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Investigating the physiological mechanisms of the oxygen consumption "slow component".

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Investigating the physiological mechanisms of the Oxygen Consumption "Slow Component"

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SOMMARIO

Lo studio delle cinetiche del consumo di ossigeno (VO_2) si focalizza sul comprendere come il metabolismo del corpo umano si adatta, durante la transizione da una condizione di riposo/esercizio ad un'altra, in modo da soddisfare le esigenze energetiche necessarie alla locomozione. Essendo un indice integrato della capacità di utilizzare l'ossigeno e di funzionalità polmonare, cardiovascolare e muscolare, l'interesse verso questa materia è cresciuto in maniera costante durante il XX e l'inizio del XXI secolo. Grazie allo sviluppo di nuove tecnologie e al crescente interesse della comunità scientifica, la conoscenza riguardante le cinetiche del VO_2 è aumentano considerevolmente. Ciò nonostante, alcuni argomenti specifici legati alle cinetiche del VO_2 rimangono dibattuti e richiedono ulteriore ricerca. Tra questi vi è sicuramente la perdita di efficienza di locomozione che si registra ad intensità metaboliche medie ed elevate, dopo il periodo di adattamento in cui un nuovo stato stazionario del VO_2 dovrebbe invece già essere stato raggiunto.

Questo fenomeno prende il nome di "componente lenta" del VO_2, e rappresenta un ulteriore aumento di consumo di ossigeno rispetto a quanto previsto per un dato stato stazionario. Chiarire le basi fisiologiche di questo fenomeno è considerato di fondamentale importanza sia per il suo collegamento diretto con l'incapacità di tollerare l'esercizio fisico, sia per raggiungere una migliore comprensione degli adattamenti che avvengono all'interno del corpo umano durante esercizio fisico. Come conseguenza, la ricerca ha cercato di chiarire quali siano i meccanismi alla base della componente lenta del VO_2 e di sviluppare strategie d'intervento volte a ridurne il manifestarsi. Non di meno, una serie di incertezze riguardo il significato fisiologico di questo fenomeno persistono e richiedono ulteriore studio.

Lo scopo di questa tesi è di cercare di colmare parte di queste lacune e di comprendere le origini della componente lenta del VO_2, così come la perdita di efficienza che essa sottintende. Nel capitolo uno è fornita una breve spiegazione della risposta del VO_2 durante esercizio, e delle attuali teorie esplicative per la componente lenta del VO_2. Nel capitolo due, sono spiegati gli scopi di ricercar di questa tesi. Il capitolo tre, quattro,

cinque, e sei illustrano i risultati di quattro diversi studi sperimentali. Infine, il capitolo sette riassume i principali risultati della tesi.

ABSTRACT

The study of the oxygen consumption (VO_2) kinetics is focused on the understanding of how human metabolism adjusts during the transition from a condition of resting/movement to another in order to satisfy the new energetic demand. As an integrated index of pulmonary, cardiovascular and muscles capacity VO_2 kinetics have gained progressively increasing interests during the XX and the early XXI centuries. Thanks to the development of new technologies as well as an always increasing community of interested scientists in this subject, the knowledge in this field has been expanded considerably. However, some of the topics related to VO_2 kinetics remain debated and call for further research. One of these topics is the loss of efficiency of human locomotion that occurs at the higher metabolic intensities, after the transitory period in which a new steady-state in VO_2 should be achieved.

This phenomenon is typically called VO_2 "slow component", as representative of a further increase in VO_2 after the expected steady-state. The importance of the VO_2 slow component lies in its link with exercise tolerance and on the understanding of the adaptations of the human body during physical activity. Therefore, researchers have tried to define the physiological underpinning of the slow component and to develop intervention strategies to reduce its amplitude. Nevertheless, a number of physiological uncertainties regarding the mechanistic bases of the slow component exist and require to be clarified.

The purpose of this thesis was to deal with this gap and to study the origins of the VO_2 slow component, and the loss of efficiency of locomotion that the slow component represents. In chapter one, a brief explanation of the VO_2 response during exercise and the current explanatory theories for the VO_2 slow component are provided. In chapter two, the experimental aims of the thesis are explained. Then, the results of four different studies are presented in chapters three, four, five, and six. Finally, chapter seven summarizes the main findings of this research work.

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CHAPTER

1

Exercise Tolerance and VO_2 Response During Constant *vs* Incremental Work rate Exercise

Exercise (in)Tolerance:

Exercise tolerance is the ability to produce the required level of force/power for an adequate time to accomplish a certain task. It can influence a broad number of activities, and have an impact in a large spectrum of contexts ranging from sport to daily life.

The inability to tolerate an adequate amount of exercise may be a major mechanism in contributing to functional decline; a key landmark on the pathway from independence to "disability", and to a significant decline in quality of life. A substantial reduction in exercise tolerance is recognized to limit mobility, particularly in sedentary people and older adults, and therefore represents a barrier to gain many of the well-studied exercise benefits (Zaleski et al. 2016).

While in the young and in the adult populations exercise intolerance can limit the amount of specific physical activities (e.g. running, cycling, walking, etc.), in the elderly, it limits the ability to perform the activities necessary to everyday life accelerates the transition from a state of independence to that of dependence (Poole et al. 1994).

During whole-body exercise, (in)tolerance is determined by a combination of factors such as *i)* the maximum oxygen uptake (VO_{2max}, i.e. the highest level of whole-body aerobic capacity); *ii)* the ratio of the gas exchange threshold (GET) and the respiratory compensation point (RCP) relative to VO_{2max}; GET and RCP represent respectively the lower boundary for fatigue accumulation and the upper intensity still compatible with a metabolic steady-state; *iii)* the speed of adjustment of metabolism during a transition from no effort/low effort to a higher exercise intensity (described by the VO_2 kinetics); *iv)* and the cost/efficiency of locomotion (defined here as the ratio of a required metabolic energy input to mechanical energy output: $\Delta VO_2/\Delta W$).

These above listed parameters (that highlight the importance of VO_2 measures) are typically obtained from two main exercise paradigms: the ramp incremental (RI) and

constant work rate tests (figure 1, CWR). RI protocols were developed at the end of the 20th century to test the physiological responses to linear and continuous increases in exercise intensity (work rate) ranging from no-effort to maximum; CWR are typically used to investigate the adjustment of VO_2 (amplitude and speed) during the transition phase to a new steady-state.

Originally, the control of pulmonary VO_2 has been characterized as a first-order linear system (i.e. to a given increase in exercise intensity correspond a given increase in VO_2: $\Delta VO_2/\Delta W$).

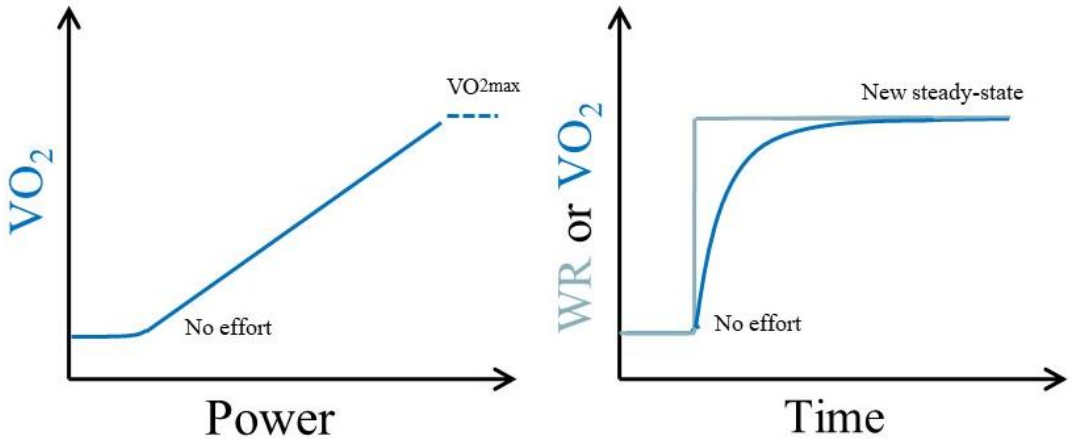

Figure 1, schematic representation of the expected VO_2 response during ramp incremental and constant workrate exercises.

However, empirical evidence (following the development of high-resolution metabolic carts) demonstrated that the VO_2 follows a more complicated response (e.g. cardiodynamic phase, slow component etc.), that this first-order model is valid only upon the reach of the intensity corresponding to GET, and when exercise intensity rises above GET, the VO_2 responses loses its linearity and leads to higher $\Delta VO_2/\Delta W$. More specifically, during RI and CWR exercises (respectively as a function of work rate or time) it is possible to discriminate a developing increase in VO_2 (figure 2), termed

"excess VO_2" during RI and VO_2 slow Component (VO_{2sc}) during CWR. Both these phenomena represent an *increased and unexpected* cost of exercise that challenges the assumptions regarding the physiological adaptations to exercise.

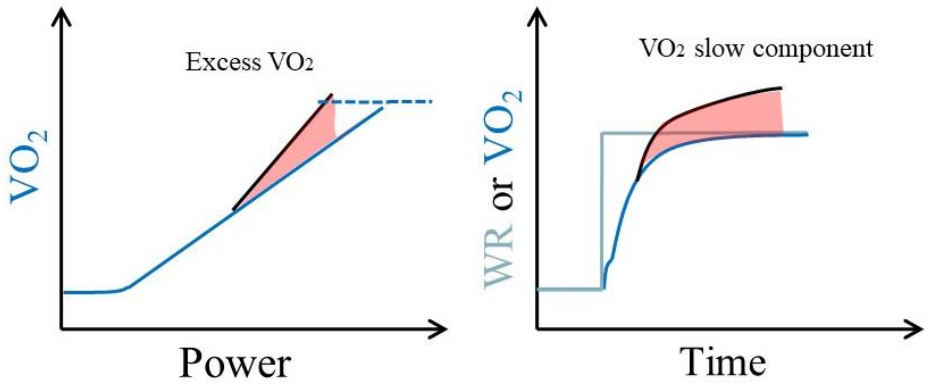

Figure 2, schematic representation of the excess VO_2 and the VO_2 slow component during ramp incremental and constant workrate exercises.

Clarifying the origin of this increased cost of locomotion is of interest because it could possibly increase the knowledge on muscle energetics, on the control of metabolism and the underpinnings of skeletal muscle contraction. Moreover, and importantly, these increases in VO_2 are typically interpreted and correlated with subjects' exercise intolerance. In this chapter, a brief explanation of the VO_2 response during RI and CWR is provided contextualizing the excess VO_2 and the VO_{2sc} as measures of loss of efficiency of human locomotion.

VO_2 response during constant workrate exercise:

In all activities of daily living there are a lot of challenges in which the energy demands of the working muscles might quickly go from rest to a new steady-state/non-steady-

state level. In this sense, the study of the dynamic adjustments of the VO_2 responses is of interest to clarify how the metabolic pathways adapt to these continuous variations (Poole and Jones 2012), and to gain an overall insight of the body's capacity to adapt to them.

After the introduction of metabolic carts with a high resolution of measurement (i.e. breath-by-breath), the pulmonary VO_2 response following the onset of exercise has been described by physiologist in three phases (Poole and Jones 2012):

- Phase I: at the onset of CWR exercise, there is an early rapid increase in VO_2 that starts with the first breath and is spurred by the quick initial elevation of cardiac output and pulmonary blood flow;
- Phase II: an exponential increase in VO_2 with a time constant of 20-45 sec in healthy adults, drives VO_2 to the actual or towards the initially anticipated steady-state values. This phase, called "primary component" reflects the arrival at the lung of venous blood draining the exercising muscles and reflects the kinetics of O_2 consumption in the exercising muscles;
- Phase III: represents the steady-state (or expected steady-state) values, and it is typically achieved within the first 2-3 min of exercise, mainly according to the subject's fitness level.

Moreover, it is broadly accepted and demonstrated that the VO_2 profile changes differently according with exercise intensity, describing three exercise domains: *moderate, heavy*, and *severe* (figure 3).

- during *moderate* exercise (below GET/lactate threshold), work rate can be sustained for about long periods and there is not a metabolic acidosis. A steady-state is usually attained in about 3 min in young and healthy adults.
- during *heavy* exercise (above GET and below RCP/Maximal Metabolic Steady State), can be identified a metabolic acidosis with an increase in lactate and H^+. A metabolic steady-state and in VO2 is obtainable but delayed to a time period

of 6-10 min, due to the appearance of the "VO_2 slow component"; Exercise in this domain is sustained with a bigger contribution of glycolysis to ATP production, but at whole-body level there is still a balance between lactate production and removal. Protracting exercise in this domain leads to extensive fatigue accumulation, mostly due to glycogen stores depletion and heat accumulation.

- during *severe* exercise (above RCP/Maximal Metabolic Steady State), VO_2 gradually increases as steady-state conditions are not achievable, eventually reaching the VO_{2max}. Since there is no more balance between production and removal of lactate, blood and muscle lactate progressively increase over time. In the severe domain, fatigue is accumulated "intensively" by depletion of the anaerobic energy sources (called W' in the Critical Power model) and increased acidosis.

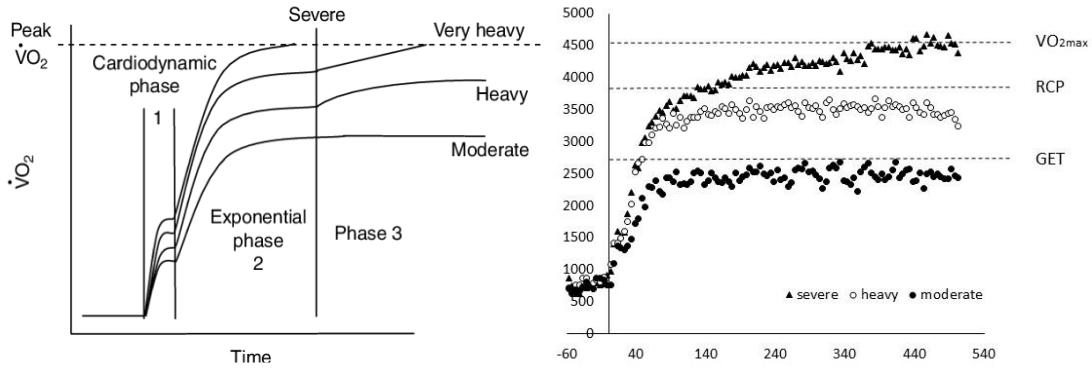

Figure 3. *On the left: schematic representation of VO_2 kinetics in response to different work-rate intensities (Poole & Jones 2012. Oxygen uptake kinetics): below the gas exchange threshold (GET, moderate exercise), above GET but below the respiratory compensation point RCP, heavy exercise) and RCP with the VO_2 projecting to VO_{2max} (severe exercise). Each curve has a Phase 1 and a phase 2 and, in addition, the >VT figure has a discernible "slow component" (Phase 3). On the right: the same representation is provided using data from a representative subject*.

