\dot{V}O_{2p}$  gain in the US ( $10.37 \pm 1.49$ ) was greater ( $p < 0.05$ ) than the LS ( $8.51 \pm 1.41$ ). The rate of adjustment of  $\dot{V}O_{2p}$  ( $\tau \dot{V}O_{2p}$ ) in the US ( $34 \pm 12$  s) was slower ( $p < 0.05$ ) compared to the lower step ( $20 \pm 8$  s).  $\tau[HHb]$  in the US ( $16 \pm 5$  s) was greater ( $p < 0.05$ ) than the LS ( $11 \pm 5$  s).  $[HHb]_{amp}$  in the US ( $3.5 \pm 2.6$  a.u) was decreased ( $p < 0.05$ ) as compared to the LS ( $4.3 \pm 3.5$ ). [PCr] degradation, [ADP<sub>free</sub>] and [P<sub>i</sub>] was increased ( $p < 0.05$ ) during the LS exercise transition (LS<sub>15</sub>) and remained elevated relative to baseline through the protocol. The calculated  $\Delta G_{ATP}$  values at the LS<sub>360</sub> and the US<sub>360</sub> were both significantly ( $p < 0.05$ ) lower than baseline but not different from each other.

### Keywords:

O<sub>2</sub> uptake kinetics; near-infrared spectroscopy; NIRS; metabolism; intramuscular metabolites

## **CO-AUTHORSHIP STATEMENT**

This study was designed by J.P Nederveen, J. M. Kowalchuk and H.B. Rossiter with input from the advisory committee (D. H. Paterson). The majority of the data were collected and analyzed by J. P. Nederveen with the assistance of J.R. Leckie (T. J. Doherty performed all needle biopsy procedures). L.L Spriet and J. Whitfield analyzed the muscle tissue from the needle biopsy procedures. J.P Nederveen wrote the original manuscript for the study and the co-authors provided financial support, lab support and editorial feedback.

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## LIST OF TERMS AND ABBREVIATIONS

[ADP] – adenosine diphosphate concentration

Amp – amplitude

[ATP] – adenosine triphosphate concentration

Bsl – baseline

CI95 – 95% confidence interval

DCA – dichloroacetate

$\Delta 50$  – in the moderate intensity domain, work rate corresponding to 50% between 20 W and 90% of the estimated lactate threshold

DS-MOD – double-step moderate-intensity exercise test

ETC – electron transport chain

fd-NIRS – multidistance frequency-domain near infrared spectroscopy

$G$  – fundamental gain in pulmonary oxygen uptake ( $\Delta \text{VO}_2/\Delta \text{WR}$ )

$\Delta G_{\text{ATP}}$  – free energy available from ATP hydrolysis

$\text{H}^+$  – hydrogen ion

[THC] – total hemoglobin concentration

[HbO<sub>2</sub>] – oxyhemoglobin, measure of muscle oxygenation concentration

[HHb] – deoxyhemoglobin; measure of muscle deoxygenation concentration

[HHb]<sub>bsl</sub> – baseline muscle deoxygenation

[HHb]<sub>ss</sub> – steady state muscle deoxygenation

HR – heart rate

$\theta_L$  – lactate threshold

$e \theta_L$  – estimated lactate threshold

LBF – leg femoral artery blood flow

LS – lower step; step increase from 20 W to moderate intensity  $\Delta 50$  work rate

MOD – moderate intensity

MRT – mean response time

PO<sub>2</sub> – partial pressure of oxygen

PCO<sub>2</sub> – partial pressure of carbon dioxide

[PCr] – phosphocreatine concentration

PDH – pyruvate dehydrogenase

[Pi] – inorganic phosphate concentration

O<sub>2</sub> – oxygen

[O<sub>2</sub>Hb] – oxyhaemoglobin concentration

RPM – revolutions per minute

SS – steady state

$\tau$  – time constant; time required to attain 63% of the steady-state response

$\tau'$  – effective time constant ( $\tau + TD$ )

US – upper step; step increase from  $\Delta 50$  to 90% estimated lactate threshold

TCA – tricarboxylic acid

%SAT – percentage of oxygen saturation

TD – time delay

VCO<sub>2</sub> – carbon dioxide output

VE – ventilation

$\dot{V}O_{2p}$  peak – maximal oxygen uptake

$\dot{V}O_{2m}$  – muscle oxygen uptake

$\dot{V}O_{2p}$  – pulmonary oxygen uptake

$\dot{V}O_{2p \text{ bsl}}$  – baseline pulmonary oxygen uptake

$\dot{V}O_{2p \text{ ss}}$  – steady-state pulmonary oxygen uptake

WR – work rate

WR<sub>max</sub> – maximal work rate attained during ramp incremental test

## PREFACE

The origins of the study of pulmonary O<sub>2</sub> uptake ( $\dot{V}O_2$ ) kinetics and metabolism are grounded in nearly hallowed antiquity. To trace the path, one must look back through the annals of both history and science to find the Greek term Μεταβολισμός; *metabolismos*, which means to ‘overthrow’, to ‘change’ or ‘alter’. Science itself is rife with ‘overthrow’. Where once the mighty Greeks trusted Empedocles, citizen of modern day Sicily, when he quantified all matter was built of ‘four elements’, the enlightened scientific body of an 18<sup>th</sup> century Europe trusted in the lauded chemist Antoine Lavoisier when he realized the transition from the inhalation of air to the exhalation of CO<sub>2</sub>. The foundation  $\dot{V}O_2$  and respiratory metabolism on which this thesis is primarily (but not solely) focused upon, began its modern life with the pioneering work of Dr. Archibald Vivian Hill. Hill’s work, derived heavily from mathematics, lead him to study and help characterize Michaelis-Menten kinetics and also reveal the exponential-like nature of  $\dot{V}O_2$  at the onset of exercise. Due to this pioneering work in both metabolic and respiratory physiology, A.V. Hill is regarded as one of the founding fathers of the field of biophysics. Building on the work of Hill, and after, of Krogh and Lindhard, the work of Dr. Brian Whipp further elucidated the effectiveness of the human physiological system to respond to a new stress with a rapid kinetic response. Furthermore, Dr. Whipp was responsible for continuing the proliferation of knowledge, concerning the accuracy and validity of making inferences of the physiological state from the exercise on-transient. It is his (and others) early and pivotal work that cemented the field of  $\dot{V}O_2$  kinetics as a standard measurement in exercise physiology. The study of  $\dot{V}O_2$  is important simply because it governs the principal method of energy generation in the human body. It falls to those delving into the field of  $\dot{V}O_2$  kinetics today to push forward, to continue to change, to add and to alter.

*‘The great book, always open and which one must make every effort to read, is the book of nature’ – Antoni Gaudi*

## CHAPTER 1

### 1 REVIEW OF THE LITERATURE

#### 1.1 INTRODUCTION

Coincident with an increase from rest or light exercise there is an instantaneous increase in the demand for adenosine triphosphate (ATP) within the working muscle (Rossiter *et al.* (1999). This instantaneous increase in demand for ATP must be met with a rapid, equivalent increase in ATP production. However, since indicators of oxidative phosphorylation such as muscle oxygen ( $O_2$ ) uptake ( $\dot{V}O_{2m}$ ) and pulmonary oxygen uptake ( $\dot{V}O_{2p}$ ), have been observed to be relatively slow (Grassi *et al.*, 1996), the initial deficit in energy production must be contributed to up-regulated substrate-level phosphorylation (degradation of intramuscular phosphocreatine (PCr), increase in glycogenolysis/glycolysis and subsequent lactate production). However, if the exercise work rate (WR) is to be maintained, the sustained ATP demand must be met through a major contribution of oxidative phosphorylation. Given that the exercise transition intensity remains beneath the lactate threshold ( $\hat{\theta}_L$ ) within the moderate-intensity domain (MOD),  $\dot{V}O_{2p}$  will be activated in an exponential fashion, creating a delay (dependent on the speed of activation) between the demand for ATP and the matched production of ATP; when the production of ATP matches the demand for ATP, this will create a steady-state (Whipp, 1971). The reaching of this new steady-state generally occurs between 120 to 240 seconds in MOD exercise, however, if the exercise intensity falls above demarcations of metabolism (i.e.,  $\hat{\theta}_L$  or critical power) which define heavy- or severe-intensity domains of exercise, the new SS will be delayed or not attained at all. The mechanism responsible for determining the rate at which oxidative phosphorylation activates has yet to be resolutely elucidated; despite much attention, the literature would suggest that there is not one exact mechanism, but instead a combination of  $O_2$  delivery factors, a “sluggish” activation of rate limiting enzymes and provision of oxidative substrate (other than  $O_2$ ) (i.e., reducing equivalents (NADH;  $FADH_2$ , ADP, Pi) to the mitochondrial TCA cycle and ETC), and cellular energetic status.

The notion that  $\dot{V}O_{2p}$  directly measures oxidative phosphorylation within the exercising muscle must be approached with considerable caution. There have been a number of modalities

to assess the *approximation* of human  $\dot{V}O_{2m}$  (Grassi *et al.*, 1996; Koga *et al.*, 2005; Krstrup *et al.*, 2009). The most common of these is the measure of  $\dot{V}O_{2p}$ , measured at the level of the mouth. In the literature,  $\dot{V}O_{2p}$  has been observed to be within approximately 10% of  $\dot{V}O_{2m}$  both *in silico* (Barstow *et al.*, 1990) and *in vivo* via measuring conduit artery blood flow and arterio-venous O<sub>2</sub> content (i.e., a-vO<sub>2</sub> difference) (Grassi *et al.*, 1996; Bangsbo, 2000). Furthermore, employment of <sup>31</sup>P-magnetic resonance spectroscopy (<sup>31</sup>P-MRS) has experimentally detected a tight matching between [PCr] degradation kinetics and the adjustment of  $\dot{V}O_{2p}$  and a similarly tight coupling between [PCr] breakdown and  $\dot{V}O_{2m}$  (Rossiter *et al.*, 1999). Therefore,  $\dot{V}O_{2p}$  kinetics remains the gold standard, non-invasive proxy for O<sub>2</sub> consumption at the level of the muscle (Rossiter *et al.*, 1999).

The application of the MOD (i.e., exercise performed below the estimated  $\hat{\theta}_L$ ) is of important to the current thesis. The  $\hat{\theta}_L$  is the metabolic rate at which the levels of blood lactate begin to increase significantly above baseline values – potentially indicating an alteration in the cellular processes required to drive  $\dot{V}O_{2p}$  (Connett *et al.*, 1990). Pulmonary gas exchange responses to incremental exercise are utilized to determine at what  $\dot{V}O_{2p}$  the  $\hat{\theta}_L$  occurs, which can then be used to determine an appropriate work rate and intensity which lies either below (MOD) or above (heavy; very heavy) this demarcation. During exercise that is performed within the MOD intensity domain,  $\dot{V}O_{2p}$  exhibits an exponential-like rise towards a higher, but well-defined steady-state. Immediately at the start of the exercise transition there is an initial rise in  $\dot{V}O_{2p}$  (Phase I, “cardio-dynamic phase”) which reflects the transit delay between the active muscle and the pulmonary circulation and consists of an increase in pulmonary blood flow (related to cardiac output) and increased venous return from the muscle due to the muscle pump that is activated by muscular contractions at the onset of exercise. The increase in  $\dot{V}O_{2p}$  during ‘Phase I’ does not represent any alteration in metabolism but only the rise in pulmonary blood flow. After an ~20 s delay there is a more rapid increase in  $\dot{V}O_{2p}$  (Phase II; fundamental or primary phase) which corresponds to the increase in  $\dot{V}O_{2m}$  (i.e., at the level of the muscle) and is associated with the appearance at the lung, of deoxygenated blood from the active muscle along with a continuing increase in pulmonary blood flow that is related to cardiac output. Within the



MOD exercise domain,  $\dot{V}O_{2p}$  continues to increase exponentially until it reaches a new steady state (Phase III). At exercise intensities above  $\theta_L$  (i.e., heavy- or severe-intensity), a complex ‘Phase III’ manifests itself as a delayed-onset, “extra”  $\dot{V}O_{2p}$  (i.e., a  $\dot{V}O_{2p}$  ‘slow’ component) which either delays the attainment of an steady-state (heavy-intensity) or results in a continued rise in the  $\dot{V}O_{2p}$  response with no evidence of a steady state (very heavy-intensity). The data herein this thesis, however, are related only to the MOD-intensity domain.

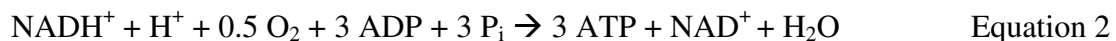
The adjustment of  $\dot{V}O_{2p}$  following a step-increase in WR performed within the moderate-intensity domain can be described by the kinetics of the phase II response. ‘Phase II’ is characterized as a mono-exponential curve (Hill, 1924; Mahler, 1985; Whipp *et al.*, 2005), the nature of which can be described by Equation 1:

$$\dot{V}O_{2p(t)} = \dot{V}O_{2p\_BSLN} + A (1 - e^{-(t-TD)/\tau}) \quad \text{Equation 1}$$

where  $\dot{V}O_{2p(t)}$  represents the  $\dot{V}O_{2p}$  at any given time ( $t$ );  $\dot{V}O_{2p\_BSLN}$  is the steady-state baseline value of  $\dot{V}O_{2p}$  before an increase in WR (given as the average  $\dot{V}O_{2p}$  value in the 80-20 s period immediately prior to a transition);  $A$  is the amplitude of the increase in  $\dot{V}O_{2p}$  above  $\dot{V}O_{2p\_BSLN}$ ;  $\tau$  is the time constant and represents the time required to attain 63% of the steady-state amplitude (with 86%, 95% and 98% of the SS achieved after  $2\tau$ ,  $3\tau$  and  $4\tau$ , respectively); and  $TD$  represents the mathematically generated time delay through which the exponential model is predicted to intersect  $\dot{V}O_{2p\_BSLN}$ . The time constant ( $\tau$ ) for the phase II  $\dot{V}O_{2p}$  response ( $\tau \dot{V}O_{2p}$ ) is believed to reflect the rate of adjustment of  $\dot{V}O_{2m}$ , and therefore acts as a proxy for the adjustment of mitochondrial oxidative phosphorylation).

## 1.2 FACTORS LIMITING OXYGEN UPTAKE KINETICS

The overall reaction describing oxidative phosphorylation can be summarized below in Equation 2:



Should any one of the substrates required for oxidative ATP production ( $O_2$ , NADH, ADP) be less than some “optimal” level, then this substrate could limit the rate of activation of oxidative phosphorylation, and thus slow  $\dot{V}O_{2p}$  kinetics. However, a delay in the  $\dot{V}O_{2p}$  response may not be due necessarily to lower concentration of a single substrate, but instead may be due to the interaction between the substrates. Hogan et al. (2005) observed that the increase in NADH was markedly greater when initiated from a point when exercise is initiated from a point of low muscle  $PO_2$  was low (Hogan *et al.*, 2005). In exercising human muscle (Haseler *et al.*, 1998), the rate of oxidative phosphorylation was well defended in the face of decreased  $O_2$  availability, which was mirrored by an increase in the concentration of [ADP]. The literature would suggest that instead of one determining factor the rate of  $\dot{V}O_{2p}$  adjustment, there are a series of interacting aspects. Despite this, there exist two hypotheses concerning the limiting of the rate of adjustment of  $V\dot{\square}O_{2p}$ ; (1) phase II  $\dot{V}O_{2p}$  kinetics are limited by  $O_2$  delivery to the exercising muscle; (2) they are slowed by a sluggish activation of enzymes and provisions of substrates for oxidative phosphorylation

### **1.3 REGULATION OF $V\dot{\square}O_{2p}$ KINETICS BY $O_2$ DELIVERY IN MODERATE EXERCISE**

There is a considerable amount of evidence to suggest that  $O_2$  delivery is the defining limitation to  $\dot{V}O_{2p}$  kinetics. In the literature, experiments have been performed that impair  $O_2$  transport and delivery either via pharmacological means, modifying body position or by altering the inspired fraction of  $O_2$  (i.e., normoxia, hypoxia and hyperoxia) in order to study the impact on  $\tau \dot{V}O_{2p}$ . Beta-adrenergic receptor blockade (which reduces cardiac output) and the supine posture (reducing perfusion pressure) have been shown to prolong  $\tau V\dot{\square}O_{2p}$  (Hughson & Kowalchuk, 1991; MacDonald *et al.*, 1998). Impairment of  $O_2$  delivery by acute bouts of hypoxia ( $FiO_2$  10-14%), and therefore the reducing of arterial  $PO_2$  ( $PaO_2$ ) have been employed to slow  $\tau \dot{V}O_{2p}$  during transitions within the moderate-intensity domain (Murphy *et al.*, 1989; Hughson & Kowalchuk, 1995; Engelen *et al.*, 1996). Furthermore, combining interventions that augment convective  $O_2$  delivery and metabolic substrate provision (i.e., the MOD-HVY-MOD protocol) with the addition of hypoxia (which presumably increases  $O_2$  delivery but impairs  $PaO_2$  through alterations in  $FiO_2$ ) have resulted in a lengthened  $\tau \dot{V}O_{2p}$  (Spencer *et al.*, 2012b).

These results may suggest that O<sub>2</sub> delivery is the major factor in the limitation of the rate of oxidative phosphorylation. However, when attempting to increase O<sub>2</sub> availability, especially in the moderate-intensity domain, there seems to be no impact on  $\tau \dot{V}O_{2p}$ . In the pump-perfused dog hindlimb model, Grassi and colleagues showed no speeding on  $\dot{V}O_{2p}$  kinetics despite improving bulk, convective blood flow (Grassi *et al.*, 1998) and peripheral, diffusive O<sub>2</sub> delivery (Grassi *et al.*, 1998). Work in the animal model (e.g., dog gastrocnemius muscle is highly oxidative and capillarized, and demonstrated rapid blood flow distribution), (Delp & Duan, 1996; Leek *et al.*, 2001) have yielded similar results as in the human model. Hyperoxia (FiO<sub>2</sub> >50%), has often failed to produce any speeding of  $\dot{V}O_{2p}$  kinetics (Hughson & Kowalchuk, 1995; Macdonald *et al.*, 1997). However, inferences from the lack of speeding with improved FiO<sub>2</sub> must be taken with caution. Hyperoxia also causes systemic vasoconstriction so that blood flow is adjusted (i.e., reduced) to maintain total O<sub>2</sub> delivery (Macdonald *et al.*, 1997). Expectations of an altered  $\tau \dot{V}O_{2p}$  following changes in O<sub>2</sub> delivery may be specific to the environment to which they are applied; in other words, initial  $\tau \dot{V}O_{2p}$  will likely play a role in determining whether or not and to what extent the rate of adjustment of  $\dot{V}O_{2p}$  is sped following any intervention which may improve O<sub>2</sub> delivery to the working muscle. Poole *et al.* (2008) proposed there is a “point” beyond which  $\dot{V}O_{2p}$  kinetics is O<sub>2</sub> dependent such that the provision of O<sub>2</sub> becomes limiting and thus  $\tau \dot{V}O_{2p}$  is lengthened – though implications have been made that this occurs only in elderly or diseased populations (Poole *et al.*, 2007; Poole *et al.*, 2008; Poole & Musch, 2010). In contrast, recent data have suggested that this O<sub>2</sub> dependent point exist in young healthy adults as well, at least when the severity of a O<sub>2</sub> delivery limitation is capable of impacting the  $\tau \dot{V}O_{2p}$  (Murias *et al.*, 2011). As such, interventions that affect provision of O<sub>2</sub> to the active muscles (via convective and/or diffusive O<sub>2</sub> delivery) are likely to alter the dynamic adjustment of oxidative phosphorylation even in young healthy populations – if the initial  $\tau \dot{V}O_{2p}$  is slow, and therefore can be altered by an improved O<sub>2</sub> delivery.

### *Near-infrared spectroscopy*

In order to effectively measure tissue oxygenation, the use of near-infrared spectroscopy (NIRS) has become a preferred method in the non-invasive observation of microvascular blood flow.

The NIRS measurements are based on the absorption of light wavelengths within the NIR range (~700-900nm) due to the differences in light absorption characteristics displayed by oxygenated hemoglobin [HbO<sub>2</sub>] and deoxygenated hemoglobin [HHb]. NIR light is projected from an emitting diode to a light-detecting optode (optical sensor device) after passing through tissue. Kallikoski *et al.* (2006) outlines that the penetration depth is approximately half the distance of the inter-optode spacing (Kalliokoski *et al.*, 2006). [HHb] and [HbO<sub>2</sub>] are distinct based on their different light absorption characteristics, so by emitting light at several specific wavelengths in the NIR spectrum, the precise determination of the amount within the tissue under the area of interrogation is possible. The quantification of specific changes in [HbO<sub>2</sub>] and [HHb] via the NIRS method has been utilized to provide an index of O<sub>2</sub> extraction during transitions from baseline to a new metabolic work rate. While the NIRS does not provide an estimate of a-vO<sub>2</sub>diff, as circulation within the microvasculature cannot be concretely assessed: the [HHb] has been observed to match microvascular partial pressure (PO<sub>2mv</sub>) through the on-transient of exercise (Grassi *et al.*, 2003). The NIRS method provides an insight into the local, microvascular O<sub>2</sub> delivery to the working muscle and the rate of O<sub>2</sub> utilization.

#### **1.4 REGULATION OF V̇O<sub>2p</sub> KINETICS BY SUBSTRATE PROVISION**

For oxidative phosphorylation to increase in the on-transient following a step-increase in WR, there must be an increase in provision of electrons and reducing equivalents (i.e., NADH; FADH<sub>2</sub>) to the mitochondrial electron transport chain (ETC). For the concentration of NADH to rise there must be an increase in either the breakdown of fat (via beta oxidation) or the production of pyruvate (stemming from glycolytic pathways). The production of pyruvate subsequently increases the tightly regulated pyruvate dehydrogenase (PDH) facilitated production of acetyl-CoA and flux through the tricarboxylic acid (TCA) cycle. Proponents of the hypothesis of a sluggish response in terms of metabolic activation that causes a slowing of V̇O<sub>2p</sub> kinetics would suggest that if augmenting O<sub>2</sub> delivery causes no perceptible changes in τ V̇O<sub>2p</sub>, then the limitation must lie within the metabolic pathways. PDH has been studied as a potential site of regulation for oxidative phosphorylation. The PDH complex exists in two forms: an inactive phosphorylated form and an active dephosphorylated form. Regulation of PDH is determined by enzymes PDH kinase (PDK) and PDH phosphatase (PDP) – increased activity of

PDK inhibits the activity of PDH (by phosphorylates the enzyme) while increased activity of PDP activates PDH (via dephosphorylation of the enzyme). The mitochondrial PDH complex catalyzes the oxidative decarboxylation of pyruvate, with production of Acetyl CoA, NADH and  $H^+$ , and  $CO_2$ , and thus is responsible for regulating the entry of carbohydrate-derived substrate into the TCA, therefore, the provision of reducing equivalents to the ETC. Conflicting evidence lies in the literature in both human and canine models; by increasing the activation of PDH via a pharmacological intervention (dichloroacetate, DCA) (Timmons *et al.*, 1996), there was a marked reduction of the contribution of substrate-level phosphorylation during moderate exercise (i.e., 65%  $\dot{V}O_{2p}$  max) (Howlett *et al.*, 1999). These findings would suggest that the reduction of substrate-level phosphorylation would stem from a more rapid activation of both oxidative phosphorylation and muscle  $O_2$  utilization. However, experiments in humans performed by Rossiter *et al.* (2003) in the heavy-intensity domain (Rossiter *et al.*, 2003) and Koppo *et al.* (2004) in the moderate-intensity domain failed to demonstrate faster  $\dot{V}O_{2p}$  kinetics following prior PDH activation by DCA supplementation. Also, Grassi *et al.* (2002) using an isolated dog gastrocnemius muscle preparation showed that despite an improved metabolic efficiency (i.e., '[PCr] sparing'), there were no effect on  $\tau \dot{V}O_{2p}$  (Grassi *et al.*, 2002). The different findings in the literature may stem from differences in experimental models, techniques or inaccuracies in the modeling of  $\tau \dot{V}O_{2p}$  data, but also may stem from a limitation to reducing equivalent production *elsewhere* in the complex oxidative pathway (i.e., one of a number of enzymatic relationships found in the TCA).

In order for oxidative phosphorylation to increase during the on-transient from one metabolic demand to another, ADP will increase in response to the breakdown of ATP; and thus, control of oxidative phosphorylation may reside within the creatine kinase (CK) reaction. As ATP is broken down at the level of the muscle, the instantaneous increase of the products of the hydrolysis (ADP, Pi) are moderated by the CK reaction. Chance & Williams (1955) proposed that within isolated mitochondrial preparations, increases in concentration of both free (unbound) adenosine diphosphate ( $[ADP_{free}]$ ) and  $[P_i]$  would cause an increase in  $\dot{V}O_{2m}$  (Chance *et al.*, 1955). Interestingly, when CK was inhibited in a single isolated frog myocyte, there was a rapid fall in intracellular  $PO_2$  (which the authors believed was indicative of a rapid  $\tau \dot{V}O_{2m}$ )

(Kindig *et al.*, 2005). The inference here is that there is a high correlation between the flux of CK and oxidative phosphorylation; the breakdown of PCr serves as a “buffer” against the increase in ADP and slows the further activation of oxidative phosphorylation. The authors suggested that the PCr pool may act as a potential ‘dampening system’ that protects oxidative phosphorylation from sudden swings in ATP requirement (Kindig *et al.*, 2005). When the PCr system is no longer capable of buffering the rising  $ADP_{free}$ ,  $ADP_{free}$  would likely be the driving force to increase oxidative phosphorylation.

### 1.5 SLOWED $\dot{V}O_{2p}$ KINETICS IN TRANSITIONS FROM RAISED METABOLIC RATES

During exercise performed below  $\hat{\theta}_L$ , the steady-state  $\dot{V}O_{2p}$ -WR relationship is linear (Whipp & Wasserman, 1972). The kinetic adjustment during transitions between one WR and another gives a glimpse into possible mechanisms controlling  $O_2$  uptake. Hughson & Morrissey (1982) experimentally identified gas exchange kinetics (including  $\dot{V}O_{2p}$ ) when transitioning to a new WR from an elevated WR (relative to rest) (Hughson & Morrissey, 1982). The authors confirmed the observation that there exists a ‘non-linear’ relationship in the relationship between WR and  $\dot{V}O_{2p}$  during exercise transitions. The data from Hughson and Morrissey (1982) indicated not only a slower rate of adjustment of  $\dot{V}O_{2p}$ , but also an increase  $O_2$  cost per unit increase in WR (i.e.,  $\dot{V}O_{2p}$  gain;  $\Delta \dot{V}O_{2p} / \Delta WR$ ). Since the identification of this non-linear relationship, the control mechanism that governs such a relationship has received much attention. Using similar protocols these observations have been reproduced on a number of occasions, highlighting the relative “robustness” of these responses. A modified “double-step” protocol was introduced by Brittain *et al.* (2001) which involved two step-transitions of equal magnitude (i.e., identical  $\Delta WR$ ) performed sequentially, with a ‘lower-step’ (LS) initiated from a light-intensity WR (often using baseline leg cycling of 20W) to a higher WR midway between the baseline WR and a WR corresponding to  $\sim 90\% \hat{\theta}_L$  (i.e., WR50) followed by an ‘upper-step’ (US) transition from WR50 to a WR corresponding to  $\sim 90\% \hat{\theta}_L$  (WR90) (where  $\Delta WR_{LS} = \Delta WR_{US}$ ) (Figure I) (Brittain *et al.*, 2001). In this study, and in others (Brittain *et al.*, 2001; Spencer *et al.*, 2012; Williams *et al.*, 2013), despite the identical  $\Delta WR$  transitions (reflecting possibly a similar increase in ATP turnover rate) maintained within the MOD-intensity domain,

slower  $\dot{V}O_{2p}$  kinetics (i.e., a greater  $\tau \dot{V}O_{2p}$ ) and a greater  $\dot{V}O_{2p}$  gain (i.e., larger  $\Delta \dot{V}O_{2p} / \Delta WR$ ) are reported consistently for the US compared to the LS. The exact mechanism(s) responsible for this dynamic “non-linearity” currently are unknown. Hughson and Morrissey (1982) proposed that “bulk” blood flow was slowed in the US as evidenced by slower heart rate kinetics (due to the relative sluggishness of sympathetic drive at the initiation of the US versus the rapid withdrawal of the parasympathetic system at the onset of the LS). This notion was supported by MacPhee *et al.* (2005) who reported slower heart rate (HR) and femoral (conduit) artery blood flow kinetics initiated from a high versus light baseline metabolic rate performed during alternate-leg knee-extension exercise however the relationship between blood flow and oxygen uptake remains unclear; in the same study, the rate of adjustment of muscle oxygen extraction (as measured by the near infrared spectroscopy (NIRS)-derived [HHb] signal) was slowed in the US, suggesting that the delivery of  $O_2$  to the working muscle was sufficient and mirrored a slowed rate of  $\dot{V}O_{2p}$  adjustment. (MacPhee *et al.*, 2005). Brittan *et al.* (2001) proposed that the possible fiber-type recruitment strategy in the LS (primarily type I slow-twitch, highly oxidative fibers) versus those recruited in the US (“less efficient” fibers with slower  $\dot{V}O_{2p}$  kinetics and greater  $\dot{V}O_{2p}$  gain). Bowen *et al.* (2011) attempted to dissociate the effects of muscle fibre recruitment from the effects of metabolic rate by having subjects perform a step-transition from a baseline of light-intensity exercise to a WR corresponding to 90%  $\hat{\theta}_L$  (WR90), allowing subjects a brief recovery last 30 s at the light-intensity WR which allowed  $\dot{V}O_{2p}$  to recovery partially, and then transitioning back to WR90. In this protocol  $\dot{V}O_{2p}$  kinetics remained slow despite the step-transition being initiated from a light-intensity WR (i.e., similar muscle fibre recruitment but elevated metabolic rate (high  $\dot{V}O_{2p}$ ) and muscle blood flow) (Bowen *et al.*, 2011). These data suggest that differences in the adjustment of  $\dot{V}O_{2p}$  (and oxidative phosphorylation) within the “double-step” protocol may not due to muscle fibre type inefficiencies or to attenuated muscle blood flow and  $O_2$  delivery, but instead suggest that the metabolic status of the working muscle may play a role.