Whereas the VO_2 response below GET is described by a mono-exponential function, the VO_2 response above GET, has been shown to be better described by bi-exponential processes which represents the amplitude of the VO_{2sc} (figure 3). This suggests an intensity-dependent *loss of muscle efficiency* as high intensity exercise proceeds. Moreover, during CWR at higher power outputs, the VO_2 causes an "excess" of oxygen consumption and brings the subjects to VO_{2max}. Therefore, the subject in unable to sustain exercise, or progressively decreases the power output in order to oppose the development of VO_{2sc}. In the field of physiology, the study of VO_{2sc} is of interest to enhance basic understanding of muscle energetics, metabolic control and the determinants of the efficiency of skeletal muscle contraction and the underpinnings of exercise intolerance.

VO_2 response during ramp incremental exercise:

RI test is characterized by a linear and continuous increase in work rate. In this protocol, the specific non steady-state conditions provides information on the ability of the aerobic system to adjust to continuously changing metabolic demand (Poole and Jones 2012). During the initial phase of ramp test, despite the linear increase in work rate, the pulmonary VO_2 response lags the metabolic demand by a time interval, typically defined as mean response time (MRT). The MRT is quantified as the time interval between the onset of the ramp and the intersection of the extrapolation of the baseline VO_2 and the backwards extrapolation of the linear VO_2/time relationship below the GET (Boone and Bourgois 2012). Following this initial delay, VO_2 increases linearly with time (t) and work rate (W). Below the GET, the linear phase of the VO_2 response to ramp exercise has described by the following equation:

$y = ax + b$

where a is the relationship between + VO_2 and work rate ($\Delta VO_2/\Delta W$) and b is the y-intercept or VO_2 at baseline work rate determined from the linear VO_2/W relationship.

The $\Delta VO_2/\Delta W$ represents an expression of delta efficiency. This parameter is used to quantify mechanical muscle efficiency and is one of the main parameters considered in planning endurance (long duration) activities and events. Above GET, also the anaerobic metabolism is involved into exercise, leading to a slow VO_2 response as similar as VO_{2sc} in constant work rate ((figure 4 (Boone and Bourgois 2012)). This suggests that in high intensity exercise the VO_2/W linearity is lost; the upward deflection in the VO_2/W, named "excess" VO_2, due to a delayed additional increase in VO_2 is ascribed to a drop in mechanical muscle efficiency, that is typically considered equivalent to the VO_{2sc} which occurs during constant work rate (Grassi et al. 2015) Finally, it should be considered that a clear emergence of the excess VO_2 its emergence in not always guaranteed, and can be influenced by both subjects' fitness level and by the RI protocol (e.g. excess VO_2 manifest more during "slow" $W*min^{-1}$ ramps) (Boone and Bourgois 2012; Iannetta et al. 2019a).

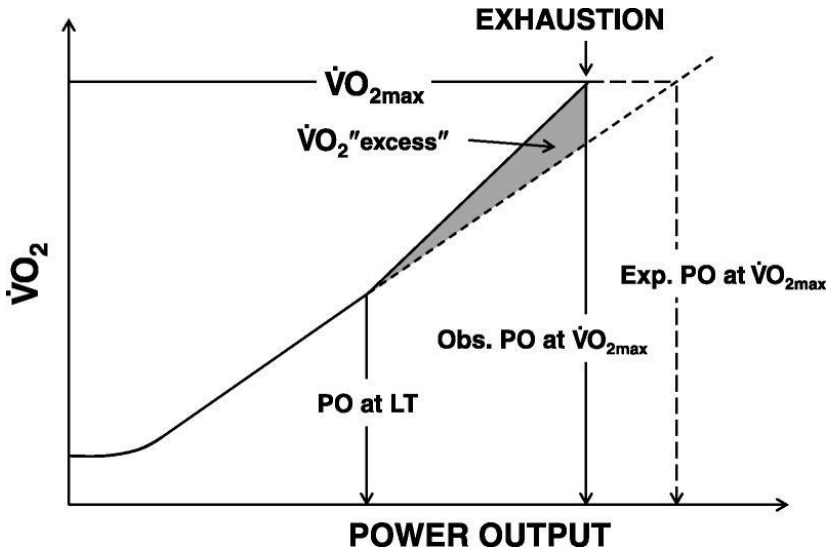

Figure 4. *From Grassi et al. 2015.VO_2 response during incremental ramp test when intensity exercise is above GET/lactate threshold. The relationship between power output and VO_2 is linear until GET; than linearity is lost and the VO_2 response is greater in compared with power*

output leading early exhaustion. The upwards curvilinear deflection leads to excess VO_2 caused by a drop in mechanical efficiency.

Loss of efficiency:

The most immediate consequence of the arise of the VO_{2sc} during CWR and the excess VO_2 during RI (Jones et al. 2011), compared to situations in which these phenomena do not manifest, is the early exhaustion of the subject as well of a bigger accumulation of fatigue-related processes leading to metabolic instability and recruitment of additional muscle fibres (Jones et al. 2011). Considering that the magnitude of the VO_{2sc} can exceed 1L of extra O_2, the impact of loss of efficiency cannot be underestimated, especially in populations with already low levels of VO_{2max} such as patients. Moreover, the loss of the VO_2/W linearity has also more practical consequences such as the not straightforward translation of a work rate measured during RI (the typical test for cardiopulmonary evaluation) to CWR (the easiest protocol for training interventions) (Keir et al. 2018a).

For these reasons, and to gain a better insight into the metabolic control during exercise, the causes of the loss of efficiency have been studied for decades using different approaches and models (Jones et al. 2011). Nowadays, it is commonly accepted that the loss of efficiency could be caused by: *i)* a progressive *recruitment* over time of new muscle fibres (Poole and Jones 2012) *ii)* metabolic instability within the fibres that requires a higher energetic input to sustain the same mechanical output *iii)* a complex interaction of both these processes. A schematic representation of the factors/mechanisms possibly causing the loss of efficiency linked with the VO_{2sc} in presented in the figure below (figure 5).

i) *Recruitment hypothesis*: a number of studies have tested the hypothesis that a progressive recruitment of low-efficiency, glycolytic, type II muscle fibres is the main mechanism responsible for the reduced efficiency at exercise intensities above the lactate threshold (Jones et al. 2011). VO_{2sc} or excess VO_2 have been hampered by the poor temporal and spatial resolution of the available techniques (i.e. Electromyography, Magnetic Resonance Imaging) leading to conflicting results (Vanhatalo et al. 2011).

ii) *Metabolic instability hypothesis*: as an alternative hypothesis, the genesis of the reduced efficiency has been attributed to fatigue of the working fibres (mostly type I) (Woledge 1998). Fatigue itself causes an increase in the ATP and/or O_2 cost of exercise during exercise due to the accumulation of metabolites (P_i, IMP, AMP, H^+, K^+) in fatiguing muscles that affect Ca^{2+} dynamics, troponin sensitivity to Ca^{2+} and the contraction force of the cross-bridges attachment (Grassi et al. 2015).

iii) *Recruitment-instability hypothesis*: an alternative or complementary explanation is that VO_{2sc} and excess VO_2 arise due to the combined effects of fatigue on the initially recruited fibres (both type I and II fibres) and the recruitment of new, less efficient fibres. Type I fibres might be increasingly activated at a high contraction velocity that is sub-optimal in terms of force production. This would require the activation of additional motor units (mainly type II fibres that due to the higher recovery cost, require increasing energy (ATP) and O_2 over time) (Jones et al. 2011).

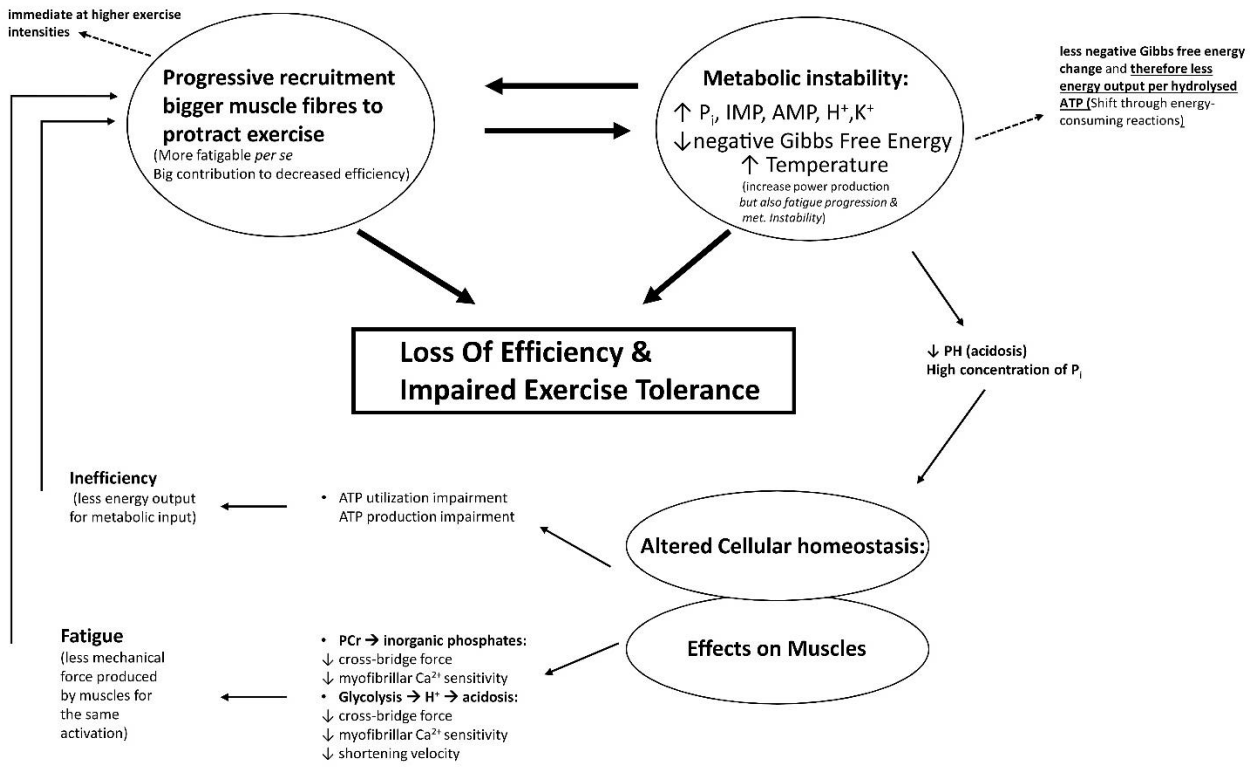

Figure 5. *Adapted from Grassi et al. 2015, factors/mechanisms possibly causing the loss of efficiency linked with the* VO_{2sc}.

State of the Art, methodological discrepancies and new theories:

To date, the recruitment of new muscle fibres and/or metabolic instability within the already working fibres are considered the two phenomena that interplay to the loss of efficiency and the VO_{2sc}. Specifically, during exercises in which the level of muscle contraction is not maximal, it is thought that the VO_{2sc} mostly originates from the recruitment of additional muscle fibres (Jones et al. 2011), but experimental evidences also showed that VO_{2sc} may manifest within the already active fibres (Zoladz et al. 2008; Vanhatalo et al. 2011). Nevertheless, the magnitude of this interaction in terms

of the relative contribution to the VO_{2sc} (%), as well as how this interaction changes over time or according to exercise intensity (e.g. is not clarified if the recruitment of new muscle fibres can be considered the main mechanism to VO_{2sc} both in the heavy and severe domains) has still to be quantified and described.

In addition, the interpretation of results of previous researches on the VO_{2sc} is not always straightforward due to some methodological discrepancies that can somehow mislead the researchers. For example, the VO_{2sc} is reported as an increase in VO_2 after the moment in which a metabolic steady-state should be achieved, the VO_{2sc} in the heavy exercise domain is considered "physiologically equivalent" to the VO_{2sc} in the severe domain. However, there is no direct evidence for this to be. Also, some investigations are performed at intensities corresponding to the boundary between the heavy and severe domains (e.g. Δ50% between GET and VO_{2max}) (Saunders et al. 2003), but without accounting for methodological pitfalls that may have elicited the prescription of exercise in the wrong exercise domain (Iannetta et al. 2019b).

Another problem when investigating the nature of VO_{2sc} is that only the VO_2 measured at the level of the mouth is considered, while the other energetic contributors to exercise (i.e. glycolysis), as well as the ventilatory portion of the VO_{2sc} (typically 15 to 20% of the VO_{2sc} (Poole et al. 1991)) are usually not or scarcely considered. This may be particularly important in studies that investigated how the VO_2 response is affected by interventions such as training (Saunders et al. 2003), priming, or glycogen depletion (Bouckaert et al. 2004; Carter et al. 2004; Krustrup et al. 2004b; Korzeniewski and Zoladz 2015). In fact, in all these scenarios the phase II of VO_2 can be fasten-up or slowed by the enhancement/impairment of the contribution of aerobic metabolism to exercise, in turn affecting the subsequent VO_{2sc}. Pertinently, a recent study (O'Connell et al. 2017) refuted that the energy demand of a constant, high-intensity exercise changes over time. By subtracting the VO_2 cost of ventilation and accounting for the contribution of the glycolytic energy sources (di Prampero et al. 1999) O'Connell quantified the total, "adjusted" metabolic cost of muscle exercise over time during a constant work rate trial in the severe domain. It was concluded that the oxygen cost of

locomotion does not increase over time, other than what required by the augmenting cost of ventilation. In other words, these findings suggest that VO_{2sc} may in fact not represent a loss of efficiency as a function of time but rather a delayed adjustment of the oxidative metabolism.

This thesis will try to deal with some of the aspects discussed in this chapter. Specifically, the first part will focus on the role of muscle recruitment in increasing the loss of efficiency, while the second part will investigate across the different exercise intensity domains i) the bioenergetic contributors to exercise and the loss of efficiency and ii) by applying non-invasive measures of muscle metabolism and activation will evaluate the contribution of metabolic instability and muscle recruitment to the loss of efficiency.

CHAPTER

2

Experimental Aims

Purposes and Research Questions:

Part 1

Clarifying the origin of the loss of efficiency linked to the rise of the oxygen consumption (VO_2) slow component (VO_{2sc}), and the excess of VO_2 is of interest to increase the knowledge on muscle energetics and the control of muscle metabolism. Moreover, this loss of efficiency is typically interpreted and related with subjects' exercise intolerance, and the understanding of its mechanisms could represent the starting point through the development of better training and intervention strategies.

The two main putative mechanisms of the loss of efficiency are metabolic instability within the working muscle fibres or recruitment of new less efficient fibres. However, given the difficulty to selectively affect either one of these contributors, the exact physiological mechanisms underpinning the loss of efficiency remains elusive.

The first part of this thesis will focus on the possible role of increased muscular activation (necessary to maintain the same work rate when fatigued) as explanatory theory of the loss of efficiency. In particular, the two studies presented in Part 1 will explore the effect of acute, non-metabolic fatigue interventions, on the loss of efficiency during RI and CWR exercises:

- *Study 1*: the main objectives of this study will be i) evaluating two acute, non-metabolic fatiguing interventions to impair maximal force and possibly augment muscle recruitment at a given absolute work rate ii) testing the physiological response to acute fatigue in non-steady-state conditions as during RI exercise.
- *Study 2*: the most "effective" fatiguing intervention detected in study 1 (i.e. the intervention that elicits the highest force impairment) will be applied during constant workrate cycling in the severe exercise intensity domain to test the impact of acute fatigue on the VO_{2sc}.

The underlying hypothesis of both these studies is that the loss of muscle strength induced with the fatiguing interventions will translate in increased muscle activation at a given absolute work rate; in turn, increased muscle activation will increase the VO_2 cost of locomotion and reduce exercise tolerance

Part 2

A well-established concept on the VO_{2sc} is that roughly 85 % of it origins from the working muscles, while the remaining 15% is ascribable to the VO_2 cost of ventilation. Most of the studies concerning the VO_{2sc} ignore this distinction. Interestingly, a recent research from O'Connell et al. (2017) evaluated these two components together with the anaerobic contribution to ATP resynthesis and suggested that the VO_{2sc} may in fact represent a shift in energetic sources and increasing cost of ventilation over time rather than loss of efficiency.

Applying the same approach, and implementing measures of peripheral muscular activation and metabolism, this part of the thesis will:

- In *Study 3*, evaluate the bioenergetics of the VO_{2sc} in the three exercise domains.
- In *Study 4*, test the contribution of both metabolic instability and fiber recruitment to the VO_{2sc} in the three exercise domains

It was hypothesized that the contributors to the VO_{2sc} will differ between different exercise domains.

CHAPTER

3

Response to Acute Non-Metabolic Fatigue During Ramp Incremental Exercise

Based on the article published in Respiratory Physiology and Neurobiology 270 (2019) 103281, doi: 10.1016/j.resp.2019.103281.

Alessandro L Colosio, Emmanuele Baldessari, Enrico Basso, Silvia Pogliaghi.

Abstract:

We tested the hypothesis that acute, non-metabolic fatigue, by reducing maximal power output and possibly increasing muscle recruitment at a given exercise intensity, will reduce indexes of exercise tolerance during incremental cycling. Ten subjects performed three ramp incremental tests respectively after static stretching (STRC), dropjumps (DJ) or control (CTRL). Fatigue was assessed as reduction in maximal power output ($_{sprint}$PO) during isokinetic sprints. During the ramps we measured: oxygen consumption (VO_2), power output (PO), and surface electromyography. $_{sprint}$PO was reduced after STRC and DJ ($p=0.007$) yet not after CTRL. During the ramps, the interventions augmented muscle excitation *vs* CTRL ($p\leq0.001$). Peak PO and VO_2 were reduced after STRC (302±39W $p=0.033$, 3365±465 $ml*min^{-1}$ $p=0.015$) and DJ (300±37W $p=0.023$, 3413±476 $ml*min^{-1}$ $p=0.094$) *vs* CTRL (314±41W, 3505±486 $ml*min^{-1}$). Interventions were associated with early occurrence of the ventilatory thresholds and increased VO_2 *vs* CTRL ($p=0.029$). The physiological response after acute non-metabolic fatigue suggests a link between exercise intolerance and the decreased ability to produce force.

Introduction:

Exercise tolerance, *i.e.* the ability to sustain a specific amount of force/power to complete a movement task, is fundamental in maintaining independence (for work, sport and leisure activities) and quality of life (American College of Sports Medicine 2017). During whole-body exercise above the Gas Exchange Threshold (GET) and in particular above the Critical Power, exercise intolerance is associated with an increased cost of locomotion expressed as augmented gain of oxygen consumption (VO_2) for a given gain in absolute work rate, compared to below threshold intensities. This loss of efficiency of locomotion is experimentally described as "excess-VO_2" during incremental exercise, and "VO_2 Slow Component" during constant work rate exercise (Jones et al. 2011; Grassi et al. 2015). Previous studies determined that roughly 85% of this phenomenon originates from the contracting muscles, while the remaining 15% corresponds to the increased VO_2 cost of ventilation (Poole et al. 1991). Several researchers focused on the possible causes of the muscular component of the loss of efficiency using a series of approaches (Jones et al. 2011 for a summary) and two main theories have been proposed: *i) decreased metabolic stability of type I muscle fibres*, caused by the negative effect of physiological metabolites (P_i, IMP, AMP, H^+, K^+), associated with increased O_2 cost of ATP resynthesis and/or increased ATP cost of contraction (Jones et al. 2011; Grassi et al. 2015) *ii) recruitment of fast-fatigable intrinsically inefficient type II muscle fibres,* to obtain/maintain the external power output above a certain intensity threshold (e.g. Critical Power) (Jones et al. 2011; Poole and Jones 2012; Grassi et al. 2015). However, the exact physiological mechanisms underpinning the loss of efficiency remain elusive, one of the reasons being the difficulty to selectively affect either metabolic stability or type II fibres recruitment in human models. In fact, the different manipulations used in interventional studies (e.g. speed of movement, intensity modulation, aerobic training, priming exercise, nutritional interventions) affect to some extent both metabolic stability and fibres recruitment (Jones et al. 2011).

In this context, different authors explored the effect of acute, non-metabolic fatigue interventions, on the loss of efficiency. These interventions reduce the ability of muscles to produce force without inducing metabolic changes within the fibres. As such, non-metabolic fatigue interventions, should elicit increased muscle activation at a given absolute intensity (recruitment theory), while avoiding the confounding effect of intracellular homeostasis perturbations (metabolic instability theory). Among these authors, Hopker et al. (Hopker et al. 2016) evaluated the effect of 100 dropjumps (DJ) on VO_2 Slow Component during cycling exercise at the heavy-to-severe boundary. Notwithstanding a significant acute fatigue, the authors found no effect of DJ on VO_2. On the contrary, Esposito et al. (2012) found that when maximal force was acutely reduced following stretching (STRC) manoeuvres, the oxygen cost of locomotion increased both during ramp incremental and constant work rate exercises (Esposito et al. 2012; Limonta et al. 2015). However, given that measures of muscles recruitment (e.g. electromyography, EMG) are missing in the above cited studies, a link between muscle recruitment and loss of efficiency remains to be conclusively demonstrated/dismissed.

Considering the above contrasting results with acute, non-metabolic fatiguing interventions, further research is needed to investigate the possible link between fatigue, muscle excitation and augmented VO_2 at a given absolute work rate.

Accordingly, this study was designed to investigate the effects of acute, non-metabolic fatigue induced by either DJ or STRC interventions on muscle excitation (*i.e.* EMG) and oxidative metabolism (*i.e.* VO_2) during incremental cycling. Considering the possible role of increased muscular activation (necessary to maintain the same work rate when fatigued) as explanatory theory of the loss of efficiency phenomena, we hypothesis that both STRC and DJ *i)* will reduce maximal muscle force; *ii)* in turn, force loss will translate in increased muscle excitation at a given absolute work rate; *iii)* finally, increased muscle excitation will impair maximal and submaximal indexes of exercise tolerance and reduced efficiency during a ramp incremental test performed to exhaustion.

Methods:

Participants:

Ten active men gave written informed consent to participate in the study (25±4 years age, 80±13 kg body mass 176±8 cm stature, 25.6±2.3 BMI). Inclusion criteria were male sex and age between 20 and 35 years; exclusion criteria were smoking and any condition that could influence the physiological responses during testing. The study was approved by Departmental Ethics Committee and adhered to the principles of the declaration of Helsinki. All participants were instructed to avoid caffeine consumption and physical activity respectively for at least 8 h and 24 h before each testing session.

Protocol:

Subjects visited the laboratory on five occasions within a maximum of three weeks. In the first two occasions, they were familiarized with the experimental procedure (isokinetic sprinting and incremental cycling) and the position on the ergometer was recorded for the successive appointments. On the third, fourth and fifth visit, separated by no less than 2 days of recovery, subjects performed the following identical protocol:

i) PRE-isokinetic cycling sprints to measure maximal power output at baseline

ii) a 40-minutes intervention (either STRC, DJ, or control (CTRL)), with the DJ always executed as last session to avoid interference due to the long lasting effects of eccentric exercises (Twist and Eston 2005))

iii) POST-isokinetic cycling sprints

iv) a ramp incremental test to exhaustion.

A schematic representation of the protocol is presented in figure. 1 (panel A).

Tests were conducted at the same time of the day in an environmentally controlled laboratory (22-25°C, 55-65% relative humidity), after a standardised meal as previously described in (Keir et al. 2015).

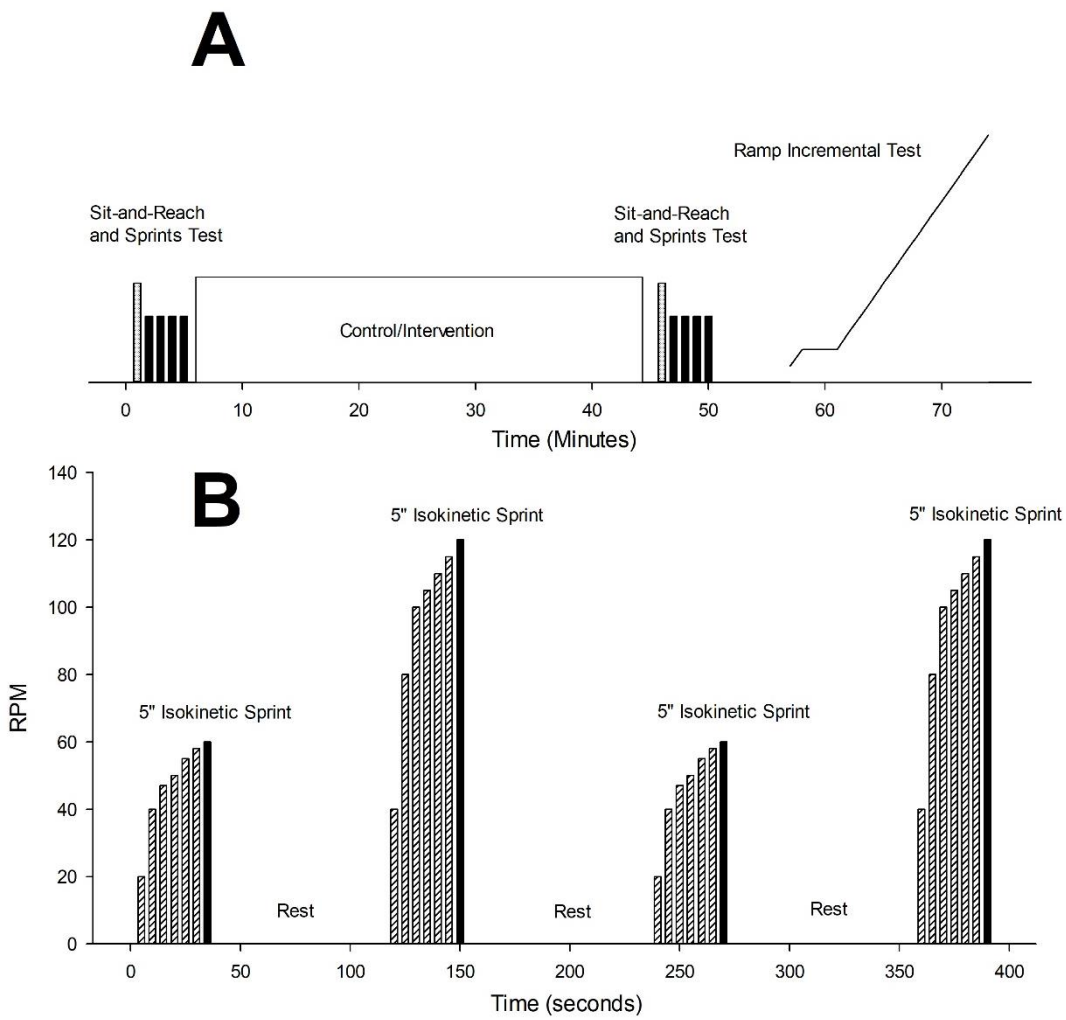

Figure 1, *Panel A: session protocol; Panel B: Isokinetic sprints test protocol; the black bar represents the 5 sec sprints while the shaded bars are the 30 sec of freewheeling cycling necessary to approach the required number of rpm.*

Isokinetic Sprints

Isokinetic maximal sprints were performed on an electromagnetically braked cycle-ergometer equipped with a pedal force measurement system and controlled by computer (Sport Excalibur PFM, Lode, Groningen, NL). The ergometer was set up into an isokinetic mode that limited the peak pedalling frequency and used to measure maximal force expressed on the pedals. Frequencies of 60 and 120 rpm were chosen to measure velocity-specific peak power as proposed by Cannon et al. (Cannon et al. 2011), and controlled by the electromagnetic breaking system of the flywheel. Each sprints session was composed of 4, five-seconds maximal sprints alternating between 60-120-60-120 rpm. The 4 maximal sprints were separated by a 2-min passive rest, to maximise recovery while limiting the total duration of the sprints session. For each five-seconds sprint, participants started to cycle with the bike set at freewheeling, gradually attaining the required rpm within 30 sec. Sprints procedure (schematised in figure 1, panel B) was completed within 9 minutes.