During transitions from low to higher intensities of exercise the metabolic stability is not maintained but coincident with decreases in local muscle [PCr] and [ATP], increases in [Pi], [ADP<sub>free</sub>], [AMP<sub>free</sub>], [IMP<sub>free</sub>], and reductions in Gibbs free energy of ATP hydrolysis ( $\Delta G_{ATP}$

$= [\text{ADP}_{\text{free}}] \cdot [\text{Pi}]/[\text{ATP}]$ ). What is meant by the term metabolic stability is that the ratios of high energy phosphates are similar to those found at, in this case, baseline cycling. With respect to the “double-step” protocol, metabolic “stability” will be similar to baseline during the LS (initiated from a light-intensity baseline metabolic rate) than in the US (initiated from a higher-intensity baseline metabolic rate), with the fall in metabolic “stability” being exacerbated during the transitions when muscle energy production relies more heavily on substrate-level, rather than oxidative, phosphorylations. The raised metabolic rate elicited by the LS potentially reduces the cellular energetic state within the working muscles (i.e., a reduction in  $\text{PO}_2$ , [PCr] and increased  $[\text{ADP}_{\text{free}}]$  would lead inexorably to a diminished Gibb’s free energy ( $\Delta G_{\text{ATP}}$  of ATP degradation) (Meyer, 1988) which has been shown both *in silico* (Kemp, 2008), *in vivo* (Barstow *et al.*, 1994), and *in vitro* (Glancy *et al.*, 2008). The implications of the diminished Gibb’s free energy would be that the energy released per hydrolysis of ATP would be diminished, perhaps leading to a greater ATP turnover for a given energetic output.

The primary purpose of the study herein was to examine; (1) the rate of adjustment of  $\dot{V}\text{O}_{2\text{p}}$ , muscle deoxygenation and heart rate during transitions from low and elevated baseline metabolic rates within the MOD exercise domain; (2) the relationship between metabolites (i.e.,  $[\text{ADP}_{\text{free}}]$ , [ATP], [PCr],) measured via needle biopsy and  $\tau \dot{V}\text{O}_{2\text{p}}$ .

It was hypothesized (1) that  $\dot{V}\text{O}_{2\text{p}}$  kinetics would be comparatively slower and that the  $\dot{V}\text{O}_{2\text{p}}$  gain would be greater during exercise transitions initiated from a higher metabolic rate; and (2) that muscle [PCr] would be lower,  $[\text{ADP}_{\text{free}}]$  and [Pi] would be higher, and the calculated  $\Delta G_{\text{ATP}}$  would be lower with exercise initiated from a higher metabolic rate; and (3) the  $\tau \dot{V}\text{O}_{2\text{p}}$  would be inversely related to  $\Delta G_{\text{ATP}}$ . Furthermore, bulk blood flow (as measured by HR) during exercise transitions initiated from an elevated metabolic rate will be slowed due to the lengthened rate of adjustment of oxidative phosphorylation, furthermore, the  $\text{O}_2$  extraction (as measured by the NIRS-derived [HHb] signal) will also be slowed.



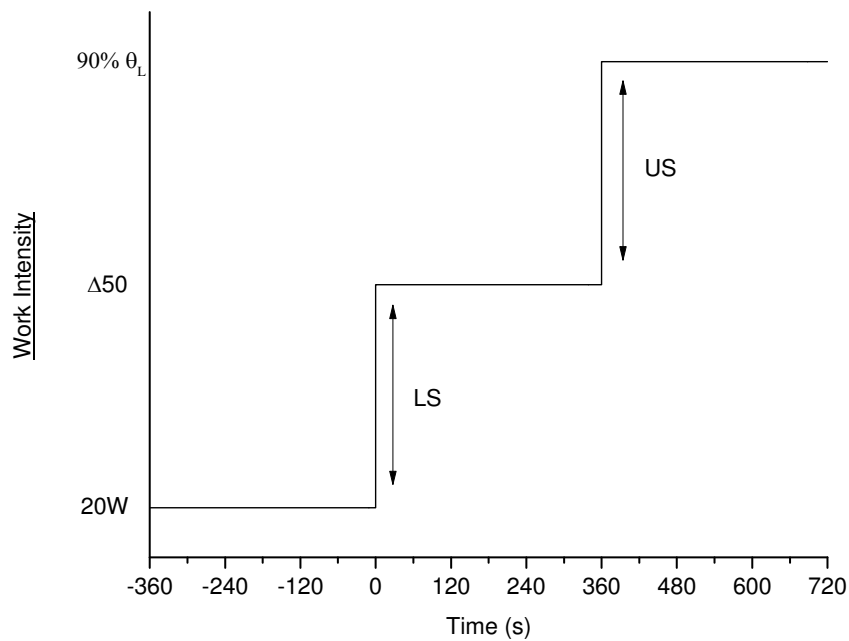


Figure I: Schematic representation of the double-step, constant-load moderate intensity exercise test. The lower step (LS) involves a transition from light-intensity exercise (20 W) to a work rate representing 50% of the difference between 20 W and the estimated lactate threshold  $\theta_L$ , ( $\Delta 50$ ); the upper step (US) involves a transition from  $\Delta 50$  to  $90\% \theta_L$ .

## 2 ADJUSTMENT TO PULMONARY $\dot{V}O_{2p}$ , MUSCLE DEOXYGENATION AND METABOLISM DURING MODERATE INTENSITY TRANSITIONS INITIATED FROM LOW AND ELEVATED BASELINE METABOLIC RATES

### 2.1 INTRODUCTION

During an abrupt step-transition from rest to exercise, there is an immediate increase in energy turnover in the working skeletal muscle (Rossiter *et al.*, 1999). In order to prevent a fall in intracellular [ATP], the increased ATP demand is met immediately by the provision of ATP via substrate-level phosphorylation (PCr degradation) and by an increase in flux through the glycolytic pathway resulting in increased [lactate] (La-). Coincident with this, the rate of mitochondrial oxidative phosphorylation increases in a time-dependent manner, becoming the principal method of sustained ATP production, thereby reducing the reliance on substrate-level phosphorylations. The rise in muscle  $O_2$  uptake ( $\dot{V}O_{2m}$ ), and thus increase in oxidative phosphorylation, does not increase instantaneously with the immediate rise in ATP demand but instead, increases exponentially towards a new steady-state. The rate of  $\dot{V}O_{2m}$  increase at the muscle is reflected closely by the increase in pulmonary  $O_2$  uptake ( $\dot{V}O_{2p}$ ) (Grassi *et al.*, 1996; Koga *et al.*, 2005), although the pulmonary expression of the  $\dot{V}O_{2m}$  is shifted in time owing to the intervening muscle-to-lung blood volume and modulated by the ongoing increase in central and peripheral blood flow. Despite this, the time course of  $\dot{V}O_{2m}$  and the time course of the phase II  $\dot{V}O_{2p}$  were shown to agree within ~ 10% (Barstow *et al.*, 1994; Grassi *et al.*, 1996; Koga *et al.*, 2005). Should this abrupt increase in work rate (WR) lie within the moderate-intensity domain (MOD),  $\dot{V}O_{2p}$  will eventually (within ~2-3 min) realize and maintain a steady-state (Whipp & Wasserman, 1972)

During MOD exercise it has been demonstrated that the rate of adjustment of  $\dot{V}O_{2p}$  (determined by the  $\dot{V}O_{2p}$  time constant ( $\tau \dot{V}O_{2p}$ ) is lengthened, and the  $\dot{V}O_{2p}$  gain (i.e.,  $\Delta \dot{V}O_{2p} / \Delta WR$ ; reflecting the  $O_2$  cost of a given WR change) is greater when exercise is initiated from a higher compared to lower baseline metabolic rate in healthy young (Hughson & Morrissey, 1982; Brittain *et al.*, 2001; MacPhee *et al.*, 2005; Bowen *et al.*, 2011), elderly (Spencer *et al.*, 2011) and despite training (Williams *et al.*, 2013). That the  $\dot{V}O_{2p}$  kinetics response is dependent

on the baseline metabolic rate suggests a ‘dynamic nonlinearity’ within the moderate-intensity domain - i.e, a different “output” response ( $\dot{V}O_{2p}$ ) despite the same “input” WR change.

There have been a number of proposed mechanisms contributing to the slowed time course of adjustment of  $\dot{V}O_{2p}$  during transitions from an elevated metabolic rate. Hughson and Morrissey (1982) proposed that the rate of convective and diffusive transport of  $O_2$  was responsible for the slowed  $\dot{V}O_{2p}$  adjustment in transitions initiated from a raised WR. Slower adjustment of central blood flow was due to the relatively slow activation of the sympathetic system when compared to the rapid adjustment of heart rate (HR) seen due to withdrawal of parasympathetic neural activity. Consistent with this MacPhee et al. (2005) reported a slowed adjustment of conduit artery blood flow and slowed HR kinetics. The limitation of  $\dot{V}O_{2p}$  via slowed bulk blood flow ( $O_2$  delivery) or from poor matching of  $O_2$  distribution to the working muscles within the microvasculature remains to be elucidated. Williams et al. (2013) suggested that following the abolishment of the ‘overshoot’ of the HHb-to- $\dot{V}O_{2p}$  ratio (as reflecting the abolishment of a greater reliance upon muscle  $O_2$  extraction to  $O_2$  consumption) following training made no impact on the slowed adjustment of  $\dot{V}O_{2p}$  in transitions made from an elevated metabolic baseline.

Alternatively, the slowed rate of adjustment of  $\dot{V}O_{2p}$  may be related to muscle fibre recruitment and differences in kinetic and metabolic characteristics of the muscle fibres recruited during exercise transitions from a lower compared to higher WR and metabolic rate (Brittain *et al.*, 2001). Muscle fibers recruited during lower compared to higher exercise intensities, even within the same exercise intensity domain, may differ in contractile characteristics, metabolic efficiency (ATP cost of force production and/or  $O_2$  cost of ATP production), mitochondrial content, capillarity and blood flow responses. Brittain et al. (2001) speculated that the motor unit pool from which fibers would be recruited in the low work rates would be more oxidative and ‘efficient’ as compared to those recruited at a raised WR.

Furthermore, Bowen et al. (2011) observed that when exercise transitions were initiated from a common light-intensity WR but with either a low or high  $\dot{V}O_{2p}$ , and thus different “baseline” metabolic rates,  $\dot{V}O_{2p}$  kinetics were slower when exercise was initiated from the

higher baseline  $\dot{V}O_{2p}$ . This suggests that energetic state of the recruited muscle may impact the adjustment of  $\dot{V}O_{2p}$  (and  $\dot{V}O_{2m}$  and mitochondrial oxidative phosphorylation) during an exercise transition - e.g., transitions from a higher baseline WR and/or metabolic rate would be expected to have lower muscle [PCr] and local [ATP]; greater [ADP<sub>free</sub>] and [Pi], and lower Gibbs free energy of ATP hydrolysis ( $\Delta G_{ATP}$ ) (Korzeniewski & Zoladz, 2006; Zoladz *et al.*, 2006). A decrement in the cellular energetic status has been shown to be capable of slowing the rate of oxidative phosphorylation in ensuing transitions in healthy young (Barstow *et al.*, 1994), isolated mitochondria *in vitro* (Glancy *et al.*, 2008; Kemp, 2008). Furthermore, while there have been studies that have examined transitions from the moderate-intensity domain into the heavy-intensity domain (Jones *et al.*, 2008; Dimenna *et al.*, 2010b) the muscle metabolic response during a “double-step” protocol performed within the MOD exercise has not been examined.

Therefore, the primary purpose of the study was to examine; (1) the rate of adjustment of  $\dot{V}O_{2p}$  and muscle deoxygenation during transitions from a low and elevated baseline metabolic rates performed within the MOD exercise domain; (2) the muscle metabolic response during these transitions using direct muscle sampling; and (3) the relationship between  $\dot{V}O_{2p}$  kinetics and changes in specific muscle metabolites determining the muscle energetic state (i.e., [ADP<sub>free</sub>], [ATP], [PCr], [Pi]). It was hypothesized 1) that  $\dot{V}O_{2p}$  kinetics would be slower and that the  $\dot{V}O_{2p}$  gain would be greater during exercise transitions initiated from a higher metabolic rate; and 2) that muscle [PCr] would be lower, [ADP<sub>free</sub>] and [Pi] would be higher, and the calculated  $\Delta G_{ATP}$  would be lower with exercise initiated from a higher metabolic rate; and 3) the  $\tau \dot{V}O_{2p}$  would be inversely related to  $\Delta G_{ATP}$ .

## 2.2 METHODS

*Participants.* Young, healthy men (n = 10; 25 ± 2 yr; mean ± SD) volunteered and gave written informed consent to participate in this study. All procedures were approved by The University of Western Ontario’s Research Ethics Board for Health Sciences Research Involving Human Subjects. Participants were recreationally active and non-smokers, and had no known cardiovascular, respiratory, metabolic or musculoskeletal disease, nor were they taking medications that might affect the cardiorespiratory and hemodynamic responses to exercise.

Participants were instructed not to consume food or caffeine two hours prior to visits to the laboratory for data collection.