Ramp incremental tests

The ramp incremental tests were performed on an electromagnetically braked cycle ergometer (Sport Excalibur, Lode, Groningen, NL) and consisted of a 4-min baseline cycling at 20 W, followed by a 25-W*min^{-1} increase in power output (PO) until volitional exhaustion. Participants were asked to pick a self-selected cadence in the range of 70-90 rpm and to maintain it throughout all tests. Breath-by-breath pulmonary gas exchange, ventilation and heart rate were continuously measured using a metabolic cart (Quark B^2, Cosmed, Italy) as previously described (De Roia et al. 2012). Surface EMG of the left *vastus lateralis* muscle was continuously recorded by means of a wireless system (Wave wireless EMG, Cometa, Milan, Italy). A pair of surface Ag/AgCl electrodes (Blue sensor, Ambu®, Ballerup, Denmark) was attached to the skin with a 2-cm inter-electrode distance. The electrodes were placed longitudinally with respect to the underlying muscle fibres arrangement, according to the recommendations by Surface EMG for Non-Invasive Assessment of Muscles (Hermens et al. 2000). Before electrode application, the skin was shaved, scratched

with sandpaper and cleaned with alcohol in order to minimize impedance. Semi-permanent ink marks allowed consistent re-positioning of the electrodes between sessions. The EMG transmitter connected to the electrodes was well secured with adhesive tape to avoid movement-induced artifacts.

Capillary blood samples (20 µl) were drawn from the ear lobe before and at the 1^{st},3^{rd}, 5^{th} and 7^{th} min after exhaustion. Samples were immediately analysed using an electro-enzymatic technique (Biosen C-Line, EKF Diagnostics, Barleben, Germany) and the highest value was considered as the peak of blood lactate accumulation for the incremental test.

Interventions

CTRL consisted in 40 minutes of resting in a sitting position under the control of the examiner.

STRC: six cycles of STRC, in which each position was maintained for 80 sec, were used to maximise acute force reduction (Behm et al. 2016). The standardised stretching protocol sequentially involved the quadriceps of the right leg, the right hamstrings, left quadriceps, left hamstrings, with no recovery between exercises. Subjects were continuously encouraged to stretch muscles to the point of discomfort. The total duration of the STRC intervention was about 40 minutes. STRC effectiveness in increasing flexibility was measured pre and post STRC and CTRL (before the isokinetic sprints) using a sit-and-reach test (Limonta et al. 2015).

DJ: participants dropped 100 times from a 40-cm high platform down to 90° knee angle, with a resting period between each DJ of 20 sec (Skurvydas et al. 2000; Hopker et al. 2016). While the original version of this protocol entails a maximal jump after each drop, this part of the protocol was omitted to avoid the confounding effect of metabolic fatigue being added to the non-metabolic fatigue induced by eccentric exercise. DJ intervention lasted 40 minutes. In order to confirm minimal metabolic activation during DJ (Hopker et al. 2016), capillary blood samples were drawn before

and at the 1st,3rd, 5th and 7th minutes after the end of the 100-DJ to determine peak lactate accumulation.

Data analysis

Isokinetic Sprints test: Crank torque was measured independently from the two crank arms by strain gauge transducers (maximal recordable force 2000 N, <0.5 N resolution and measurement uncertainty of <3%). Angular velocity of the crank was recorded every 2 degrees using three independent sensors sampling in series with uncertainty of measurement <1%. Overall power for each pedaling cycle was calculated as the sum of the left and the right crank as resulted by the pedal force measurement analysis software. The initial and the last pedaling cycles of each sprint were excluded from computation. Then, maximal power expressed during each pedaling cycle was detected and cycles were averaged to obtain a mean peak power output for every sprint. Finally, mean peak power output ($_{sprint}$PO) of the two repetitions of the 60 and 120 rpm sprints performed either pre-or post-intervention were averaged to increase measure reliability and the relative change between pre and post conditions. (Δ_{sprint}PO) was calculated as follows:

$\Delta_{sprint}PO = [(_{sprint}PO\ post - {}_{sprint}PO\ pre) / {}_{sprint}PO\ pre) * 100]$

Ramp incremental test: The raw EMG signal was rectified and smoothed using a fourth-order band-pass Butterworth digital filter with a frequency range set between 20 and 500 Hz. Root mean square (RMS) was calculated every second and averaged at 5 sec intervals from the raw signal and was used as an index of the total muscle excitation (Moritani et al., 1986; Ryan & Gregor, 1992). The RMS recorded during the last 2 minutes of 20 W baseline for each test was used to normalize the ramp portion of the tests (Iannetta et al. 2017).

Gas exchange variables and heart rate were sampled breath by breath; aberrant data-points that lay 3 SD from the local mean were removed and thereafter data were interpolated at 5 sec intervals; finally, gas exchange threshold (GET), respiratory

compensation point (RCP), maximum VO_2 (VO_{2max}) and peak PO (PO_{peak}) were determined as previously described (Fontana et al. 2015). Briefly, VO_{2max} was determined as the highest VO_2 obtained over a 10s interval and PO_{peak} was defined as the highest mechanical power output achieved upon exhaustion during the RI exercise. GET and RCP were estimated by visual inspection from gas exchange variables by three blinded expert reviewers (Beaver, 1985; Whipp, 1989).

In addition, VO_2 and RMS signals obtained during CTRL, DJ and STRC were compared at the same absolute work rate by performing a linear interpolation every 10% of the PO_{peak} reached during CTRL. Finally, RMS and VO_2 changes as a function of work rate (% of the PO_{peak} reached during CTRL) were expressed as multiples of 20 W baseline values and the RMS/VO_2 ratio was calculated using these normalized units.

Statistics

After assumptions verification (*i.e.*, normality, homogeneity of variance), repeated measures ANOVA was applied to compare flexibility values (pre and post sit-and-reach after CTRL/STRC) and blood lactate accumulation before and after the DJ and CTRL.

Pre and post $_{sprint}$PO at 60 and 120 rpm were compared within and between STRC, DJ, and CTRL conditions using a two-way repeated measures ANOVA (time x condition); Δ_{sprint}PO (condition x pedalling frequency) among interventions at the two speeds were compared by two-way repeated measures ANOVA.

A two-way repeated measures ANOVA was performed to compare VO_2, RMS and RMS/VO_2 ratio between conditions over different work rate percentages (work rate x condition). Finally, a one-way repeated measures ANOVA was used to compare PO_{peak}, VO_{2max}, GET, RCP, incremental Lactate peak, ventilation, heart rate and post-DJ Lactate peak between conditions.

Data are presented as means ± SD. 95% Confidence intervals around mean differences (95% ΔCIs [lower limit, upper limit]) and effect sizes of those differences (Cohen's *d*, ranked as trivial (0-0.19), small (0.20-0.49), medium (0.50-0.79) and large (0.80 and

greater) (Cumming 2014a)) are also reported as objective and standardized measures to quantifying the magnitude of difference after intervention vs control condition (Winter, 2014). In effect size calculation, the SD in the control condition was used to standardize the mean difference for each contrast (Field, 2012).

All statistical analyses were performed using Sigmaplot version 12 and α was set in advance at the 0.05 level; statistical significance was accepted when $p < \alpha$.

Results:

Flexibility, as measured by sit-and-reach test, was not significantly different at baseline between CTRL and STRC and significantly improved only after STRC (+0.4±7.6 cm *pre* vs +5.9±6.5 cm *post* STRC, p<0.001, *d*=0.847 , 95%CI=+1.881, +9.969; +0.9±5.2 cm *pre* vs +0.9±5.3 cm *post* CTRL, p=0.832, *d*=0.009 , 95%CI=-2.389, +4.189). Blood lactate concentration was not significantly different at baseline between CTRL and DJ and was not significantly affected by either DJ protocol (1.0±0.3 mmol*L^{-1} *pre* vs 1.1±0.2 mmol*L^{-1} *post* DJ, p=0.110, *d*=0.349, 95%CI=0.305, 1.660) or CTRL (1.0±0.2 mmol*L^{-1} *pre* vs 1.1±0.3 mmol*L^{-1} *post* CTRL, p=0.274, *d*=0.405, 95%CI=0.338, 1.690).

During isokinetic sprints, A significant interaction between "condition" and "time" was detected for the 60 (p=0.002) and the 120 (p=0.008) rpm. Post-hoc analysis revealed that $_{sprint}$PO was significantly reduced by DJ and STRC during the lower speed, 60 RPM sprints and during the higher speed, 120 RPM sprints (table 1). On the contrary, no changes were found between pre and post after CTRL for both the pedaling frequencies (table 1). Regarding Δ_{sprint}PO, a main effect of "condition" was detected (p=0.007), while there was no main effect of "pedaling frequency" (p=0.532). No interaction was found between "condition" and "pedaling frequency" (p=0.097).

During the ramp incremental tests, muscle excitation increased as a function of work rate during CTRL; both the interventions significantly affected muscle excitation (i.e. RMS, main effect: p≤0.001) that was increased at a given absolute work rate vs CTRL

(figure 2). Post-hoc analysis revealed significantly higher muscle excitation for DJ compared to CTRL at work rate ≥ 20% of CTRL peak power output; furthermore, a significantly higher muscle excitation was observed for STRC compared to CTRL at work rate ≥ 40% of the CTRL peak power output.

Acute fatigue and increased muscular excitation translated in reduced peak power output after DJ compared to CTRL and reduced peak power output and VO_{2max} after STRC. Moreover, both thresholds occurred at a lower W and VO_2 after DJ and STRC. These data are presented extensively in table 2 together with the blood lactate, ventilation and heart rate values measured in different conditions.

A significant main effect of intervention was detected for VO_2 ($p=0.029$) as a function of work rate; both interventions resulted in an increased VO_2 at a given absolute work rate *vs* CTRL (figure 2).

Finally, the stability of RMS/VO_2 ratio as a function of exercise intensity in all conditions indicates that muscle activation relative to metabolic intensity was not affected by work rate (main effect of intensity: $p=0.375$, figure 2). On the contrary, a significant main effect of interventions was demonstrated on RMS/VO_2 ratio (main effect: $p=0.023$) (figure 2). However, post-hoc analysis revealed significant increases in muscle activation relative to metabolic intensity only at < 40% of CTRL PO_{peak} following DJ and at 90% of CTRL PO_{peak} following STRC compared to CTRL (figure 2). Between 40 and 80% of CTRL PO_{peak} the increase in muscle activation was matched by an equivalent increase in VO_2.

Table 1, Mean ± SD peak power output during isokinetic sprints pre and post conditions

		pre (W)	post (W)	*p*	*d*	95%CI [LL, UL]	Δ (W)	Δ (%)	*p*
Control	**60 RPM**	640 ± 54	649 ± 60	0.334	+0.167	610, 691	+4 ± 15	+0.6 ± 3.8	*CTRL vs DJ*
	120 RPM	949 ± 169	942 ± 171	0.469	-0.056	868, 1016	-7 ± 19	-0.7 ± 2.4	0.019
Dropjumps	**60 RPM**	**653 ± 48**	**621 ± 49**	**0.002**	**-0.668**	**589, 654**	-32 ± 30	-5.2 ± 3.1	*CTRL vs STRC*
	120 RPM	**929 ± 114**	**908 ± 96**	**0.034**	**-0.185**	**846, 971**	-21 ± 32	-2.3 ± 3.5	0.011
Stretching	**60 RPM**	**663 ± 59**	**631 ± 84**	**0.003**	**-0.536**	**590, 671**	-38 ± 29	-5.4 ± 4.7	*STRC vs DJ*
	120 RPM	**973 ± 97**	**944 ± 95**	**0.003**	**-0.294**	**881, 1008**	-29 ±10	-2.9 ± 3.0	0.634

Isokinetic sprints peak power output is presented for the 60 and 120 RPM pedalling frequencies pre and post each intervention with p-values, confidence intervals and Cohen's effect size (d). Bolded values represent significant differences between pre and post absolute values. Δ *represents the mean difference between pre and post sprints. Regarding* Δ_{sprint}*PO, a main effect of "condition" was detected (p=0.007), while there was no main effect of "pedaling frequency" (p=0.532) nor interaction between "condition" and "pedaling frequency" (p=0.097).*

Table 2, Mean ± SD cardiorespiratory data during the 20W warm-up, at peak, Gas Exchange Threshold (GET), and Respiratory Compensation Point (RCP).

		Control	95%CI [LL,UL]	Dropjumps	*p*	*d*	95%CI [LL,UL]	Stretching	*p*	*d*	95%CI [LL,UL]
Warm-up	VO_2 ($ml*min^{-1}$)	1103 ± 320	904, 1301	1167 ± 289	0.612	+0.221	988, 1346	1088 ± 339	0.833	-0.044	878, 1298
	HR ($b*min^{-1}$)	107 ± 14	98, 116	103 ± 10	0.321	-0.421	97, 109	98 ± 18	0.149	-0.467	87, 110
	VE ($L*min^{-1}$)	16 ± 2	15, 18	16 ± 3	0.997	0.000	14, 18	16 ± 2	0.999	0.000	15, 17
Peak	W	314 ± 41	289, 340	**300 ± 37**	**0.023**	**-0.380**	**277, 323**	**302±39**	**0.033**	**-0.314**	**278, 326**
	VO_2 ($ml*min^{-1}$)	3505 ± 486	3204, 3806	3413 ± 476	0.094	-0.194	3118, 3707	**3365 ± 465**	**0.015**	**-0.301**	**3077, 3653**
	HR ($b*min^{-1}$)	183 ± 11	176, 189	179 ± 11	0.131	-0.298	173, 186	181 ± 9	0.804	-0.132	176, 187
	VE ($L*min^{-1}$)	151 ± 17	141, 162	154 ± 24	0.688	+0.125	139, 169	154 ± 17	0.578	+0.176	140, 162
	$[La^-]$ ($mmol*L^{-1}$)	12 ± 1	11, 13	**10 ± 2**	**0.038**	**-0.941**	**9, 11**	10 ± 1	0.247	-1.189	10, 11
	Time (sec)	704 ± 98	643, 765	**671 ± 87**	**0.022**	**-0.379**	**617, 725**	679 ± 82	0.073	-0.305	628, 730
GET	W	153 ± 44	125, 180	136 ± 52	0.070	-0.325	104, 128	136 ± 48	0.054	-0.363	106, 165
	VO_2 ($ml*min^{-1}$)	2150 ± 464	2133, 2167	**1998 ± 524**	**0.050**	**-0.291**	**1673, 2322**	**1955 ± 484**	**0.018**	**-0.403**	**1655, 2255**
	HR ($b*min^{-1}$)	142 ± 17	131, 152	**133 ± 19**	**0.027**	**-0.480**	**121, 144**	134 ± 17	0.051	-0.452	124, 144
	VE ($L*min^{-1}$)	56 ± 12	49, 63	53 ± 17	0.572	-0.176	42, 64	52 ± 14	0.460	-0.286	49, 63
RCP	W	236 ± 44	209, 263	**205 ± 45**	**0.003**	**-0.692**	**177, 233**	**209 ± 34**	**0.007**	**-0.796**	**188, 230**
	VO_2 ($ml*min^{-1}$)	2883 ± 488	2580, 3185	**2585 ± 451**	**0.004**	**-0.659**	**2305, 2865**	**2615 ± 402**	**0.006**	**-0.666**	**2366, 2864**
	HR ($b*min^{-1}$)	164 ± 14	155, 173	**153 ± 16**	**0.002**	**-0.710**	**163, 173**	159 ± 15	0.091	-0.340	150, 168
	VE ($L*min^{-1}$)	87 ± 18	76, 99	**76** ± 17	**<0.001**	**-0.647**	**65, 87**	83 ± 22	0.126	-0.182	76, 98

Acronyms represent: W: power measured in watts, VO_2: oxygen consumption, HR: heart rate, VE: ventilation, $[La^-]$: peak blood lactate concentration. Bolded values represent significant differences versus control, no significant differences were found between DJ and STRC.

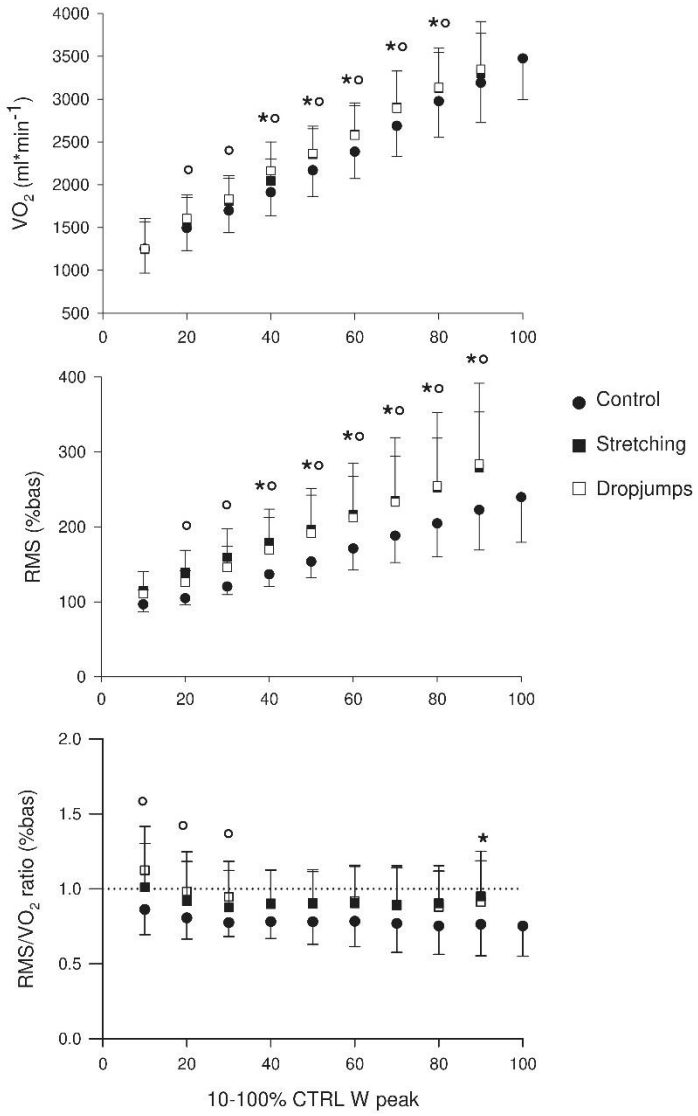

Figure 2, *Mean ± SD VO_2 (top panel) and Root Mean Square (RMS, medium panel) values and RMS/VO_2 ratio every 10-100% of Control W are presented. Symbols represent: black dots= control condition, black squares (Stretching), white squares (dropjumps). Significant main effect of intervention was detected for VO_2 ($p=0.029$) and RMS ($p\leq0.001$). Statistical differences resulting from the post-hoc analysis are represented by blank dots (DJ vs CTRL) and asterisks (STRC vs CTRL).*

Discussion:

In this study, we tested the physiological response to ramp incremental exercises performed in separated days in CTRL conditions and after two distinct acute fatiguing interventions (*i.e.* DJ and STRC). Both interventions caused acute, non-metabolic fatigue (detected as reduced maximal cycling power); in turn, acute fatigue augmented muscle recruitment for a given absolute work rate during ramp incremental cycling, reduced maximal (peak power output and VO_{2max}) and submaximal indexes of exercise tolerance (GET, RCP), compared to CTRL. Moreover, both acute fatiguing interventions were associated with metabolic loss of efficiency (i.e. higher VO_2 at an identical submaximal work rate). These findings suggest a possible link between exercise intolerance/loss of efficiency and the observed decreased ability to produce force as a result of acute, non-metabolic fatigue interventions.

The isokinetic sprints test used in this investigation was able to detect a velocity-specific peak power output impairment after both interventions compared to CTRL. No changes in velocity-specific peak power output occurred after CTRL (CTRL: 60 rpm +1.3±3.8 %, 120 rpm: -0.7±2.4 %). On the contrary, maximal $_{sprint}$PO at both velocities was significantly reduced by both interventions (STRC: 60 rpm: -4.8±4.7 %; 120 rpm: -2.9±3.0 %; DJ: 60 rpm: -4.9±3.1 %; 120 rpm: -2.3±3.5 %). The amplitude of $_{sprint}$PO impairments after STRC measured in our study were consistent with previous investigations that used a variety of techniques to quantify maximal strength/power (Behm and Chaouachi 2011; Behm et al. 2016). On the contrary, force reduction after DJ was lower than the 10% reduction reported by Hopker et al. (Hopker et al. 2016), during 6 seconds isokinetic sprints at 90 rpm. Their protocol included maximal jumping after dropping while our protocol did not; this is the likely cause of the larger fatigue effect reported by Hopker et al. compared to our study. Furthermore, our study was the first to investigate fatigue at two sprint velocities following DJ and STRC, in the attempt to detect a possible differential impairment of fast vs slow motor units. Previous studies had proposed that STRC (Limonta et al. 2015) may preferentially affect fast motor units, and therefore the higher sprint velocity. However,

our data did not demonstrate differences in the velocity-specific peak power output at higher compared to lower pedaling frequency (Cannon et al. 2011). This finding favours the idea that STRC and DJ induce acute, non-metabolic fatigue to a similar extent in both fast and slow motor units.

This is the first time that acute fatiguing interventions were used to impair maximal force while contextually measuring metabolism and muscular excitation. The values measured during CTRL condition revealed an average aerobic fitness of our sample similar to the reference, sedentary population of young male adults (absolute VO_{2max} 3505±486 ml*min^{-1}; normalized VO_{2max}: 43.9±6 ml*min^{-1} *kg^{-1} corresponding at the 50° percentile of the ACSM's guidelines) (American College of Sports Medicine 2017), and a mean peak power output of 314±41 W. Both the interventions caused a small impairment of VO_{2max} (STRC: 3365±465 ml*min^{-1} (≈ -4% vs CTRL, p=0.015); DJ: 3413±476 ml*min^{-1} (≈ -2.6% vs CTRL, p=0.094)) and significant impairments of peak power output both after STRC and DJ (STRC: 302±39 W (≈ -4% vs CTRL, p=0.033); DJ: 300±37 W (≈ -4.5% vs CTRL, p=0.023)).

Impairment of maximal indexes of performance was accompanied by a higher metabolic activation at the same absolute work rate compared to the control condition (figure 2) throughout the incremental test. In specific, a statistically significant loss of efficiency was identified at absolute work rate in the range of 40 to 80 % of CTRL-peak power output after STRC and of 20 to 80 % of CTRL-peak power output after DJ. Our results agree with previous work (Limonta et al. 2015) that reported a raised VO_2/W ratio during ramp incremental exercises performed after passive STRC. On the contrary, our data are in contrast with Hopker et al., that, in spite of a reduced exercise tolerance following DJ, reported similar VO_2 values for a given intensity (Hopker et al. 2016). Unfortunately, in the above investigations, a direct measure of muscular excitation was lacking, making it difficult to establish a clear relationship between possible alterations of muscle recruitment and VO_2 at a given work rate. In our study, the RMS/VO_2 ratio data suggest that, between 40 and 80% of CTRL PO_{peak}, the increase in VO_2 observed following the fatiguing interventions was proportional to the

augmented muscle excitation. It should also be noted that the fatiguing interventions did not alter the ventilation patterns during the different ramp incremental tests (table 2), supporting the idea that the changes in VO_2 found after DJ and STRC were mostly due to a loss of efficiency in the working muscles rather than to an increased cost of ventilation (Coast et al. 1993).

This is also indirectly supported by the occurrence of GET (STRC: -195 $ml*min^{-1}$; DJ: - 162 $ml*min^{-1}$) and RCP (STRC: -268 $ml*min^{-1}$; DJ: - 298 $ml*min^{-1}$) at lower absolute power outputs compared to the control condition. These thresholds represent the boundaries of the "heavy" and "very heavy" exercise domains (Keir et al. 2015), and consequently the edge of an augmented involvement of type two muscle fibres (Poole and Jones 2012). Importantly, the "shift" of these boundaries towards lower power outputs could lead to fatigue and exercise intolerance for a previously well tolerated work rate (Keir et al. 2015, 2016). Therefore, our results suggest a link between the ability of the body to maintain metabolic stability and the muscle's absolute capacity to produce force. Moreover, given that changes in VO_2/W ratio during ramp incremental exercise (i.e. "excess" VO_2) are considered equivalent to the VO_2 slow component measured during constant work rate cycling (Grassi et al. 2015), it is reasonable to speculate that fatiguing interventions would elicit increased muscular excitation and metabolic activation also when cycling at a fixed work rate.

The main cause of STRC-induced loss of performance has been suggested to be the reduction in neural drive caused by prolonged periods of sensory stimulations (possibly at peripheral, spinal or supra-spinal level), rather than the accumulation of metabolites within the muscles (Behm et al. 2016; Trajano et al. 2017). In addition, low lactate values measured following DJ intervention by this and other investigation confirmed low to null metabolic activation (Hopker et al. 2016). However, the exact physiological mechanisms underlying the impairment of force production after both STRC and DJ have not been clearly identified (Skurvydas et al. 2000; Twist and Eston 2005; Trajano et al. 2017). Therefore, we cannot exclude that some extent of metabolic activation may be present also in these mostly non-metabolic fatiguing interventions.

Conclusion:

Two acute, non-metabolic fatiguing interventions significantly reduced maximal cycling power output while augmenting muscle recruitment during ramp incremental cycling. Augmented muscle recruitment impaired maximal and submaximal indexes of exercise tolerance and led to metabolic inefficiency. Although further studies are warranted to identify a direct cause-effect relationship, these findings suggest a possible link between exercise intolerance/loss of efficiency and the observed decrease in the ability to produce force as a result of acute, non-metabolic fatigue.

CHAPTER

4

Response to Acute Non-Metabolic Fatigue During Constant Work Rate Exercise

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Abstract:

We tested the hypothesis that static stretching, an acute, non-metabolic fatiguing intervention, reduces exercise tolerance by increasing muscle activation and affecting muscle bioenergetics during cycling in the "severe" intensity domain. Ten active men (24±2 years, 74±11 kg, 176±8 cm) repeated an identical constant work rate cycling test, two tests were done in control conditions and two after stretching, that caused a 5% reduction of maximal isokinetic sprinting power output. We measured: *i)* oxygen consumption (VO_2); *ii)* electromyography: *iii)* deoxyhemoglobin *iv)* blood lactate ($[La^-]$); *v)* time to exhaustion (TTE) *vi)* perception of effort. Finally, VO_2 and deoxyhemoglobin kinetics were determined. Force reduction following stretching was accompanied by augmented muscle excitation at a given work rate ($p=0.025$), and a significant reduction in TTE ($p=0.002$). The time to peak of VO_2 was reduced by stretching ($p=0.034$), suggesting an influence of the increased muscle excitation on the VO_2 kinetics. Moreover, stretching was associated with a mismatch between O_2 delivery and utilization during the on-kinetic, increased perception of effort and $[La^-]$, that are all compatible with an increased contribution of the glycolytic energy system to sustain the same absolute intensity. These results suggest a link between exercise intolerance and the decreased ability to produce force.

Introduction:

During whole-body exercise at constant work rate in the moderate domain, oxygen consumption (VO_2) adapts to the energetic demands of locomotor and ventilatory muscles within 3 minutes (Poole and Jones 2012). If relative intensity rises above the gas exchange threshold (GET), approximately after the third minute of exercise VO_2 displays a "slow component" (VO_{2sc}) that is typically interpreted as an increased cost of locomotion for a given exercise intensity (Poole and Jones 2012). In particular, when exercise is performed between the metabolic rates associated to the GET and the respiratory compensation point (RCP; i.e. heavy intensity domain) (Keir et al. 2015, 2018b) VO_{2sc} tends to a steady-state; however, when effort rises above RCP (i.e. severe exercise domain) a steady-state is no longer achievable and VO_2 increases over time tending to the maximum oxygen consumption (VO_{2max}) (Jones et al. 2011). The magnitude of VO_{2sc} is considered linked with exercise intolerance and fatigue (Grassi et al. 2015). Therefore, during the past forty years many researchers have focused their attention on clarifying its physiological bases (Jones et al. 2011). Two main theories have been proposed to explain the physiological origin of VO_{2sc}: *i) decreased metabolic stability of type I muscle fibres* associated with increased O_2 cost of ATP resynthesis and/or increased ATP cost of contraction (Jones et al. 2011; Grassi et al. 2015) and/or *ii) recruitment of fast-fatigable intrinsically inefficient type II muscle fibres* to obtain/maintain the external power output above a certain intensity threshold (e.g. RCP) (Jones et al. 2011; Poole and Jones 2012; Grassi et al. 2015). However, the exact physiological mechanisms underpinning VO_{2sc} remain elusive, one of the reasons being the difficulty to selectively affect either metabolic stability or type II fibre recruitment in human models. In fact, the different manipulations used in interventional studies (e.g. speed of movement, intensity modulation, aerobic training, priming exercise, nutritional interventions) affect to some extent both metabolic stability and fibre recruitment (Jones et al. 2011).