*Exercise protocol.* At the start of the study, participants reported to the laboratory to perform a ramp incremental exercise test (20-25 W/min) to the limit of tolerance on a cycle ergometer (model: H-300-R Lode; Lode B.V., Groningen, Holland) for determination of peak  $\dot{V}O_{2p}$  ( $\dot{V}O_{2p\text{ peak}}$ ) and estimated lactate threshold ( $\hat{\theta}_L$ ); the ramp portion of the protocol was initiated after 4 min baseline cycling at 20 W. Participants were asked to maintain a cycling cadence between 60-70 rpm. The  $\hat{\theta}_L$  was estimated by visual inspection using standard gas exchange and ventilatory measures as described previously (Beaver *et al.*, 1986) and using the following criteria: the  $\dot{V}O_{2p}$  at which  $\dot{V}CO_{2p}$  CO<sub>2</sub> output ( $\dot{V}CO_{2p}$ ) began to increase out of proportion in relation to  $\dot{V}O_{2p}$ , with a systematic rise in minute ventilation ( $\dot{V}_E$ )-to- $\dot{V}O_{2p}$  ratio and end-tidal PO<sub>2</sub>, whereas the  $\dot{V}_E$ -to- $\dot{V}CO_{2p}$  ratio and end-tidal PCO<sub>2</sub> remained stable (Beaver *et al.*, 1986). From the results of the ramp incremental test, a moderate-intensity WR (WR90) was selected to elicit a  $\dot{V}O_{2p}$  equivalent to ~ 90% of the  $\dot{V}O_{2p}$  at  $\hat{\theta}_L$ . Subjects returned to the laboratory on five separate occasions and performed a “double-step” exercise protocol which consisted of: 6 min baseline cycling at 20 W, followed by a lower step-transition (LS) from 20 W to a WR (WR50) midway between 20 W and the WR90 ( $\Delta WR_{LS}$ ), and then followed immediately by an upper step-transition (US) from WR50 to WR90 ( $\Delta WR_{US}$ ); both step transition were performed consecutively and the change in WR was identical for both transitions (i.e.,  $\Delta WR_{LS} = \Delta WR_{US}$ ). Transitions lasted 6 min to establish a well-defined  $\dot{V}O_{2p}$  state-state ( $\dot{V}O_{2ss}$ ). Participants maintained a cadence of ~ 70 rpm during these tests, which they monitored on a screen in front of them.

*Muscle sampling.* During an additional visit to the laboratory, muscle biopsies were obtained from the vastus lateralis muscle using the needle biopsy technique (Bergstrom, 1975) as the subjects performed ‘double-step’ exercise transitions, consistent with the above protocol. Before exercise, five biopsy sites (three on the left leg and two on the right leg) were prepared under local anesthesia (2% lidocaine without epinephrine) by making small incisions through the skin to the deep fascia. The subject then moved to the cycle ergometer and began baseline cycling at

20 W. A muscle biopsy sample was taken after 4 min baseline cycling and again at 15 s and 6 min during each of the step-transitions to LS. Two minutes of cycling were added following the needle biopsy at the steady-state in LS prior to the biopsy taken during the transition from LS to US to allow for the re-establishment of steady-state in the LS. Muscle biopsy samples were frozen immediately in liquid N<sub>2</sub> (<10 s from the cessation of exercise), removed from the needle while frozen, and stored in liquid N<sub>2</sub> until later analysis.

*$\dot{V}O_{2p}$  measurement.* Gas exchange measurements were similar to those previously described (Babcock *et al.*, 1994). Briefly, inspired and expired volumes were measured using a low dead space (90 mL) bidirectional turbine (Alpha Technologies VMM 110) which was calibrated before each test using a syringe of known volume. Inspired and expired gases were continuously sampled (50 Hz) at the mouth and analyzed for concentrations of O<sub>2</sub>, CO<sub>2</sub>, and N<sub>2</sub> by mass spectrometry (Innovision, AMIS 2000, Lindvedvej, Denmark) after calibration with precision-analyzed gas mixtures. Changes in gas concentrations were aligned with gas volumes by measuring the time delay for a square-wave bolus of gas passing the turbine to the resulting changes in fractional gas concentrations as measured by the mass spectrometer. Data were transferred to a computer, which aligned concentrations with volume information to build a profile of each breath. Breath-by-breath alveolar gas exchange was calculated by using algorithms of Beaver *et al.* (1981).

*Near-infrared spectroscopy.* Local muscle deoxygenation ([HHb]) of the quadriceps vastus lateralis muscle was monitored continuously with a frequency-domain multi-distance near-infrared spectroscopy (NIRS) system (Oxiplex TS, Model 95205, ISS, Champaign, IL, USA) as described elsewhere (Spencer *et al.*, 2012a). The probe was placed on the belly of the muscle, midway between the lateral epicondyle and greater trochanter of the femur; it was secured in place with an elastic strap tightened to prevent movement and covered with an optically-dense, black vinyl sheet, thus minimizing the intrusion of extraneous light and loss of NIR light. An elastic bandage was applied to further minimize intrusion of extraneous light and probe movement. NIRS measurements were collected continuously for the entire duration of each trial. Briefly, the system was comprised of a single channel consisting of eight laser diodes operating at two wavelengths ( $\lambda = 690$  and 828 nm, four at each wavelength) which were pulsed in a rapid

succession (110 MHz) and a photomultiplier tube. The lightweight plastic NIRS probe (connected to laser diodes and photomultiplier tube by optical fibers) consisted of two parallel rows of light emitter fibers and one detector fiber bundle; the source-detector separations for this probe were 2.0, 2.5, 3.0, and 3.5 cm for both wavelengths.

The NIRS was calibrated at the beginning of each testing session following an instrument warm-up period of at least 20 min (as recommended by the manufacturer). Calculation of [HHb] reflected continuous measurements of a reduced scattering coefficient ( $\mu_s'$ ) made throughout each testing session (i.e., constant scattering value not assumed). Data were stored online at an output frequency of 25 Hz, but were reduced to 1 s bins for all subsequent analyses within the present study.

*Heart-rate measurement.* HR was monitored continuously by electrocardiogram (three-lead arrangement) using PowerLab (ML132/ML880; ADInstruments, Colorado Springs, CO) and was calculated (using a 5 s rolling average) based upon the R-R interval. Data were recorded using LabChart v6.1 (ADInstruments, Colorado Springs, CO) on a separate computer.

*Modelling of the  $\dot{V}O_{2p}$ , HR and NIRS deoxygenation responses.* Breath-by-breath  $\dot{V}O_{2p}$  data were edited by removing data that lay outside 4 standard deviations of the local mean (Lamarra *et al.*, 1987) The remaining data were interpolated to 1 s intervals, and time-aligned so that time zero represented the initiation of the step-increase in WR.

The ensemble-averaged, second-by-second  $\dot{V}O_{2p}$  and HR responses were further time-averaged into 5 s bins. The on-transient responses for  $\dot{V}O_{2p}$  and HR were modeled using the following equation:

$$Y_{(t)} = Y_{\text{BSLN}} + A (1 - e^{-(t-\text{TD})/\tau}) \quad \text{Equation 1}$$

where  $Y_{(t)}$  represents the  $\dot{V}O_{2p}$  or HR at any given time ( $t$ );  $Y_{\text{BSLN}}$  is the steady-state baseline value of  $Y$  before an increase in WR (given as the average  $Y$  value in the 80-20 s period immediately prior to a transition);  $A$  is the amplitude of the increase in  $Y$  above  $Y_{\text{BSLN}}$ ;  $\tau$  is the time constant and represents the time required to attain 63% of the steady-state amplitude; and

TD represents the mathematically generated time delay through which the exponential model is predicted to intersect  $Y_{BSLN}$ . Data were modeled from Phase I  $\dot{V}O_{2p}$  in the fitting of Phase II while still allowing TD to vary freely (in order to optimize accuracy of parameter estimates).  $\dot{V}O_{2p}$  data were modeled to 4 min (240 s) of the step-transition; this ensured that each subject had attained a  $\dot{V}O_{2p}$  steady-state, yet did not bias the model fit during the on-transient (Bell *et al.*, 2001). HR data were modeled from exercise onset to the end of the 6 min exercise transition with TD constrained to  $\geq -10$  s and baseline fixed as described above (i.e., 60 s average during the 80-20 s period prior to a transition). The model parameters were estimated by least-squares nonlinear regression (Origin, OriginLab Corp., Northampton, MA, USA) in which the best fit was defined by minimization of the residual sum of squares and minimal variation of residuals around the Y-axis ( $Y = 0$ ). The 95% confidence interval ( $CI_{95}$ ) for the estimated time constant was determined after preliminary fit of the data with  $Y_{BSLN}$ , A, and TD constrained to the best-fit values and the  $\tau$  allowed to vary.

The mean response time (MRT) (Linnarsson, 1974) of  $\dot{V}O_{2p}$  described the overall time course of  $\dot{V}O_{2p}$  during the exercise transition and was estimated using the function described in Eq. 1, but with inclusion of all  $V\dot{\square}O_{2p}$  data from the onset of exercise, and the TD constrained to 0 s. This approach allowed for an estimate of the  $O_2$  deficit (Rossiter *et al.*, 1999) for each WR transition. The  $O_2$  deficit provides information on non-oxidative energy transfer and was calculated as:

$$O_2 \text{ deficit (l)} = \text{MRT (s)} * \Delta \dot{V}O_{2p_{ss}} \text{ (L/min)} * \text{min}/60\text{s} \quad \text{Equation 2}$$

The functional gain of the fundamental  $V\dot{\square}O_{2p}$  response was calculated as  $\Delta \dot{V}O_{2p_{ss}}/\Delta WR$  ( $\text{ml}\cdot\text{min}^{-1}\cdot\text{W}^{-1}$ ), where  $\dot{V}O_{2p_{ss}}$  is steady-state increase in  $O_2$  uptake above baseline.

The NIRS-derived [HHb] profiles were time-aligned and ensemble-averaged into 5 s bins to yield a single response time for each subject. The time-course of adjustment for the [HHb] profile has been previously described as consisting of a time delay at the onset of exercise, with a subsequent “exponential-like” increase in the signal with time of exercise (DeLorey *et al.*, 2003). The time delay for the [HHb] response (TD[HHb]) was determined visually using the second-by-



second data and corresponded to the time between the step-increase in WR and the time where the [HHb] signal began a systematic increase from its nadir value. Determination of the TD[HHb] was made on individual response profiles, averaged for the five trials and reported as the WR-specific response for each subject. The ensemble-averaged [HHb] responses were modeled from TD[HHb] to 90 s of the transition, with a monoexponential function of the form in Eq. 1 to determine the time course of muscle [HHb] ( $\tau$ [HHb]). A systematic decline in [HHb] did not occur prior to 90 s of the transition in any subjects. Baseline [HHb] ( $[\text{HHb}]_{\text{BSLN}}$ ) values were fixed as the mean value in the 80-20 s period leading up to a transition, as described above with  $\dot{V}\text{O}_{2p}$  and HR. Whereas the  $\tau$ [HHb] describes the time course for the increase in [HHb], the effective time constant, or mean response time, of [HHb] ( $\tau'[\text{HHb}] = \text{TD}[\text{HHb}] + \tau[\text{HHb}]$ ) described the overall time course of the [HHb] from the onset of each step transition.

*Muscle analysis.* Small pieces of frozen wet muscle (55-60 mg) were removed from each biopsy under liquid N<sub>2</sub> for the determination of PDH activity as described elsewhere (Putman *et al.*, 1993). An aliquot of each muscle enzyme homogenate was then extracted with 0.5 mol.L<sup>-1</sup> perchloric acid (HClO<sub>4</sub>), containing 1 mmol.L<sup>-1</sup> EDTA, and neutralized with 2.2 mol.L<sup>-1</sup> KHCO<sub>3</sub>. The supernatant from the extracts was used for the enzymatic spectrophotometric determination of total creatine. Enzyme measurements were normalized to the highest total creatine content measured from all biopsies from each subject. A portion of muscle from each biopsy was freeze dried, dissected free of visible blood and connective tissue, and powdered for metabolic and glycogen analyses. An aliquot of freeze-dried muscle (10-12 mg) was extracted with 0.5 mol.L<sup>-1</sup> perchloric acid, containing 1 mmol.L<sup>-1</sup> EDTA, and neutralized with 2.2 mol.L<sup>-1</sup> KHCO<sub>3</sub>. The supernatant was used to determine creatine, [PCr], ATP, and lactate levels, with enzymatic spectrophotometric assays (Bergmeyer, 1974). Muscle glycogen content was determined from 2 aliquots of freeze-dried muscle from all biopsies, as described elsewhere (Bergmeyer, 1974). Muscle metabolites were corrected for total creatine, as described above.

*Calculations.* Muscle contents of free [ADP] ( $[\text{ADP}]_{\text{free}}$ ) and AMP were calculated by assuming equilibrium of the creatine kinase and adenylate kinase reactions, respectively (Dudley *et al.*, 1987). The  $[\text{ADP}]_{\text{free}}$  was calculated by using the measured [ATP], [Cr], [PCr], estimated  $[\text{H}^+]$  concentration, and the creatine kinase equilibrium constant of  $1.66 \times 10^6$ ;  $[\text{H}^+]$  concentration was

calculated from the measured pyruvate and lactate contents as described by Sahlin et al. (1976) (Sahlin *et al.*, 1976). Free inorganic phosphate (Pi) in exercise was calculated by assuming an estimated free phosphate content at rest of 10.8 mmol/kg dry wt (Dudley *et al.*, 1987) and adding this value to the difference in [PCr] content relative to the baseline value (Harris *et al.*, 1974). The Gibbs free energy change for ATP hydrolysis ( $\Delta G_{ATP}$ ) was calculated as described previously (Meyer, 1988).

*Statistics.* Values are presented as mean  $\pm$  SD. Parameter estimates for  $\dot{V}O_{2p}$ , NIRS-derived [HHb] and HR, and muscle metabolite data were analyzed using repeated-measures ANOVA with time as within-subject factor using SPSS version 20.0 (SPSS, Chicago, IL). Statistical significance was accepted at  $p < 0.05$ .

### 2.3 RESULTS

*Absolute and relative workloads of US and LS.* The  $\Delta WR$  from the baseline to the LS was  $62 \pm 16$  (LS WR =  $82 \pm 16$  W) was  $63 \pm 0.1\%$  of the  $\hat{\theta}_L$ , and  $35 \pm 0.1\%$  of the  $\dot{V}O_{2p\text{peak}}$ . The  $\Delta WR$  from the LS to US was  $62 \pm 16$  (LS WR =  $143 \pm 43$  W) was  $89 \pm 0.1\%$  of the  $\hat{\theta}_L$ , and  $50 \pm 0.1\%$  of the  $\dot{V}O_{2p\text{peak}}$ . A summary of subject characteristics are presented in Table 1.