An interesting approach to selectively augment fibre recruitment while trying to avoid the perturbation of metabolic stability is acute, non-metabolic fatigue that reduces the

ability of muscles to produce force. Among the interventions able to cause acute, non-metabolic fatigue, a promising model could be static stretching, that can impair force production as result of prolonged nervous stimulation (Trajano et al. 2017). It was broadly documented that stretching, particularly when positions are maintained for more than 60 sec, can impair maximal force in many different tasks and conditions for a period lasting up to 1 hour (Behm et al. 2016). Given that no effort is required to perform stretching, and that force impairment after stretching is mostly caused by neural mechanisms (Trajano et al. 2017), this would be a particularly convenient model to acutely reduce force and investigate the link between muscle activity and metabolism. Indeed, recent studies (Esposito et al. 2012) documented that when maximal force was acutely reduced by stretching, the oxygen cost of locomotion increases both during ramp incremental (exercise modality in which the VO_{2sc} is defined as "excess VO_2" (Grassi et al. 2015)) and constant work rate exercises (Esposito et al. 2012; Limonta et al. 2015). However, the above studies did not specifically investigate the underpinnings of VO_{2sc} and were, therefore, lacking measures to investigate the link between muscle activation and increased VO_2 (e.g. electromyography, EMG). In a recent study from our group, the effects of stretching on the VO_2 response during ramp incremental cycling were described while also implementing measures of muscle excitation (Colosio et al. 2019). We found that when muscle force is acutely impaired by stretching also muscles excitation increases, at unison with an increased cost of locomotion (i.e. VO_2 at a given absolute work rate).

The above findings, in an incremental exercise paradigm, support the existence of a sequence of events, i.e. acute fatigue, increased muscle activation, loss of metabolic efficiency, causing the VO_{2sc} with increasing exercise intensity. In this context, constant work rate exercise represents the ideal model to determine the possible role of increased muscular activation over time (necessary to maintain the same work rate when fatigued) in the genesis of the VO_{2sc}. In fact, only under prolonged, constant work rate conditions, the increased cost of locomotion (i.e. VO_{2sc}) at a given intensity has the time to fully manifest itself. The confirmation of a connection between

fatigue/increased muscle activation and the loss of metabolic efficiency over time during a constant work rate exercise paradigm would further support the existence of a causative link.

Accordingly, this study investigated the effects of acute, non-metabolic fatigue induced by stretching on central and peripheral physiological measures (VO_2, blood lactate accumulation [LA^-], EMG, Near-Infrared Spectroscopy (NIRS)) during constant work rate cycling in the severe exercise domain. We hypothesis that stretching *i)* will reduce maximal muscle force; *ii)* in turn, force loss will translate in increased muscle excitation at a given absolute work rate *iii)* increased muscle excitation will reduce exercise tolerance and increase the VO_2 cost of locomotion. Finally, this study will provide the first comprehensive investigation on the effects of static stretching on high-intensity constant work rate cycling.

Materials and methods:

Participants:

Ten active men gave written informed consent to participate in the study (age: 24±2 years, body mass: 74±11 kg, stature: 176±8 cm). Inclusion criteria were male sex and age between 20 and 35 years; exclusion criteria were smoking and any condition that could influence the physiological responses during testing. The study was approved by Departmental Ethics Committee and adhered to the principles of the declaration of Helsinki. All participants were instructed to avoid physical activity for at least 24 h before each testing session and followed a standard and individualised food intake prescription before all the testing sessions to minimise variability of glycogen stores and glucose oxidation (i.e. 2 g of low glycemic index carbohydrates per kg of body weight, 2 hours before testing; 0.5 L of water in the 90 min before testing; restriction from caffeine during the 8 h before testing).

Experimental Protocol:

After medical clearance, participants visited the laboratory on eight occasions within a maximum of three weeks.

On the first two visits, subjects familiarized with a test consisting of isokinetic sprints for the determination of the maximal cycling power output. On the third appointment, isokinetic sprints were performed pre and post either the control condition (i.e. 40 min of seated rest, control) or 40 min of stretching, to determine the effect of stretching on the maximal cycling power output. On the fourth visit, subjects performed a ramp incremental test to exhaustion for the determination of the GET, the RCP and the VO_{2max}. Then, during the last four visits participants repeated four identical constant work rate trials in the severe exercise intensity domain (at a power output corresponding to Δ60% between GET and VO_{2max}). Randomly, 2 of the constant work rate trials were done in control conditions and 2 after 40 min of stretching. A schematic representation of the protocol is provided in figure 1.

All the tests were conducted at the same time of the day in an environmentally controlled laboratory (22-25°C, 55-65% relative humidity), on an electromagnetically braked cycle ergometer (Sport Excalibur, Lode, Groningen, Netherlands). Ergometer position was chosen during the first familiarization visit and recorded for the successive appointments.

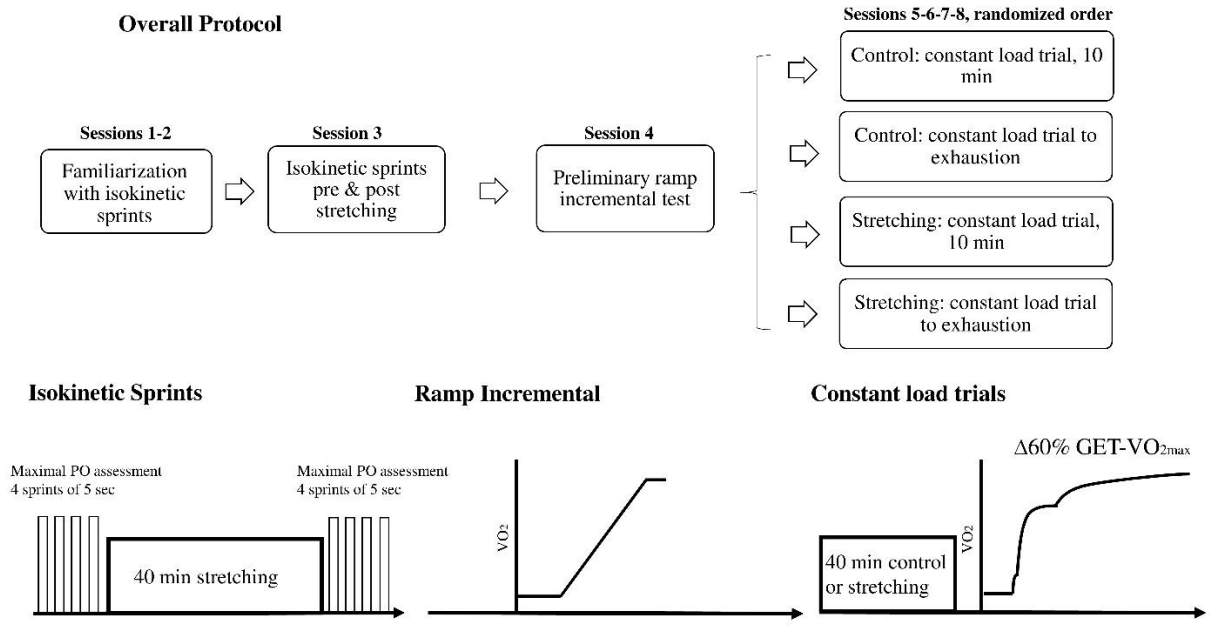

Figure 1, *Schematic representation of the overall protocol (above), and of the single testing sessions (below).*

Stretching procedure

Control consisted in 40 minutes of resting in a sitting position under the examiner's surveillance.

Six cycles of stretching, were used to maximise acute force reduction (Behm et al. 2016). The standardised stretching cycle sequentially involved i) the quadriceps of the right leg, ii) the right hamstrings, iii) left quadriceps, iv) left hamstrings. Each position was maintained for 80 sec with no recovery between positions. Subjects were continuously encouraged to stretch muscles to the point of discomfort. The total duration of the stretching intervention was about 40 minutes. Stretching effectiveness in increasing flexibility was measured pre and post stretching and control using a sit-and-reach test (Limonta et al. 2015).

Isokinetic maximal sprints

To assess force reduction after stretching, isokinetic maximal sprints were performed on an electromagnetically braked cycle-ergometer in isokinetic mode equipped with a pedal force sensor (Sport Excalibur PFM, Lode, Groningen, NL) as previously described (Colosio et al. 2019). In brief, two pedalling frequency (60 and 120 rpm) were used to measure velocity-specific peak power as proposed by Cannon et al. (Cannon et al. 2011). Each sprints session was composed of 4, five-seconds maximal sprints alternating between 60-120-60-120 rpm. The 4 maximal sprints were separated by a 2-min passive rest, to maximise recovery while limiting the total duration of the sprints session.

Ramp incremental test

The ramp incremental test consisted of a 4-min baseline cycling at 20 W, followed by increases in power output (PO) ranging from 17.5 to 25 $W*min^{-1}$ according to individuals' predicted fitness level with the aim of obtaining a time to exhaustion around 8-12 minutes (American College of Sports Medicine 2017) using a method extensively described elsewhere (Pogliaghi et al. 2014). Participants were asked to pick

a self-selected cadence in the range of 70-90 rpm and to maintain it throughout all tests. Failure to maintain the indicated cadence within 5 rpm (for longer than 5 sec) during testing despite strong verbal encouragement was considered as the criterion for exhaustion. Breath-by-breath pulmonary gas exchange, ventilation and heart rate were continuously measured using a metabolic cart (Quark B^2, Cosmed, Italy) as previously described (De Roia et al. 2012).

Constant work rate trials

After the preliminary ramp incremental test, subjects completed 4 constant work rate trials at the power output corresponding to the 60%Δ between GET and VO_{2max}. Two of the constant work rate trials were performed after stretching and 2 after control in a randomized order. In each condition, one constant work rate trial lasted 10 min while the other one was performed to exhaustion to allow recording of time to exhaustion (TTE) after control and stretching. Constant work rate trials were preceded by a 4-min warm-up at 20 W in which cycling cadence was limited to 30 rpm to minimize any metabolic activation that could influence the effects of stretching and the physiological response at the onset of exercise. Throughout the test, subjects kept the same, constant rpm selected during the ramp incremental test and the same bike position selected during the sprints test.

Surface EMG of the right vastus lateralis and biceps femoris muscles were continuously recorded by means of a wireless system (Wave wireless EMG, Cometa, Milan, Italy). A pair of surface Ag/AgCl electrodes (Blue sensor, Ambu®, Ballerup, Denmark) was attached to the skin with a 2-cm inter-electrode distance. The electrodes were placed longitudinally with respect to the underlying muscle fibres arrangement, according to the recommendations by Surface EMG for Non-Invasive Assessment of Muscles (Hermens et al. 2000). Before electrode application, the skin was shaved, scratched with sandpaper and cleaned with alcohol in order to minimize impedance. Semi-permanent ink marks allowed consistent re-positioning of the electrodes between

sessions. The EMG transmitter connected to the electrodes was well secured with adhesive tape to avoid movement-induced artifacts.

VO_2, ventilation (VE), respiratory exchange ratio (RER), and heart rate (HR) data were measured with the same method described for the ramp incremental test. During each constant work rate trial, capillary blood samples (20 µl) were drawn from the earlobe in the last 30 sec of warm-up, during the 1^{st},3^{rd}, 5^{th}, 7^{th}, 10^{th} and then every 5 min of the trial to exhaustion. Moreover, blood samples were drawn at the 1^{st},3^{rd}, 5^{th} and 7^{th} min after exhaustion. Samples were immediately analysed to measure [La^-] (Biosen C-Line, EKF Diagnostics, Barleben, Germany).

Deoxygenation of the left vastus lateralis was evaluated in microcirculation using a quantitative near-infrared spectroscopy system (Oxiplex TSTM, ISS, Champaign, USA) that provided continuous measurement (sampling frequency 1 Hz) of the absolute concentrations (µM) of deoxyhemoglobin ([HHb]). After shaving, cleaning and drying of the skin area, the NIRS probe was positioned longitudinally on the belly of the vastus lateralis muscle ~15 cm above the patella, attached to the skin with a bi-adhesive tape and secured with elastic bandages around the thigh. The device was calibrated before each test after a warm-up of at least 30 minutes as per manufacturer recommendations.

Finally, perceptual responses to exercise was monitored using a 0-100 rating perceived exertion (RPE) scale (Borg and Kaijser 2006). The scale was displayed to the participants during baseline, every five minutes during the constant work rate trials and immediately after exhaustion.

Data analysis

Isokinetic Sprints test: Crank torque was measured independently from the two crank arms by strain gauge transducers (maximal recordable force 2000 N, <0.5 N resolution

and measurement uncertainty of <3%). Angular velocity of the crank was recorded every 2 degrees using three independent sensors sampling in series with uncertainty of measurement <1%. Overall power for each pedaling cycle was calculated as the sum of the left and the right crank as resulted by the pedal force measurement analysis software. The initial and the last pedaling cycles of each sprint were excluded from computation. Then, maximal power expressed during each pedaling cycle was detected and cycles were averaged to obtain a mean peak power output for every sprint. Finally, mean peak power output of the two repetitions of the 60 and 120 rpm sprints performed either pre-or post-intervention were averaged and the relative % change between pre and post conditions were calculated.

Ramp incremental test: For the gas exchange variables, aberrant data-points that lay 3 SD from the local mean were removed, and trials were linearly interpolated on a 1-sec basis and then averaged every 5 sec. VO_{2max} was determined as the highest VO_2 obtained over a 10-sec interval (Fontana et al. 2015). GET and RCP were determined with the standard technique from gas exchange variables by three blinded expert reviewers as detailed elsewhere (Fontana et al. 2015). Briefly, GET was determined by visual inspection as the VO_2 at which CO_2 output began to increase out of proportion in relation to VO_2, with a systematic rise in the VE-to-VO_2 relation and end-tidal PO_2 whereas the ventilatory equivalent of VCO_2 (VE/VCO_2) and end-tidal PCO_2 is stable (Beaver et al. 1986). RCP was determined as the point where end-tidal PCO_2 began to fall after a period of isocapnic buffering (Whipp et al. 1989). This point was confirmed by examining VE/VCO_2 plotted against VO_2 and by identifying the sec breakpoint in the VE-to-VO_2 relation. VO_{2max} was determined as the highest VO_2 obtained over a 10-sec interval. Finally, we determined the constant work rate equivalent to the specific severe (60%Δ between GET and VO_{2max}) VO_2 target. To this aim, the VO_2/W relationship identified with the incremental test was left-shifted to account for the individual mean response time. Briefly, the mean response time was determined as the time interval between the onset of the incremental portion of the exercise (time = 0)

and the increase of the VO_2 signal above baseline. It was determined as the x coordinate of the intersection of the forward extrapolation of the baseline VO_2 and the back-wards extrapolation of the linear VO_2–time relationship below the GET (Fontana et al. 2015).

Constant Work Rate Trials

The raw EMG signal was rectified and smoothed using a fourth-order band-pass Butterworth digital filter with a frequency range set between 20 and 500 Hz. Root mean square (RMS) was calculated every second and averaged at 30 sec intervals from the raw signal and was used as an index of the total muscle excitation for vastus lateralis (RMS_{VL}) and biceps femoris (RMS_{BF}) (Moritani et al., 1986; Ryan & Gregor, 1992). Thereafter, the RMS recorded during the last 2 minutes of 20 W baseline for each test was used to normalize the constant work rate trials and expressed as multiples of baseline.

Time to exhaustion was calculated as the total duration of exercise from work rate onset to failure.

VO_2 during constant work rate trials was cleaned and interpolated using the same procedure described for the ramp incremental test. Then, data of the two constant work rate trials performed in each condition were mediated in order to reduce breath-by-breath signals variability. Finally, 30 sec means were calculated.

Net [La^-] accumulation during constant work rate trials was calculated as the difference between [La^-] at a specific timepoint and the [La^-] during cycling at 20 W. The highest value after exercise end was considered as the peak of blood lactate concentration.

NIRS derived [HHb] response during constant work rate trials was time aligned with the onset of exercise transition, treated by subtracting the steady-state value measured during the last 2 min of warm-up, and then averaged at 30 sec bins.

VO_2, [HHb] kinetics and VO_{2sc}:

Using 1 sec bins data, the on-transient responses to exercise of VO_2 was modelled as follows: first, the VO_2 response from -60 up to 180 seconds (time 0 being exercise onset) was preliminarily characterized with a two-component model (linear + exponential), integrated by a Heaviside function, after the exclusion of the data-points of the initial 20 sec of exercise that correspond to the cardiodynamic phase (Murias et al. 2011a)). With this approach, we derived the initial parameters for the primary component. Then, the complete on-transient responses to exercise of VO_2 were modelled from the onset of work rate to the end of the 10^{th} min (or to exhaustion for tests that lasted less than 10 min after stretching) using the following two-component exponential equation integrated by a Heaviside function (De Roia et al. 2012):

$$Y_{(t)} = Y_{bsln} + AMP_p \left(1 - e^{-(t - TD_p)/\tau_p} \right) + AMP_{sc} \left(1 - e^{-(t - TD_{sc})/\tau_{sc}} \right)$$

Where $Y_{(t)}$ represents the increase in VO_2 at the onset of exercise, Y_{blsn} is the baseline VO_2 value recorder during the 4 min 20 W cycling, AMP_p and AMP_{sc} represent the amplitude of the VO_2 response above the baseline value of the primary and the slow component respectively; τ_p and τ_{sc} and TD_p and TD_{sc} are the time constant and the time delay of the response for each component. The mean response time (MRT) was then calculated as the sum of τ + TD. Furthermore, we calculated the time requested to reach VO_{2max} during constant work rate trials by resolving on the individual fitting of VO_2 data for the time coordinate corresponding to VO_{2max}.

[HHb] signal was fitted on a time window of -60 to 180 sec (time 0 being exercise onset) using a two-component model (linear + exponential), integrated by a Heaviside function, as previously described (De Roia et al. 2012).

Finally, [HHb] and VO_2 data were normalized with 0% corresponding to the value recorded while cycling at 20 W baseline and 100% reflecting the maximal response in the 180 sec window and expressed as Δ[HHb] and ΔVO_2. Individualized 1 sec Δ[HHb] and ΔVO_2 were time-aligned by left-shifting the VO_2 data by 20 sec (i.e. the typical duration of the cardiodynamic phase in young individuals (Murias et al. 2011b). Then, the ratio between Δ[HHb]/ΔVO_2 was calculated during the first 180 sec of exercise to

express the fractional muscle O_2 extraction required to sustain a given net increment of VO_2 (De Roia et al. 2012). Finally, the following indexes were calculated: $\Delta[HHb]/\Delta VO_2$ AUC, as the integral of the total mismatch between O_2 delivery and utilization (i.e. index values > 1); $\Delta[HHb]/\Delta VO_2$ peak, as the maximal value reached within the 180 sec; $\Delta[HHb]/\Delta VO_2$ time to peak, as the time requested to reach the peak in $\Delta[HHb]/\Delta VO_2$. Moreover, given that these time-resolved values are typically implemented during steady-state condition, an overall quantification of the increase in fractional muscle O_2 extraction required to sustain a given net increment in VO_2 during the primary phase of exercise was calculated by dividing the amplitudes of the response in VO_2 and [HHb] between the onset of exercise and the onset of the slow component: overall $\Delta[HHb]/\Delta VO_2$ (Tam et al. 2018).

Statistics

After assumptions verification (i.e., normality, homogeneity of variance), two-way repeated measures ANOVA was applied to compare flexibility values (pre and post sit-and-reach after control/stretching). Pre and post peak power output measured during isokinetic sprints at 60 and 120 rpm were compared pre and post between stretching, using a two-way repeated measures ANOVA (time x pedalling frequency).

For constant work rate trials, two-way repeated measures ANOVAs were performed to compare VO_2, net $[La^-]$, RPE, [HHB], RMS_{VL} and RMS_{BF} between conditions over time (time x condition). Post-hoc analyses were performed using Holm-Sidak test. Student's t-test was applied to compare between conditions the time to exhaustion, parameters of VO_2 and [HHb] kinetics (τ, TD and MRT), time to VO_{2max}, $\Delta[HHb]/\Delta VO_2$ AUC, $\Delta[HHb]/\Delta VO_2$ peak, $\Delta[HHb]/\Delta VO_2$ time to peak, overall $\Delta[HHb]/\Delta VO_2$.

Data are presented as means ± SD. α was set in advance at the 0.05 level and significance was accepted when $p < \alpha$. The 95% confidence intervals of the TD_p and

TD_{sc}, τ_p and τ_{sc} of VO_2 kinetics and of TD and τ of [HHb] kinetics were calculated based on the asymptotic intervals of the non-linear parameters resulting from the fitting (Field et al. 2012). Effect sizes of the differences between control and stretching were also reported (Cohen's d, ranked as trivial (0-0.19), small (0.20-0.49), medium (0.50-0.79) and large ($\geq$ 0.80)) as objective and standardized measures to quantifying the magnitude of difference after stretching vs control (Cumming 2014b). In Cohen's effect size calculation, the SD in the control condition was used to standardize the mean difference for each contrast (Field et al. 2012). Moreover, generalized eta squared (η_G^2) were calculated to quantify the effects sizes of different independent variables during the constant work rate trials (Olejnik and Algina 2003; Bakeman 2005). Based on an expected standard deviation of breath-by-breath VO_2 measurements for steady-state exercise equal to 2.5%, and a minimum detectable change in VO_2 of 100-170 $ml \cdot min^{-1}$ at a VO_2 of 2.1 to 3.5 $L \cdot min^{-1}$ (Keir et al. 2015), the minimum sample size to obtain a power of 0.8 was 6 individuals. All statistical analyses were performed using Sigmaplot version 12.

Results:

Flexibility, as measured by sit-and-reach test, was not significantly different at baseline between stretching and control and significantly improved only after stretching (+0.3±6.5 cm *pre* vs +6.1±5.9 cm *post* stretching, p<0.001, *d*=+0.89 +0.7±5.1 cm *pre* vs +0.8±4.9 cm *post* control, p=0.784, *d*=+0.02). The peak power output measured during isokinetic sprints pre stretching was reduced, after the intervention, of ≈ 5% (table 1). ANOVA revealed a significant main effect of "time" (p≤0.001) and "pedaling frequency" (p≤0.001), whit no interaction (p=0.885). Post-hoc analysis confirmed that peak power output was significantly reduced by stretching both during the 60 RPM and the 120 RPM sprints (table 1).

Subjects mean VO_{2max} and peak PO measured at the end of the ramp incremental test were respectively 3505±375 ml*min^{-1} and 315±26 W. GET and RCP were detected at a VO_2 of 2155±355 ml*min^{-1} and 2900±472 ml*min^{-1}. The calculated target VO_2 and PO for the Δ60% constant work rate trials were 3030±411 ml*min^{-1} and 232±29 W (74±7% of the peak PO) respectively. As expected under the non-steady-state conditions of the severe intensity domain, the contribution of the VO_2 slow component raised the actual experimental VO_2 above the initially predicted target so that values close to VO_{2max} were measured in the last 20 sec of the 10-min trials. In one subject only, the target intensity turned out to fall clearly below the desired severe domain (i.e. both VO_2 and [La$^-$] were stable over time after the 10th min of exercise and time to exhaustion exceeded 40 min). Therefore, for this subject the constant work rate trials performed until that moment were repeated at +20 W after a wash-out period of three weeks in order to assure a metabolic intensity corresponding to the desired severe intensity domain.

The time to exhaustion of the control constant work rate trials was 839±200 sec (14'19''±3'20''). stretching significantly affected this parameter, that was reduced to 743±166 sec (12'23''±2'46'', p=0.002, *d*=-0.48). Reduced TTE was associated with increased levels of RMS_{VL}, [HHb], peak net [LA$^-$], and perceived exertion at exhaustion (figure 2). In fact, RMS_{VL}, [HHb], peak net [La$^-$], and perceived exertion

were significantly higher in the stretching vs control condition (main effect of "time" RMS_{VL}: p<0.00, η_G^2: 0.30, [HHb]: p<0.00, η_G^2: 0.65, peak net [La^-]: p<0.00, η_G^2: 0.99, perceived exertion: p<0.00, η_G^2: 0.990, main effect of "condition" RMS_{VL}: p=0.025, η_G^2: 0.44, [HHb]: p=0.011, η_G^2: 0.53, net [LA^-]: p=0.023, η_G^2: 0.49, perceived exertion: p=0.003, η_G^2: 0.50; "time" x "condition" interactions for RMS_{VL}: p<0.001, η_G^2: 0.70, [HHb]: p=0.007, η_G^2: 0.34, , net [LA^-]: p=0.221, η_G^2: 0.01 and perceived exertion: p=0.044, η_G^2: 0.01). On the contrary, there were no changes in VO_2, and RMS_{BF} in stretching vs control (main effect of "time" VO_2: p<0.001, η_G^2: 0.99, RMS_{BF}: p<0.001, η_G^2: 0.77; no main effect of "condition" VO_2: p=0.864, η_G^2: 0.01, RMS_{BF}: p=0.362, η_G^2: 0.09). VO_2 kinetics analysis revealed that the time required to reach the VO_{2max} during constant work rate trials was reduced by stretching (control= 709±183 vs stretching 639±197, p=0.034, *d*=-0.38).

Other parameters regarding VO_2 and [HHb] kinetics (e.g. τ, TD etc.) are presented in table 2 and figure 3 displays the mean signals during the transient phase after control and stretching. VO_2 showed no changes between control and stretching in time delay (p=0.874, *d*=+0.04), and in τ (p=0.066, *d*=-0.31, table 2). On the contrary, stretching reduced the time delay of [HHb] (p=0.023, *d*=-0.61, table 2 and figure 3), but no difference was detected in τ (p=0.690, *d*=+0.19). Finally, during the transient phase (first 180 sec of exercise), $\Delta[HHb]/\Delta VO_2$ revealed an increased mismatch in oxygen delivery and utilization at the peripheral level after stretching (figure 3), corroborated by a significant difference between conditions in $\Delta[HHb]/\Delta VO_2$ peak (p=0.038, *d*=+1.01), and a larger, yet not significant of $\Delta[HHb]/\Delta VO_2$ AUC (p=0.099, *d*=+0.59 table 2 and figure 3).

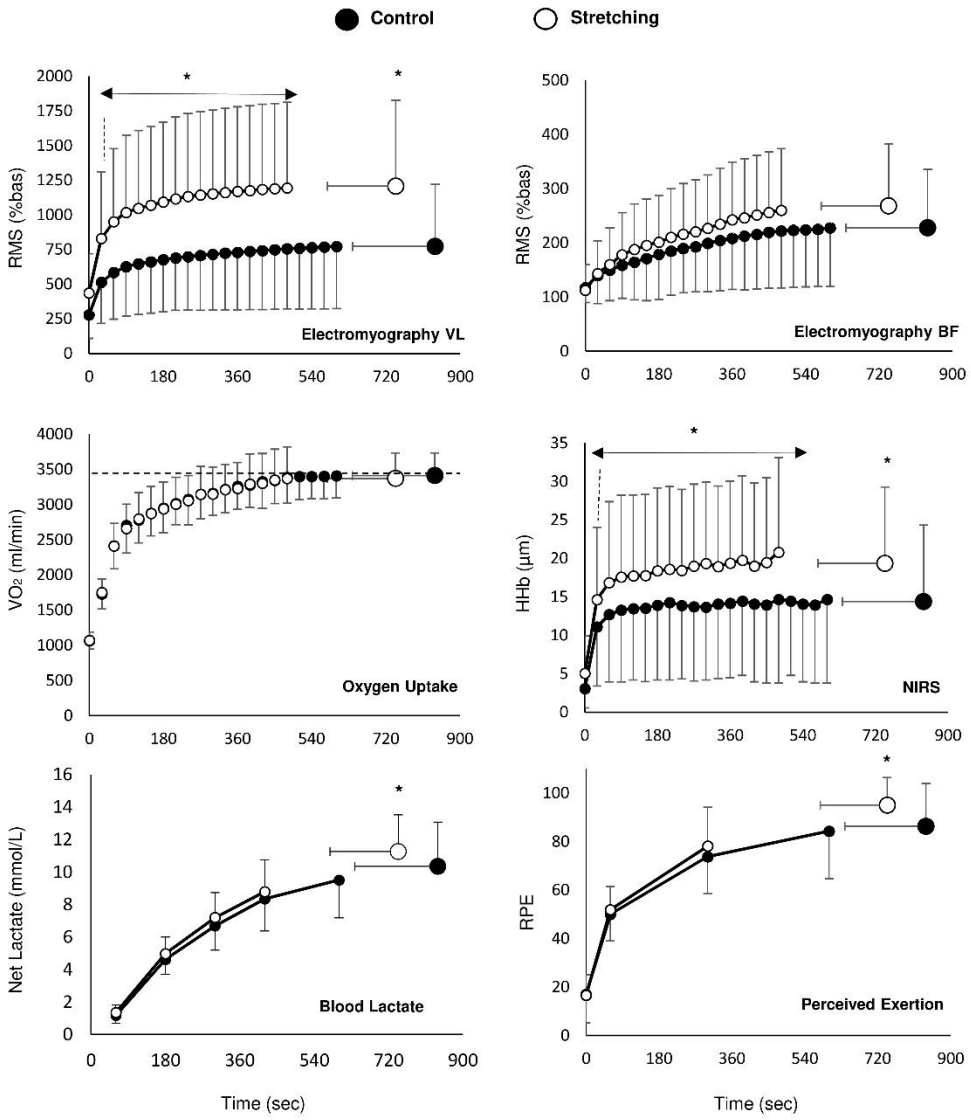

Figure 2, *The physiological responses during control (black dots) and stretching (white dots) conditions are presented in 30 sec means ±SD. * represent statistical difference between control and stretching for a given timepoint.*