*$\dot{V}O_{2p}$  kinetics.* Figure 1 displays the  $V\dot{\square}O_{2p}$  response profile for all subjects (Fig. 1A) and for a representative subject (Fig. 1B) during exercise transitions initiated from a low and higher metabolic rate and WR, including model line-of-best-fit and residuals. A summary of parameter estimates for the on-transient  $\dot{V}O_{2p}$  response for the LS and US aspects of the protocol are presented in Table 2. The  $\tau \dot{V}O_{2p}$  was greater ( $p < 0.05$ ) in the US ( $34 \pm 12$  s) compared to the LS ( $20 \pm 8$  s). Despite the identical increase in WR in LS ( $62 \pm 16$  W) and US ( $62 \pm 16$  W), the  $\dot{V}O_{2p}$  gain ( $\Delta \dot{V}O_{2p} / \Delta WR$ ) was greater ( $p < 0.05$ ) in the US ( $10.37 \pm 1.49$ ) versus that of the LS ( $8.51 \pm 1.41$ ). The calculated  $O_2$  deficit was greater in the US ( $0.48 \pm 0.25$  L) than in the LS ( $0.30 \pm 0.14$  L).

*NIRS-Derived [HHb] Kinetics and Muscle Oxygenation Parameters.* The ensemble-averaged [HHb] group response during the LS and US transitions (Fig. 2A) and the [HHb] response for a representative subject (Fig. 2B) are presented in Figure 2, including model line-of-best-fit and

residuals. The group mean profiles of total hemoglobin content ([THC]; Fig. 3A), O<sub>2</sub> saturation (%O<sub>2</sub>sat; Fig. 3B), oxygenated hemoglobin ([HbO<sub>2</sub>]; Fig. 3C), and deoxygenated hemoglobin ([HHb]; Fig. 3D) are displayed in Figure 3. A summary of parameter estimates for the on-transient [HHb] response for LS and US are presented in Table 3. The time delay (TD) before the increase in muscle deoxygenation, although not different statistically ( $p = 0.06$ ), tended to be greater in LS ( $10 \pm 3$  s) and US ( $7 \pm 4$ ). After the TD, the rate of adjustment of [HHb] ( $\tau$ [HHb]) was greater ( $p < 0.05$ ) in US ( $16 \pm 5$  s) than in LS ( $11 \pm 5$  s); the overall rate of adjustment of muscle deoxygenation from the start of the exercise transitions (i.e., effective time constant or mean response time ( $\tau'$ [HHb] =  $\tau$ [HHb] + TD) was not different in US ( $22 \pm 7$  s) and LS ( $22 \pm 7$  s). The [HHb]<sub>bsl</sub> and [HHb]<sub>ss</sub> were greater ( $p < 0.05$ ) in US than in LS, while the [HHb]<sub>amp</sub> was lower ( $p < 0.05$ ) in US (Table 3).

*Heart rate response.* A summary of parameter estimates for the on-transient HR response for LS and US are presented in Table 4. The rate of adjustment of heart rate ( $\tau$  HR) was slowed ( $p < 0.05$ ) during the transition in the US ( $29 \pm 9$  s) compared to the LS ( $17 \pm 9$  s). The HR<sub>bsl</sub>, HR<sub>amp</sub> and HR<sub>ss</sub> were elevated significantly ( $p < 0.05$ ) in the US versus the LS (Table 4).

*Muscle metabolite content.* Changes in the measured and calculated muscle metabolites are presented in Tables 5 and 6. Muscle ATP content ([ATP]) remained at the BSL<sub>20W</sub> level ( $21.2 \pm 1.0$  mmol/kg dw) during the LS and US exercise transitions (Table 5). Muscle [PCr] tended to be lower ( $p = 0.06$ ) at LS<sub>15s</sub> ( $74 \pm 3$  mmol/kg dw) compared to BSL<sub>20W</sub> ( $79 \pm 2$  mmol/kg dw), and was further reduced ( $p < 0.05$ ) at LS<sub>360s</sub> ( $67 \pm 3$  mmol/kg dw) and US<sub>360s</sub> ( $63 \pm 3$  mmol/kg dw) (Table 5). The relative decrease in [PCr] for an identical WR change ( $\Delta$ [PCr]/ $\Delta$ WR) was not different between US and LS. Muscle [Pi] tended to increase ( $p = 0.06$ ) from BSL<sub>20W</sub> ( $10.8$  mmol/kg dw) to LS<sub>15s</sub> ( $15.5 \pm 2$  mmol/kg dw), and was higher ( $p < 0.05$ ) from BSL<sub>20W</sub> and LS<sub>15s</sub> at LS<sub>360s</sub> ( $22.8 \pm 3$  mmol/kg dw) and US<sub>360s</sub> ( $26.8 \pm 3$  mmol/kg dw) (Table 5). There was an elevation ( $p < 0.05$ ) in muscle [ADP<sub>free</sub>] from the BSL<sub>20W</sub> ( $85 \pm 4$   $\mu$ mol/kg dw) at LS<sub>15s</sub> ( $101.26 \pm 6$   $\mu$ mol/kg dw) and remained elevated ( $p < 0.05$ ) from BSL<sub>20W</sub> throughout the transitions. There was also a greater ( $p < 0.05$ ) muscle [ADP<sub>free</sub>] at US<sub>360s</sub> ( $131 \pm 13$   $\mu$ mol/kg dw) than at LS<sub>360s</sub> ( $123 \pm 10$   $\mu$ mol/kg dw) (Table 5). The increase in  $\Delta$ [ADP<sub>free</sub>] during LS and US were not different. The calculated  $\Delta G_{ATP}$  at the LS<sub>360s</sub> and US<sub>360s</sub> were similar and both were lower than

the  $\Delta G_{ATP}$  at BSL<sub>20w</sub> (Table 5). There was a strong positive correlation (Pearson's  $r$  correlation coefficient = 0.62) between  $\tau \dot{V}O_{2p}$  and  $\Delta G_{ATP}$  during transitions from both low and elevated steady-states. The group, ensemble average response of  $[ADP_{free}]$  following transitions from lower and elevated metabolic baselines are illustrated in Figure 4. The steady-state and modeled transition  $\dot{V}O_{2p}$  as a function of  $[ADP_f]$  and  $[PCr]$  (Figure 5, 6 respectively) during moderate intensity double-step exercise transitions are shown.

Muscle  $[\text{lactate}^-]$  content was elevated ( $p < 0.05$ ) above BSL<sub>20w</sub> throughout the entire protocol (Table 6); the muscle  $[\text{lactate}^-]$  at US<sub>360s</sub> was greater ( $p < 0.05$ ) than at LS<sub>360s</sub>. Muscle  $[H^+]$  remained at BSL<sub>20w</sub> throughout the MOD exercise transitions.

*Table 1. Subject characteristics*

Parameter	
Age, yrs	23 ± 3
Mass, kg	86 ± 10
Height, cm	183 ± 6
$\dot{V}O_{2p\ peak}$ , L/min	4.31 ± 0.47
$\dot{V}O_{2p\ peak}$ , mL/kg/min	50 ± 4
$\hat{\theta}_L$	2.4 ± 0.27

Values are means ± SD;  $\dot{V}O_{2\ peak}$ , peak O<sub>2</sub> uptake;  $\hat{\theta}_L$ , estimated lactate threshold.

Table 2.  $\dot{V}O_{2p}$  kinetics parameters for LS and US moderate-intensity exercise transitions

Parameter	LS	US
$\dot{V}O_{2p \text{ bsl}}$ L/min	1.00 ± 0.12	1.54 ± 0.23*
$\dot{V}O_{2p \text{ ss}}$ , L/min	1.54 ± 0.23	2.20 ± 0.43*
$\dot{V}O_{2p \text{ amp}}$ , L/min	0.52 ± 0.15	0.63 ± 0.20
TD, s	15 ± 4	10 ± 7
$\tau \dot{V}O_{2p}$ , s	20 ± 8	34 ± 12*
$C_{95}$ , s	7 ± 2	7 ± 3
$\Delta \dot{V}O_{2p \text{ ss}}/\Delta WR$ , mL/min <sup>-1</sup> /W <sup>-1</sup>	8.51 ± 1.41	10.37 ± 1.49*
O <sub>2</sub> deficit	0.30 ± 0.14	0.48 ± 0.25*
$\dot{V}O_{2p \text{ ss}}$ , % $\dot{V}O_{2\text{max}}$	35.3 ± 4.4	49.9 ± 8.8*
WR, W	82 ± 16	143 ± 32*

Values are means ± SD;  $\dot{V}O_{2p \text{ bsl}}$ , baseline  $\dot{V}O_{2p}$ ;  $\dot{V}O_{2p \text{ ss}}$ , steady-state  $\dot{V}O_{2p}$ ; amp, amplitude of  $\dot{V}O_{2p}$  response; TD, time delay;  $\tau \dot{V}O_{2p}$ , time constant for  $\dot{V}O_{2p}$  response;  $C_{95}$ , 95% confidence interval of  $\tau \dot{V}O_{2p}$ ;  $\Delta \dot{V}O_{2p \text{ ss}}/\Delta WR$ , functional gain. \*Different from LS ( $P < 0.05$ ).

Table 3. [HHb] kinetics parameters for LS and US moderate-intensity exercise transitions

Parameter	LS	US
[HHb] <sub>bsl</sub> , a.u.	22.8 ± 5.6	26.6 ± 7.9*
[HHb] <sub>ss</sub> , a.u.	25.6 ± 9.6	29.7 ± 10.3*
[HHb] <sub>amp</sub> , a.u.	4.3 ± 3.5	3.5 ± 2.6*
TD, s	10 ± 3	7 ± 4 <sup>+</sup>
τ[HHb], s	11 ± 5	16 ± 5*
τ'[HHb], s	22 ± 7	22 ± 7

Values are means ± SD; [HHb]<sub>bsl</sub>, baseline; [HHb]<sub>ss</sub>, fitted steady-state; TD, calculated time delay; τ[HHb], time constant for [HHb] response; τ', effective time constant (τ[HHb] + TD);

\*Different from LS ( $P < 0.05$ ); <sup>+</sup> ( $P = 0.06$ )

Table 4. Parameter estimates for HR kinetics for LS and US moderate-intensity exercise transitions

Parameter	LS	US
HR <sub>bsl</sub> (beats/min)	89.5 ± 11	102 ± 10*
HR <sub>ss</sub> (beats/min)	103 ± 11	123 ± 13*
HR <sub>amp</sub>	15 ± 4	21 ± 7*
τHR, s	17 ± 9	29 ± 9*
C <sub>95</sub> , s	7 ± 4	6 ± 3

Values are means ± SD; HR<sub>bsl</sub>, baseline HR; HR<sub>ss</sub>, steady-state HR; amp, amplitude of HR response; TD, time delay; τHR, time constant for HR response; C<sub>95</sub>, 95% confidence interval of τHR. \*Different from LS ( $P < 0.05$ ).



Table 5. Muscle content of [PCr], [ATP], [ADP<sub>free</sub>], and [Pi] for LS and US moderate-intensity exercise transitions

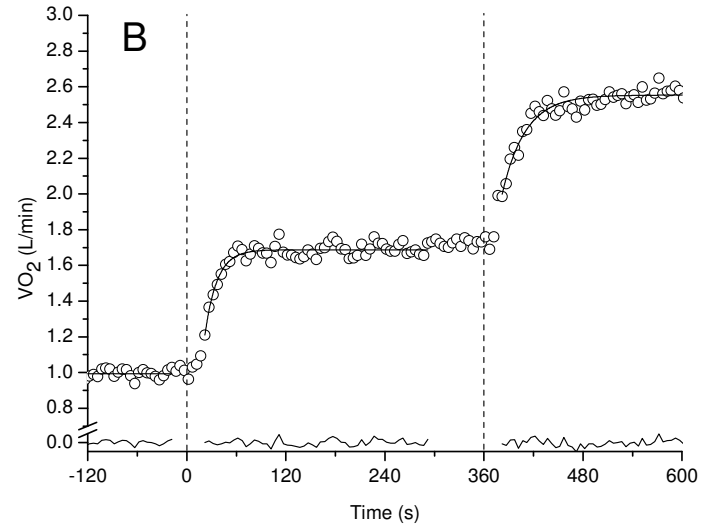
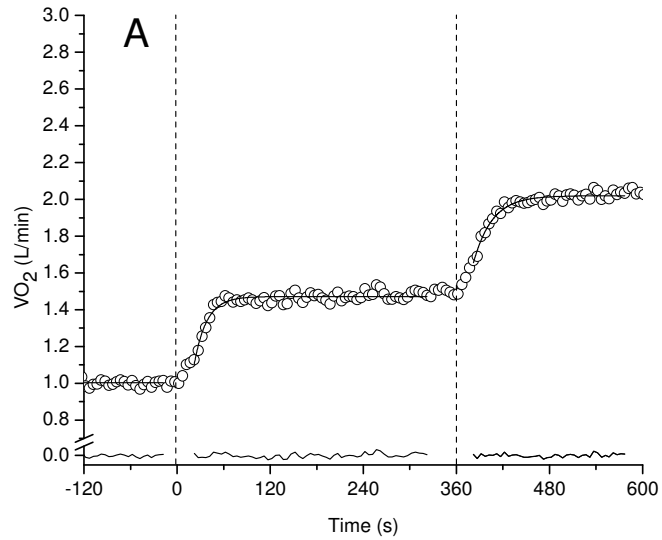
Parameter	LS			US	
	BSL <sub>20W</sub>	LS <sub>15s</sub>	LS <sub>360s</sub>	US <sub>15s</sub>	US <sub>360s</sub>
[ATP]	21.19	22.04	21.58	23.73	21.66
	±0.98	±1.27	±0.64	±0.64	±1.40
[PCr]	79.01	74.27	67.05*†	69.97*	63.40*†
	±1.51	±2.80	±2.74	±3.20	±3.38
[P <sub>i</sub> ]	10.8	15.54	22.76*†	20.29*	26.78*†
	±0	±2.44	±3.13	±3.29	±3.44
[ADP <sub>free</sub> ]	85.06	101.26*	123.32*	124.92*	131.30*†
	±4.30	±5.79	±10.42	±14.51	±13.04
ΔG <sub>ATP</sub>	-56.40	-55.31	-53.79*†	-54.86	-53.26*†
	±0.39	±1.36	±1.49	±2.33	±1.59

Values are means ± SD. Muscle biopsies samples at steady-state baseline at 20W (BSL<sub>20W</sub>), at 15 s during the exercise transition into the lower (LS<sub>15s</sub>) and upper (US<sub>15s</sub>) steps, and at steady-state after 360 s exercise in the lower (LS<sub>360s</sub>) and upper (US<sub>360s</sub>) steps. Muscle [Pi] was assumed to be 10.8 mmol/kg dw at rest (Dudley *et al.*, 1987), with the increase during exercise equivalent to the fall in muscle [PCr]. Muscle [ADP<sub>free</sub>] calculated according to (Dudley *et al.*, 1987) and Gibbs free energy (ΔG<sub>ATP</sub>) calculated according to (Meyer, 1988). Units are mmol/kg dry wt except free [ADP] ([ADP<sub>free</sub>]) which is in μmol/kg dry wt and ΔG<sub>ATP</sub>, which are in kJ/mol. *P* < 0.05, significant difference vs. BSL<sub>20W</sub> (\*), vs. LS<sub>15s</sub> (†), vs. LS<sub>360s</sub> (§), vs. US<sub>15s</sub> (§), vs. US<sub>360s</sub>

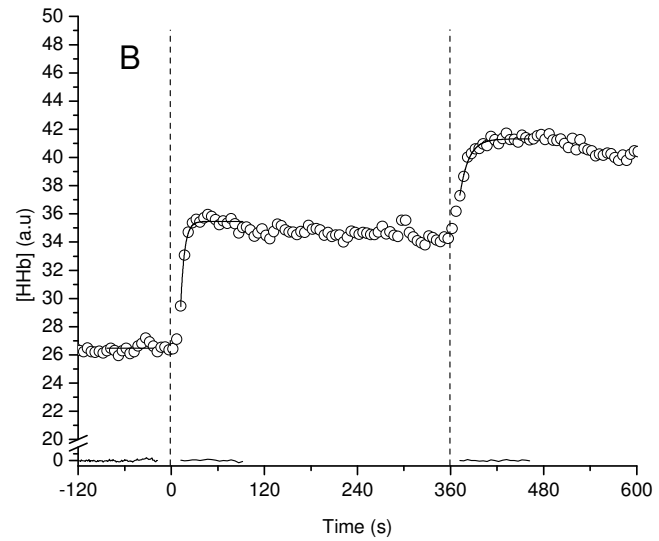
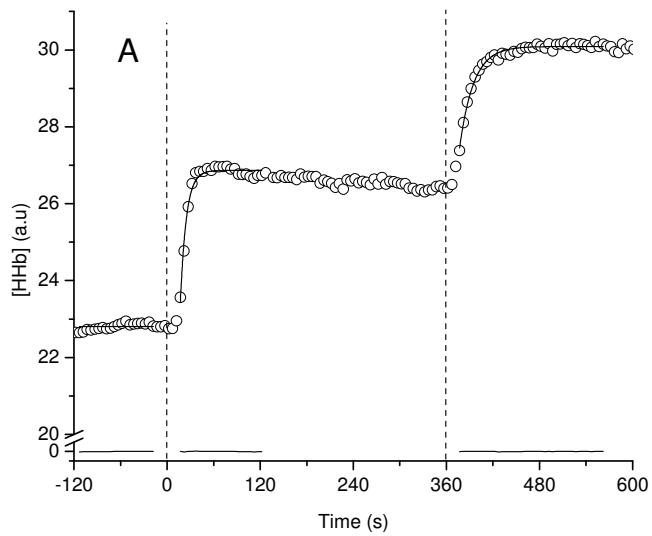
Table 6. Muscle contents of lactate<sup>-</sup> and c[H<sup>+</sup>] for LS and US moderate-intensity exercise transitions

Parameter	<i>LS</i>			<i>US</i>	
	BSL <sub>20W</sub>	LS <sub>15s</sub>	LS <sub>360s</sub>	US <sub>15s</sub>	US <sub>360s</sub>
[Lactate]	4.11	5.69*	8.20*	8.06*	14.32*†‡§
	±0.42	±0.78	±1.70	±2.15	±3.20
c[H <sup>+</sup> ]	108.85	110.42	113.04	113.66	118.91
	±1.30	±2.45	±5.66	±6.82	±10.40

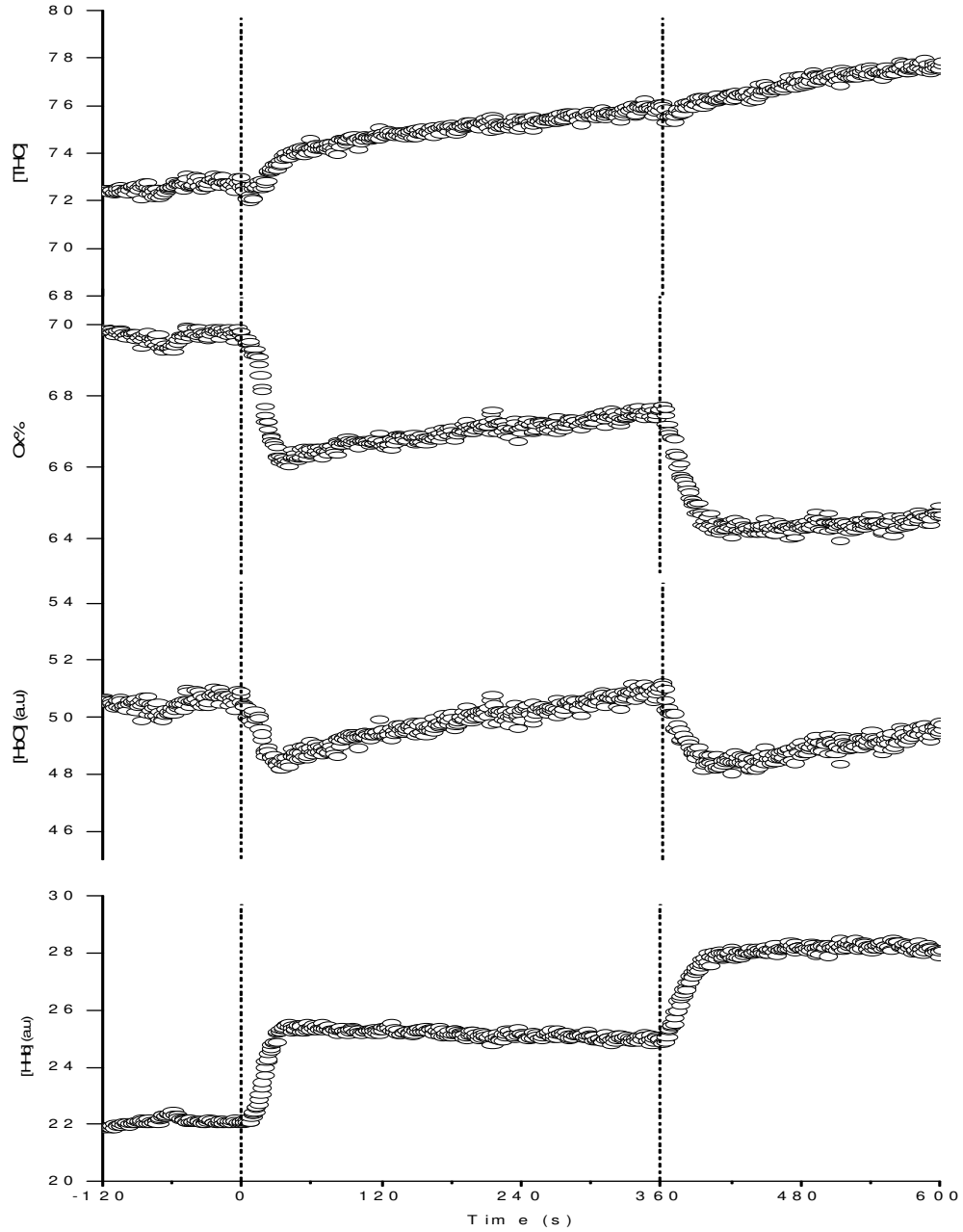
Values are means ± SD. Muscle biopsies samples at steady-state baseline at 20W (BSL<sub>20W</sub>), at 15 s during the exercise transition into the lower (LS<sub>15s</sub>) and upper (US<sub>15s</sub>) steps, and at steady-state after 360 s exercise in the lower (LS<sub>360s</sub>) and upper (US<sub>360s</sub>) steps. Muscle content values are expressed as mmol/kg dry wt except for calculated H<sup>+</sup> concentration (c[H<sup>+</sup>]), which is expressed as 10<sup>-9</sup> mol/L. *P* < 0.05, significant difference vs. BSL<sub>20W</sub> (\*), vs. LS<sub>15s</sub> (†), vs. LS<sub>360s</sub> (‡), vs. US<sub>15s</sub> (§), vs. US<sub>360s</sub>



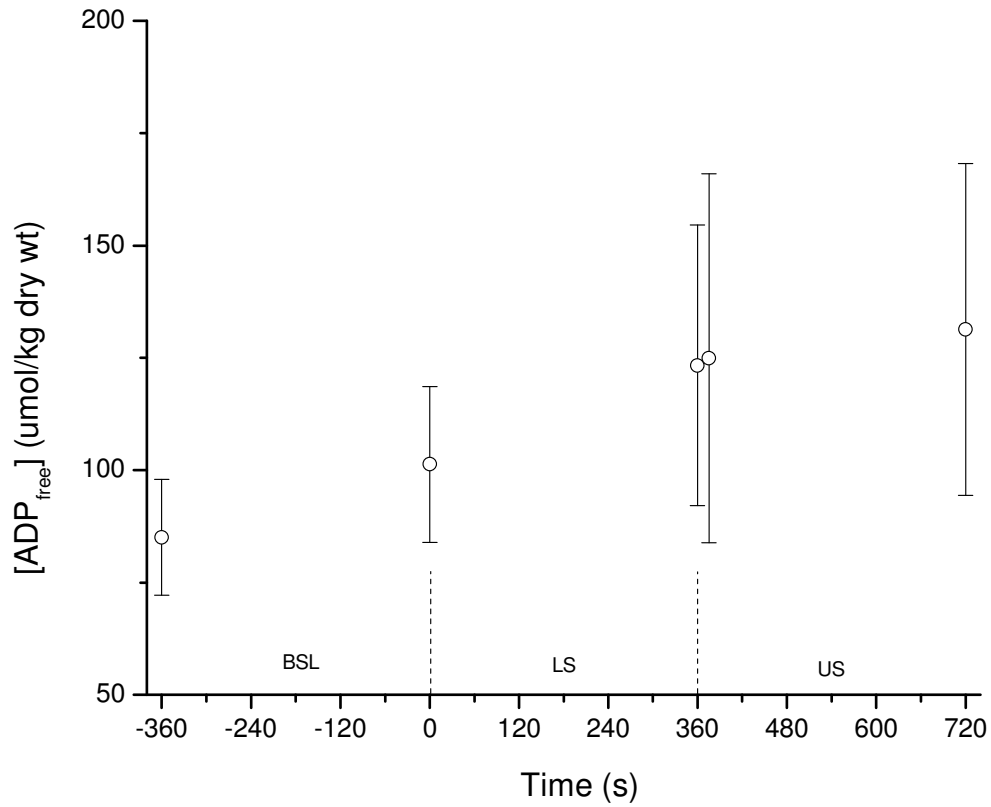
**Figure 1A.** Ensemble-averaged, group mean breath-by-breath  $\dot{V}O_{2p}$  responses to moderate intensity double-step transitions as a function of time. **Figure 1B.**  $\dot{V}O_{2p}$  response to exercise transitions in the LS and US for a representative subject showing model line-of-best-fit and residuals. Dashed vertical lines represent the time of onset of the LS and US.



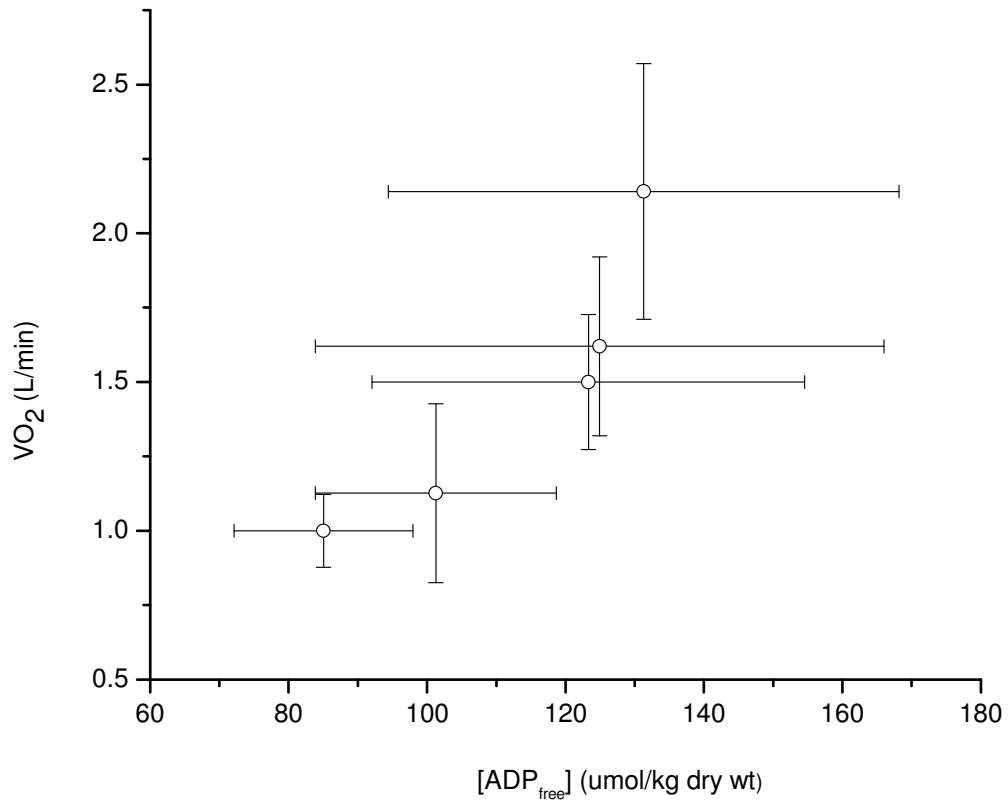
**Figure 2A.** Ensemble-averaged, group mean NIRS-derived [HHb] response to moderate intensity double-step transitions as a function of time. **Figure 2B.** NIRS-derived [HHb] response to exercise transitions in the LS and US for a representative subject showing model line-of-best-fit and residuals. Dashed vertical lines represent the time of onset of the LS and US.



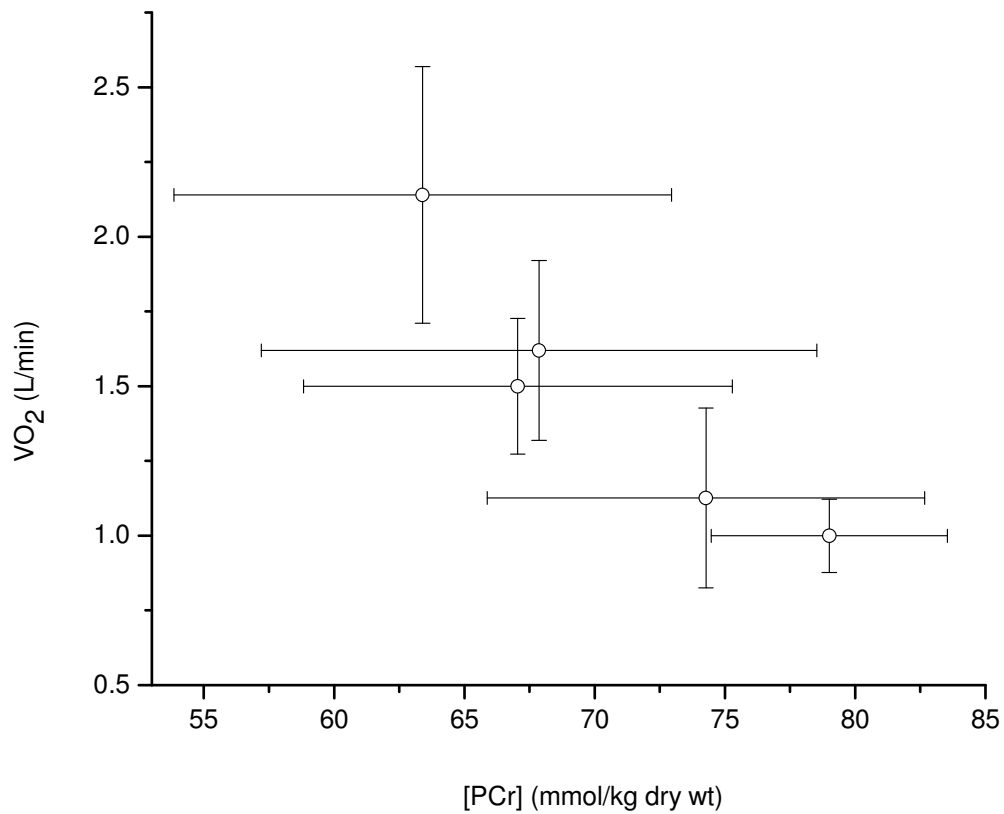
**Figure 3.** Ensemble-averaged NIRS-derived profiles for total hemoglobin content ([THC]; 3A), %O<sub>2</sub> saturation ([%O<sub>2</sub>sat];3), oxyhemoglobin content ([HbO<sub>2</sub>]; 3C), and deoxyhemoglobin content ([HHb]; 3D ), during LS and US transitions Dashed vertical lines represent the time of onset of the LS and US.



**Figure 4.** Intramuscular [ADP<sub>free</sub>] ( $\mu\text{mol/kg dry wt}$ ) at steady state baseline (BSL;20W), and at 15s and 360s during the transitions into LS and US. Values are mean  $\pm$  SD. Vertical dashed lined indicate the time corresponding to an instantaneous increase in work rate for the lower (LS; time = 0s) and upper (US; time = 360s) step-transitions.



**Figure 5.** Relationship between [ADP<sub>free</sub>] ( $\mu\text{mol/kg dry wt}$ ) and estimated muscle oxygen uptake (L/min) during lower and upper step transitions in work rate. Values are mean  $\pm$  SD



**Figure 6.** Relationship between [PCr] (mmol/kg dry wt) and estimated muscle oxygen uptake (L/min) during lower and upper step transitions in work rate. Values are mean  $\pm$  SD



## 2.4 DISCUSSION

This study investigated the adjustments to pulmonary  $\dot{V}O_2$  uptake ( $\dot{V}O_{2p}$ ), NIRS-derived muscle deoxygenation ([HHb]), and muscle metabolism during moderate-intensity step-transitions in work rate initiated for a low and elevated metabolic rate. In agreement with previous reports (MacPhee *et al.*, 2005; Bowen *et al.*, 2011; Spencer *et al.*, 2011; Williams *et al.*, 2013) when exercise was initiated from a high (US) compared to low (LS) baseline metabolic rate: (1) the rate of adjustment of  $\dot{V}O_{2p}$  was slower (i.e., the  $\tau \dot{V}O_{2p}$  was longer) and the  $\dot{V}O_{2p}$  gain ( $\Delta \dot{V}O_{2p} / \Delta WR$ ) was greater in US; (2) the delay before a rise in muscle deoxygenation (TD [HHb]) was shorter ( $p=0.06$ ) and the adjustment of [HHb] was slower (longer  $\tau$ [HHb]) in US, but the overall rate of adjustment of muscle deoxygenation ( $\tau'$ [HHb]) was not different despite slower  $\dot{V}O_{2p}$  kinetics in US; and (3) HR kinetics were slower in US. Novel findings related to the muscle metabolic response during the moderate-intensity “double-step” exercise transitions were that: (4) the increase in muscle [ADP<sub>free</sub>] and [P<sub>i</sub>], and decrease in muscle [PCr] that occurred during exercise transitions into LS, tended to be greater than seen during transitions into the US, despite identical increases in WR and slower  $\dot{V}O_{2p}$  kinetics (and thus, presumably, a slower adjustment of muscle oxidative phosphorylation); and (5) the Gibbs free energy for ATP hydrolysis ( $\Delta G_{ATP}$ ) was lower in LS and US compared to BSL<sub>20W</sub>, but was not different between LS and US.

To our knowledge, this is the first study to examine the kinetics of  $\dot{V}O_{2p}$  and muscle deoxygenation in combination with intramuscular high energy phosphate metabolism during exercise transitions constrained within the moderate-intensity exercise domain but initiated from a lower and higher baseline metabolic rates. The slower adjustment of  $\dot{V}O_{2p}$  and greater increase in  $\dot{V}O_{2p}$  for a given  $\Delta WR$  ( $\Delta \dot{V}O_{2p} / \Delta WR$ ) when exercise was initiated from the higher compared to lower baseline metabolic rate may provide information on limitation and regulation of  $\dot{V}O_{2p}$ , and thus on activation of muscle mitochondrial respiration.

Collectively, these data suggest that; (1) activation and adjustment of mitochondrial oxidative phosphorylation is slower, and the  $O_2$  cost per  $\Delta WR$  (and per  $\Delta$ [ATP requirement])

was greater when exercise is executed from a higher baseline metabolic rate; (2) the greater steady-state increase in  $\dot{V}O_{2p}$  (and mitochondrial oxidative phosphorylation) in US (0.69 L/min) compared to LS (0.54 L/min) and slower  $\dot{V}O_{2p}$  kinetics in US ( $\tau \dot{V}O_{2p}$ , 34 s vs. LS, 20 s) cannot be explained simply by the absolute increase in  $[ADP_{free}]$  (US, 8.0 vs. LS, 38.3  $\mu\text{mol/kg dw}$ ), or  $[Pi]$  (US, 4.0 vs LS, 12.0 mmol/kg dw), or by the absolute decrease in  $[PCr]$  (US, 3.7 vs. LS, 12.0 mmol/kg dw), or  $\Delta G_{ATP}$  (US, -0.53 vs. LS, -2.61 kJ/mol), which were all the same or lower in US than in LS; and (3) a slowed adjustment of  $O_2$  delivery to muscle (based on slower HR kinetics (perhaps reflecting a slower adjustment of cardiac output and central bulk blood flow; and similar overall adjustment of muscle deoxygenation (reflecting muscle fractional  $O_2$  extraction and thus the local muscle microvascular blood flow-to- $O_2$  utilization ratio).

Three hypotheses have been advanced to explain the relatively slow adjustment of  $\dot{V}O_{2p}$  (and  $\dot{V}O_{2m}$ , and thus mitochondrial oxidative phosphorylation) compared to the instantaneous increase in muscle ATP turnover that are seen during transitions to exercise. These can be summarized as: (1) an  $O_2$  delivery limitation where convective and diffusive  $O_2$  delivery to muscle and the mitochondrial cytochrome oxidase is not sufficient (Tschakovsky & Hughson, 1999); and (2) a “sluggish” activation of rate limiting enzymes and provision of oxidative substrate (other than  $O_2$ ) (i.e., reducing equivalents (NADH;  $FADH_2$ , ADP, Pi) to the mitochondrial TCA cycle and ETC (Grassi, 2000, 2001; Jones *et al.*, 2011; Rossiter, 2011); (3) that  $\dot{V}O_{2p}$  is slowed when initiated from a high compared to low baseline metabolic rate and WR is observed consistently, regardless of whether the exercise transitions are confined within the moderate-intensity domain (Hughson & Morrissey, 1982; di Prampero *et al.*, 1989; Brittain *et al.*, 2001; MacPhee *et al.*, 2005; Spencer *et al.*, 2011; Williams *et al.*, 2013), or whether the transitions are performed in both the moderate- and heavy-intensity domains (i.e., intensities below and above  $\hat{\theta}_L$ ) (Wilkerson & Jones, 2007; DiMenna *et al.*, 2010a). Studies using the double-step protocol have primarily focused upon mechanisms by which the US is slowed compared to the LS; however, to our knowledge there have been no other studies that have identified the concentrations of intramuscular metabolites during transitions from low and elevated baselines within the moderate domain. The resting  $[PCr]/[ATP]$  ratio was  $\sim 4$  and the resting  $[Pi]/[ATP]$  ratio was  $\sim 0.5$ , similar values to those reported in the literature (Kemp *et al.*, 2007; Jones *et al.*, 2008). Furthermore, when observing transitions from rest to moderate

intensity exercise, Jones et al. (2008) observed a ~15% degradation of intramuscular [PCr] (measured by  $^{31}\text{P}$ -MRS), similar to data herein which indicated a ~20% degradation of [PCr] from rest at the US. Importantly, Jones et al. (2008) also observed that when transitions were made from elevated metabolic rates versus rest, [PCr] fell to a lesser extent- similar to the data herein.

Hughson and Morrissey (1982) proposed that the slowed  $\dot{V}\text{O}_{2p}$  kinetics observed in the transition to the US was due to an  $\text{O}_2$  delivery limitation imposed by slow HR kinetics. MacPhee et al. (2005) observed a slowed rate of adjustment of blood flow both centrally (estimated via heart rate) and peripherally (measured via femoral artery blood flow) on the knee-extension ergometer. The observations in the present study are in agreement with previous data pertaining to slow HR kinetics during transitions from elevated metabolic rates (Hughson & Morrissey, 1982; MacPhee *et al.*, 2005). These data may indicate  $\text{O}_2$  delivery could be responsible for the lengthened  $\tau \dot{V}\text{O}_{2p}$  seen in transitions from the LS to the US. Also, in the present study the time delay before the increase in muscle deoxygenation (reflecting muscle  $\dot{Q}_{mv}$ -to- $\dot{V}\text{O}_{2m}$ ) was shorter in the US, and the overall rate of adjustment of muscle deoxygenation was not different between US and LS, despite the slowed  $\dot{V}\text{O}_{2p}$  kinetics, suggesting that the adjustment of microvascular blood flow and  $\text{O}_2$  delivery may be slower in the US than in the LS. This suggests that an immediate increase in  $\text{O}_2$  availability consequent to an increase in WR, via a muscle pump action, vasodilation or a combination of the two (Tschakovsky & Hughson, 1999) is more effective during transitions from a low compared to a higher metabolic and WR. However, in the present study, the steady-state end-exercise  $[\text{HHb}]_{\text{amp}}/\dot{V}\text{O}_{2p \text{ amp}}$  was lower in the US than in the LS indicating reduced reliance on  $\text{O}_2$  extraction as steady-state blood flow is achieved. These findings differ from those reported by MacPhee et al. (2005), who observed an increased in  $\Delta[\text{HHb}]/\Delta \dot{V}\text{O}_{2p}$  in the US in young men performing knee-extension exercise, and those reported by Spencer et al. (2011) who observed no difference in the steady-state end exercise  $[\text{HHb}]_{\text{amp}}/\dot{V}\text{O}_{2p \text{ amp}}$  between the two step-transitions in elderly men performing leg cycling. The unchanged mean-response time (MRT) of the [HHb] signal during transitions from the elevated metabolic demand versus that of the LS must be closely examined; despite similar MRT, the TD[HHb] in the US was faster than that in the LS, yet this is matched with a lengthened  $\tau[\text{HHb}]$ . This would

suggest that there are two distinguishable responses of microvascular  $O_2$  extraction. In the early part of the response, there is a shortened time period (TD [HHb]) in the US during which  $O_2$  extraction (as a mirrored profile of microvascular  $PO_2$  ( $PO_{2mv}$ )) is sufficient given the transition from an elevated metabolic rate perhaps due to transiently sufficient blood flow; that is to say that  $O_2$  delivery (blood flow) is sufficient for less time in the transition from LS to US versus from baseline to LS. In the late response, the slowed  $\tau$ [HHb] in the US would likely reflect a defense of the  $O_2$  driving pressure and attenuation of the drop in microvascular  $PO_2$  ( $PO_{2mv}$ ) at the onset of a transition from an elevated metabolic rate. It would seem that in the US, blood flow is initially sufficient for a lesser period of time (versus LS) when the  $\dot{V}O_{2p}$  kinetic response is being established. This is in line with slow HR kinetics during the transition from an elevated metabolic baseline. Therefore, it would appear that during transitions from elevated metabolic rates within the moderate intensity domain,  $\tau \dot{V}O_{2p}$  may be limited by an  $O_2$  delivery issue.

Brittain et al. (2001) speculated that muscle fibers active in the LS were recruited sequentially from a pool of fibers which were more oxidative and efficient (i.e., with lower  $\dot{V}O_{2p}$  gain) as compared to muscle fibres recruited in the US, which had different metabolic characteristics even if they classified within the same fibre type (based on myosin heavy chain isoforms). Lower-order fibers have lower recruitment thresholds and are activated when contractile intensity is low, while activity at greater intensities (often above  $\hat{\theta}_L$ ) require the recruitment of higher-order fibers, which have observed to yield slowed  $\dot{V}O_{2p}$  kinetics and lower efficiency in both human (Barstow *et al.*, 1996; Pringle *et al.*, 2003) and animal (Crow & Kushmerick, 1982) models. The increase in lactate observed in this study may be indicative of a reliance on less efficient, glycolytic fibers (type II) in the US as compared to the lower step.

Another possible hypothesis proposed to explain the slowed adjustment of  $V \square O_{2p}$  in transitions from an elevated metabolic baseline within the moderate intensity domain is that the metabolic rate itself (and thus, the energetic state of the active muscle) influences the rate of adjustment (Bowen *et al.*, 2011). We observed an increase in  $[ADP_{free}]$  and  $[P_i]$  during the transition from the baseline to the LS (without a change in muscle  $[ATP]$ ), which contributed a fall in calculated  $\Delta G_{ATP}$ . As a consequence, the energetic state of the exercising muscle at the onset of the transition from this elevated baseline (i.e transition to the US), was less favourable

than at the start of the lower (LS) exercise transition. The free energy released per ATP hydrolysis would likely be diminished due to the accumulation of metabolites (Zoladz *et al.*, 2006). Therefore, for a given WR, the required ATP turnover would be greater – this may help to explain the increased in  $\Delta \dot{V}O_{2p} / \Delta WR$  observed in the US. In the present study, there was a moderately strong relationship between the rate of adjustment of oxidative phosphorylation (i.e.,  $\dot{V}O_{2p}$ ) and the energetic status of the muscle (measured via  $\Delta G_{ATP}$  calculation). It has been shown that  $\dot{V}O_{2p}$  kinetics and  $\Delta G_{ATP}$  are linked, in that a fall in  $\Delta G_{ATP}$  has been observed to be related to an lengthened  $\tau \dot{V}O_{2p}$  (Barstow *et al.*, 1994; Kemp, 2008). A possible consequence of lower baseline  $\Delta G_{ATP}$  is that a greater ATP turnover (and greater  $\dot{V}O_{2m}$ ) would be needed to support a given change in WR. This notion stems from the work of Glancy *et al.* (2008), who suggested that the energy delivery to the actin-myosin crossbridges found within the active muscle is equivalent to the product of ATP production and  $\Delta G_{ATP}$ . We observed that the  $\dot{V}O_{2p}$  gain was greater in the US concomitant with a ‘less favorable’ energetic state. Therefore, a less negative  $\Delta G_{ATP}$  during US might be responsible for the higher  $\dot{V}O_{2p}$  gain ( $\Delta \dot{V}O_{2p} / \Delta WR$ ) found in the present study and in others (MacPhee *et al.*, 2005; Bowen *et al.*, 2011; Spencer *et al.*, 2011; Williams *et al.*, 2013). If correct, this would suggest that the change in ATP requirement in the US was greater than in LS, despite  $\Delta WR$  being the same. We have no evidence to support an alternate explanation that the increase in ATP turnover rate is similar for the same  $\Delta WR$  in LS and US, and it is a fall in mitochondrial ATP-to- $O_2$  coupling efficiency (P/O) that is reduced. Furthermore, it is interesting that the  $\Delta G_{ATP}$  is significantly lower even during the first transition (i.e., LS<sub>15s</sub>) as compared to the baseline, and is again lower at the LS<sub>360s</sub> as compared to the LS<sub>15s</sub> - providing insight that there may be a dynamic or transient decrease in  $\Delta G_{ATP}$  during exercise transitions in the moderate-intensity domain.

While comparisons with studies using different experimental models and interventions must be made with caution, models of respiratory control may provide an explanation for the slowed rate of adjustment of  $\dot{V}O_{2p}$  in the US. Accumulation of  $[P_i]$  and  $[ADP_{free}]$  and their links with oxidative phosphorylation have received much attention. The notion of a dynamically increasing ATP turnover for a given workload is not consistent with the model of linear capacitance proposed by Meyer (Meyer, 1988) as the model would predict that the steady-state

$\dot{V}O_{2p} - [PCr]$  relationship is linear over any given work rate. While this model has been contested (Kemp, 2008; Wust *et al.*, 2011) the observations in the present study (with limited muscle sampling) would suggest that the steady-state  $\dot{V}O_{2p} - [PCr]$  relationship is linear across both the LS and the US transitions within the moderate intensity domain (Figure 6). In terms of  $[ADP_{free}]$  accumulation, an early model (based, *in vitro*, on isolated mitochondrial preparations) proposed that  $\dot{V}O_{2m}$  was determined by  $[ADP_{free}]$  and  $[P_i]$  in a manner related to hyperbolic, first-order Michaelis-Menten enzyme kinetics (Chance *et al.*, 1955), although this relatively simple control is not as apparent in more intact muscle cells *in vivo* (Saks *et al.*, 1998). However, studies have observed that the *in vivo* ADP- $\dot{V}O_{2m}$  relationship may be too steep to be described by first-order dynamics as there is a ‘high sensitivity’ of  $\dot{V}O_{2m}$  to small changes in ADP at low  $[ADP]$  expected at rest and during the early part of the transition to a higher intensity (Jeneson *et al.*, 1996; Jeneson *et al.*, 2009). During steady-state, the relationship between  $\Delta G_{ATP}$  and oxidative phosphorylation would suggest that larger increases in  $[ADP_{free}]$  and  $[P_i]$  are required for a greater rise in oxidative phosphorylation at higher metabolic rates (Jeneson *et al.*, 1995). In the present study, muscle  $[ADP_{free}]$  already was elevated after 15 s of the LS transition, but did not increase statistically for the remainder of the LS and US transitions, despite further adjustments in  $V\dot{\square}O_{2p}$ . The  $[ADP_{free}]$  presented in the present study are consistent with values reported by Howlett and colleagues with exercise transitions from rest to either 35%, 65% or 90%  $\dot{V}O_{2p\ max}$  (Howlett *et al.*, 1998). The relationship between muscle  $[ADP_{free}]$  and  $\dot{V}O_{2p}$  is presented for the present study in Figure 5. Unlike the models and data showing large increases in  $\dot{V}O_{2m}$  with relatively small changes in  $[ADP_{free}]$  (see; Dudley *et al.*, 1987; Katz *et al.*, 1989; Jeneson *et al.*, 1996; Kemp, 2008; Wust *et al.*, 2011), the curvilinear relationship shown for the present study suggests that sensitivity of whole body  $\dot{V}O_{2p}$  to muscle  $[ADP_{free}]$  is lower during the transition in LS than during the transition to US. However, the time and intensity dependent increase in “apparent  $ADP_{free}$  sensitivity” (i.e., for very small increases of  $[ADP_{free}]$ , there is a large increase in  $\dot{V}O_{2m}$ ) for whole body  $\dot{V}O_{2p}$  more likely reflects the overall up-regulation of oxidative phosphorylation consequent to provision of other substrates to the mitochondria, and to a complex up-regulation, via covalent and allosteric regulation, of TCA cycle and ETC rate-limiting enzymes which is beyond the scope of the present study. Activation the mitochondrial PDH complex by pharmacological interventions (dichloroacetate) or ‘priming’ exercise (Gurd *et*

*al.*, 2009) has been shown to reduce the O<sub>2</sub> deficit and reliance on substrate-level phosphorylation as shown by an attenuation of muscle PCr and glycogen breakdown, and lower accumulating content of [P<sub>i</sub>], [ADP<sub>free</sub>], [lactate<sup>-</sup>], and [H<sup>+</sup>], metabolic responses consistent with a greater activation of mitochondrial oxidative phosphorylation.

### *Limitations*

One limitation in this study is related to the protocol involving needle biopsies. To reduce the discomfort of the subject, we opted to take three biopsies from one leg, and two from the other. The variability of not only tissue within one leg, but between legs themselves, may serve as a confounding factor. Furthermore, while we had hoped to capture the alterations in the metabolites following transitions to a new steady state, the timeline may be too quick (i.e., 15s) to observe a large increase. The timeline was designed in order to capture the increase of PDH – as it has been observed that after 30s, PDH is already at steady state (Gurd *et al.*, 2009). Another limitation is related to the use of the NIRS, as the area of muscle under the optodes (i.e., the area of “interrogation”) represents only a small region of active muscles, and may not necessarily quantify the contribution of all the working fibers, nor all the working muscles, in the examination of the rate of adjustment of [HHb]. Finally, we must make the assumption that  $\dot{V}O_{2p}$  is accurately reflecting what is occurring at the level of the muscle ( $\dot{V}O_{2m}$ ) (Barstow *et al.*, 1990).

## **2.5 CONCLUSION**

In summary, this study presented novel insight into the control of oxygen uptake during transitions from lower and elevated metabolic rates in the moderate intensity exercise domain. Slowed  $\dot{V}O_{2p}$  kinetics and a greater  $\dot{V}O_{2p}$  gain were observed in transitions from an elevated metabolic rate, coincident with slow HR kinetics (reflecting slow central (bulk) blood flow adjustments) and similar overall muscle deoxygenation kinetics (implying inadequate microvascular blood flow adjustments) during the same transitions. Additionally, despite slower  $\dot{V}O_{2p}$  kinetics in the US compared to the LS, the fall in muscle [PCr] and calculated  $\Delta G_{ATP}$ , and rise in [Pi] and [ADP<sub>free</sub>] during the US was less than the changes observed during the LS transition. While the lower  $\Delta G_{ATP}$  observed during the LS steady state offers a possible explanation for the slower  $\dot{V}O_{2p}$  kinetics and greater  $\dot{V}O_{2p}$  gain observed in the US, the

magnitude of the changes in the US of high-energy phosphates were (unexpectedly) small and did not seem consistent with metabolic changes expected based on a slow adjustment of  $\dot{V}O_{2p}$  (and muscle oxidative phosphorylation).

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# APPENDIX A: ETHICS APPROVAL NOTICE



**Use of Human Participants - Ethics Approval Notice**

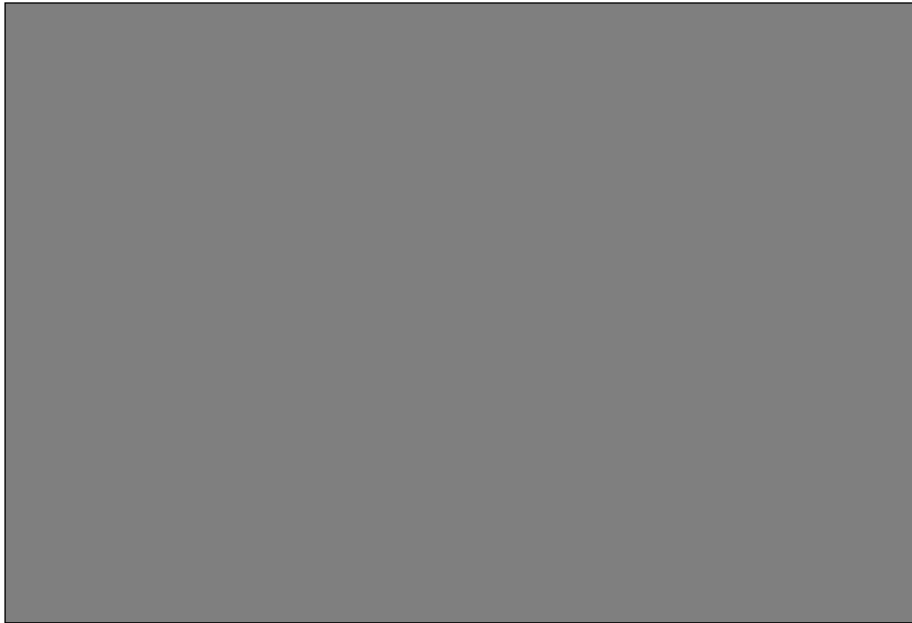
**Principal Investigator:** Dr. John Kowalchuk  
**File Number:**6085  
**Review Level:**Delegated  
**Approved Local Adult Participants:**0  
**Approved Local Minor Participants:**0  
**Protocol Title:**Adaptation of pulmonary O2 uptake and muscle pyruvate dehydrogenase (PDH) activity during the transitions from the lower and upper regions of the moderate-intensity domain (REB #16003)  
**Department & Institution:**Health Sciences/Kinesiology,Western University  
**Sponsor:**  
**Ethics Approval Date:**June 21, 2012 **Expiry Date:**December 31, 2014  
**Documents Reviewed & Approved & Documents Received for Information:**

Document Name	Comments	Version Date
Change in Study Personnel	Addition of: J. Nederveen, D. Kier & L. Spriet Removal of: A. Marjerrison & S. Peters	

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced revision(s) or amendment(s) on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the University of Western Ontario Updated Approval Request Form.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.



# CURRICULUM VITAE

**Name:** Joshua Nederveen

**Post-secondary Education and Degrees:** McMaster University  
Hamilton, Ontario, Canada  
2005-2009 B.Sc

The University of Western Ontario  
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2011-2013 M.Sc

**Related Work Experience:** Teaching Assistant  
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## Published Articles:

Spencer, M.D., Keir, D.A., **Nederveen, J.P.**, Murias, J.M., & Paterson, D. H. Moderate-intensity, steady-state VO<sub>2</sub>, [HHb] and HR remain elevated 6 and 20 minutes following heavy-intensity priming exercise *Applied Physiology, Nutrition and Metabolism*. September 2012 (*in press*)

Pemberton J, **Nederveen J**, Lamond A, Bailey K, Ratcliffe E, Walton JM. Feasibility of conducting a prospective cohort study in pediatric surgery: Introducing the CARE study. *Journal of Pediatric Surgery*. May 2012 (*in press*).

## Published Abstracts:

McLay, K.M., Murias, J.M., **Nederveen, J.**, Paterson, D.H. Day-to-day reliability of flow-mediated dilation measures in the popliteal artery. *Medicine & Science in Sports & Exercise*: 2013 (In press). Abstract

McLay, K.M., Murias, J.M., **Nederveen, J.**, Paterson, D.H. Test-to-test repeatability of FMD measures in healthy young adult males. *Applied Physiology, Nutrition, & Metabolism*: 2013 (In press). Abstract

**Nederveen J**, Murias J.M., Paterson, D.H, Kowalchuk, J.M. Effect of eccentric muscle damage on O<sub>2</sub> uptake kinetics and muscle deoxygenation during moderate-intensity cycling exercise. *Medicine & Science in Sports & Exercise*: 2014. Abstract

**Nederveen J**, Love, L, Paterson, D.H, Rossiter, H, Kowalchuk, J.M. Effect of heavy-intensity 'priming' exercise on pulmonary O<sub>2</sub> uptake and muscle deoxygenation kinetics during moderate-intensity step-transitions initiated from an elevated metabolic rate. *Applied Physiology, Nutrition, & Metabolism*: 2014. Abstract