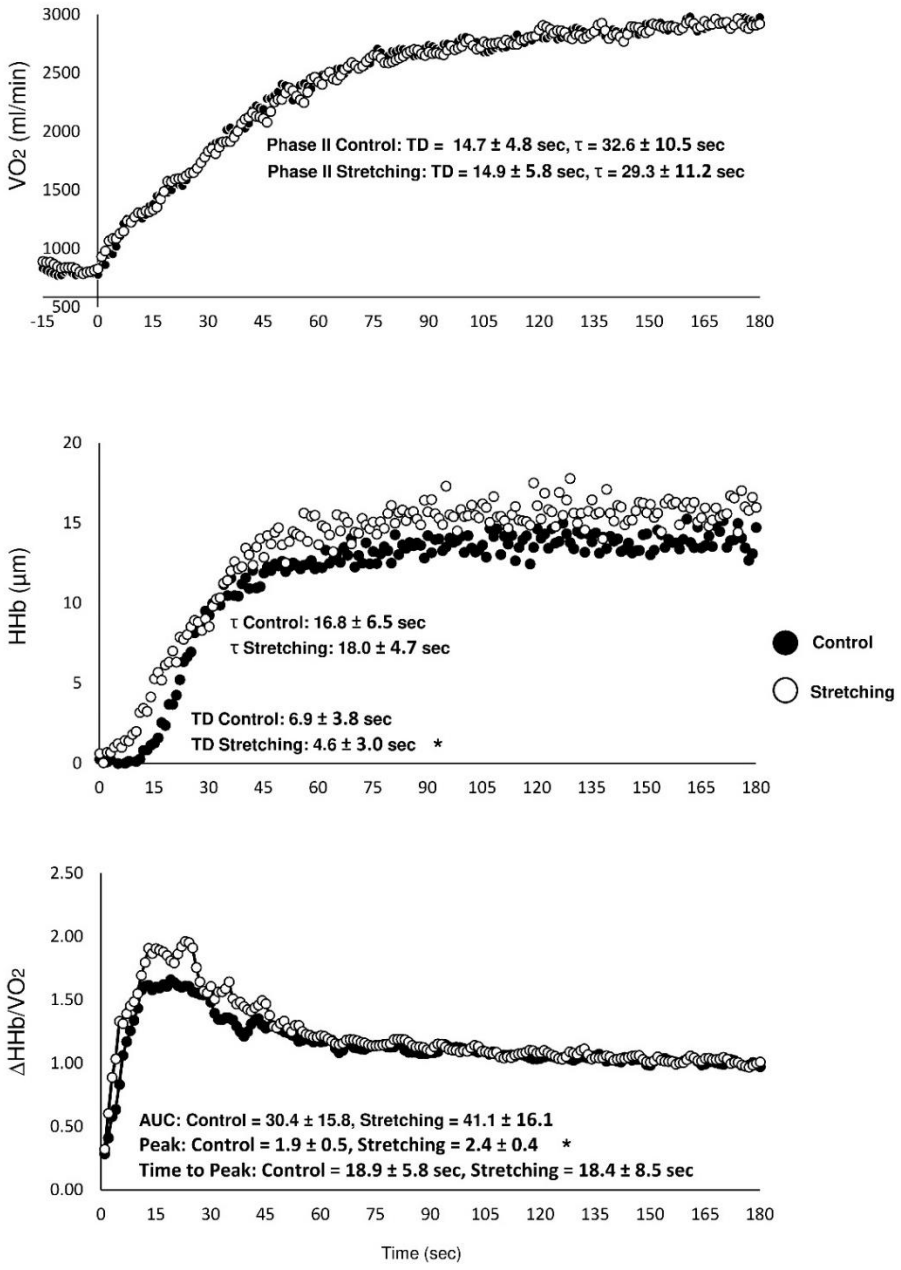

Figure 3, *The VO_2 (top panel), [HHb] (medium panel), and Δ[HHb]/ΔVO_2 (bottom panel) kinetics during the first 180 sec after exercise onset are presented during control (black dots) and stretching (white dots). * represent statistical difference between control and stretching for a given value of the kinetics. Acronyms represent: TD = time delay, τ = time constant of response, MRT = mean response time, AUC = area under the curve.*

Table 1, Mean ± SD peak power output during isokinetic sprints pre and post static stretching

	Pre stretching **(W)**	Post stretching **(W)**	**Δ%**	***p***	***d***
60 RPM	670 ± 132	626 ± 107 *	- 6.0 ± 5.3	0.009	- 0.33
120 RPM	903 ± 86	863 ± 112 *	- 4.6 ± 4.8	0.014	- 0.46

*Isokinetic sprints peak power output is presented for the 60 and 120 RPM pedalling frequencies pre and post STRC with p-values. * represents significant differences between pre and post values.*

Table 2, Mean ± SD kinetics parameters for oxygen consumption and deoxyhemoglobin

	Control	Stretching	p	*d*
VO_2				
Baseline (ml*min^{-1})	787.1 ± 174.6	790.7 ± 105.9	0.938	+ 0.02
AMP_p (ml*min^{-1})	2015.4 ± 480.1	2045.2 ± 509.9	0.607	+ 0.06
TD_p (sec)	14.7 ± 4.8	14.9 ± 10.7	0.873	+ 0.04
TD_p 95% CI (sec)	13.2 – 16.2	13.6 – 16.1		
τ_p (sec)	32.6 ± 10.5	29.3 ± 11.2	0.066	- 0.31
τ_p 95% CI (sec)	29.8 – 35.4	26.9 – 31.8		
MRT_p (sec)	47.3 ± 10.7	44.2 ± 12.3	0.116	- 0.29
AMP_{sc} (ml*min^{-1})	752.1 ± 386.9	756.1 ± 272.8	0.207	+ 0.01
TD_{sc} (sec)	152.6 ± 37.5	155.3 ± 25.6	0.803	+ 0.05
TD_{sc} 95% CI (sec)	146.5 – 158.7	147.1 – 163.4		
τ_{sc} (sec)	321.9 ± 149.7	282.9 ± 111.5	0.333	- 0.26
τ_{sc} 95% CI (sec)	251.9 – 392.0	147.1 – 163.4		
[HHb]				
Baseline (µM)	24.7 ± 9.4	27.3 ± 10.3	0.138	+ 0.38
AMP (µM)	14.8 ± 9.4	17.3 ± 12.2	0.359	+ 0.26
TD (sec)	6.9 ± 3.8	4.6 ± 3.0	0.023	- 0.61
TD 95% CI (sec)	6.2 – 7.7	4.0 – 5.3		
τ (sec)	16.8 ± 6.5	18.0 ± 4.7	0.689	+ 0.19
τ 95% CI (sec)	15.7 – 17.9	16.8 – 19.2		
MRT (sec)	23.7 ± 9.0	22.6 ± 7.5	0.767	- 0.12
Δ[HHb]/ΔVO_2				
AUC	30.4 ± 15.8	41.1 ± 16.1	0.099	+ 0.75
Peak	1.9 ± 0.5	2.4 ± 0.4	0.038	+ 1.01
Time to Peak (sec)	18.9 ± 5.8	18.4 ± 8.5	0.891	- 0.09
Overall Δ[HHb]/ΔVO_2 ratio (µM*ml*min^{-1})	0.011 ± 0.008	0.014 ± 0.012	0.275	+ 0.28

Values for VO_2, [HHb] and Δ[HHb]/ΔVO_2 kinetics are presented. Acronyms represent: AMP = amplitude, TD = time delay, τ = time constant of response, MRT = mean response time, AUC = area under the curve. Subscripts in VO_2 kinetics represent: p = primary component, sc = slow component.

Discussion:

This investigation evaluated the physiological effects of acute non-metabolic fatigue induced by static stretching on high-intensity constant work rate cycling performance. We had hypothesized that stretching, through a sequence of events going from a reduction of maximal muscle force, to an increase in muscle excitation at a given absolute work rate, to an increase the VO_2 cost of locomotion would ultimately reduce exercise tolerance. In agreement with our hypothesis, stretching caused a significant fatigue, as indicated by the reduction of the maximal power output during isokinetic sprints. In turn, force reduction was accompanied by an augmented muscle excitation at a given work rate. Finally, the above sequence of events was associated with a significant reduction in time to exhaustion. Contrary to our hypothesis, no changes were detected between control and stretching in the magnitude of VO_{2sc} in the time window in which VO_{2sc} is typically investigated (from the $\approx 2^{nd}$-3^{rd} to the 9^{th}-10^{th} min). However, the time required to reach VO_{2max} was reduced by stretching, suggesting an influence of the increased muscle excitation on the dynamics of VO_2 during constant-work rate exercise in the severe intensity domain. In support of this, stretching was associated with an increased metabolic instability/mismatch between O_2 delivery and O_2 utilization during the on-kinetic, increased perception of effort and an increased blood lactate accumulation, that are all compatible with an increased contribution of the glycolytic energy system to sustain the same absolute intensity.

The effectiveness of stretching in acutely impairing maximal force/power was previously proven during cycling-specific (Colosio et al. 2019) and non-specific tasks (Behm et al. 2016). In agreement with the above findings, our study confirmed a reduction of the maximal cycling PO following stretching intervention of a similar order of magnitude (i.e. ≈ 5%) (table 1) of that previously described. The physiological causes of the impairment of maximal PO documented after repeated bouts of prolonged stretching remain to be elucidated and supraspinal, spinal or muscle-related mechanisms have all been proposed as possible explanatory causes of reduced maximal force (Trajano et al. 2017). Independently from the cause, a reduction of maximal force

may translate in the requirement of higher levels of relative force to maintain the same absolute work rate. In turn, this could augment the recruitment of high-order, fast fatiguing, motor units (progressive recruitment theory (Henneman et al. 1965)) or the frequency of activation of motor units, at the same absolute power output. Both mechanisms are likely to cause an increase in the overall electrical activity of the muscle, as evaluated by surface EMG (Vigotsky et al. 2018).

To our knowledge, our study was the first to examine EMG after stretching during cycling. According to our hypothesis, the impairment of maximal PO documented after stretching translated in augmented muscular excitation of the vastus lateralis (figure 2); this finding supports the hypothesis that either the recruitment of higher order motor units or an increased activation frequency were necessary to sustain exercise at the same absolute work rate compared to the control condition (figure 2). The increase in muscle activation (vs control) manifested clearly within the first minute after the onset of exercise, with no further effect over time. This would support the idea that the increased muscle excitation following stretching was a result of the acute loss in maximal PO rather than to progressive fatigue. Interestingly, our intervention affected the muscle excitation of the vastus lateralis to a larger extent than that of the biceps femoris (figure 2). We speculate that this difference may be due to a larger effectiveness of stretching on the extensor of the knee compared to the flexor (i.e. mostly for the different anatomical insertion of these muscles). Moreover, a smaller contribution of the biceps femoris than the vastus lateralis during cycling, particularly in a sample of non-cyclist subjects, may have influenced these results.

Contrary to our hypothesis, increased muscle excitation did not influence the VO_{2sc}. Previous studies on the VO_{2sc} response following fatigue have led to inconsistent results: the VO_{2sc} was augmented (Colosio et al. 2019), unaffected (Hopker et al. 2016), or even diminished (Deley et al. 2006). This may be explained by the heterogeneity of the fatiguing protocols adopted in the different studies (e.g. stretching, dropjumps, electrical stimulation, etc.), by the exercise domain investigated (heavy/steady-state vs severe/non-steady-state) and by the time window considered and the analysis strategy

(e.g. fitting, integral, etc.). Furthermore, in the non-steady-state, severe-intensity exercise used in our study, VO_2 rapidly projects to VO_{2max} (figure 2) reaching this upper ceiling within ~10 min. Under these conditions the potential for an increase in the VO_{2sc} (i.e. the difference in VO_2 between the ≈ 2^{nd}-3^{rd} and the 9^{th}-10^{th} min) in response to stretching may have been too small to be measurable. In support of this view, the magnitude of the stretching effect measured in our study is consistent with the rather small increase in VO_2 (around 100-150 ml*min^{-1}) reported by Esposito et al. (Esposito et al. 2012) following stretching. Interestingly however, our data showed a reduction in the time necessary to reach VO_{2max} (of ≈ 70 sec, ≈ 10%) and augmented peak [La^-] and peak RPE (figure 2) following stretching compared to the control condition. These findings appear compatible with a faster projection of VO_2 towards VO_{2max} following stretching, possibly driven by the increased amount of muscle fibres necessary to sustain the same absolute work rate, as indicated by the increased EMG_{VL} in the fatigued condition.

Finally, a mirror intervention of acute-fatigue that could provide complementary information on the link between muscle recruitment and VO_{2sc} is represented by strength training. In this context, Tam et al. trained for two months a group of older people with either interval training or resistance training (Tam et al. 2018). A direct comparison with this study is not fully applicable due to the lack of EMG measures and to the fact that anatomical and functional adaptations other than an isolated improvement of muscle strength occurred in Tam's study. Still, the increased muscle strength reported together with a slight decrease of the VO_{2sc} amplitude seem to corroborate a possible role of muscle activation in the genesis of the VO_{2sc} (Tam et al. 2018).

Regarding the metabolic impact of stretching documented with NIRS (figure 2), a reduced time delay of the [HHb] kinetics at the onset of exercise (figure 3) and an augmented peak [HHb]/VO_2 ratio (figure 3, table 2) were observed. These indexes indicate an earlier mismatch between oxygen delivery and oxygen utilization within the working muscles and a larger mismatch during the on-kinetics, respectively (Grassi

et al. 2003). Both findings are compatible with either an increase in oxygen extraction and/or a lower oxygen delivery in the working muscles. While this is the first study that measured tissues oxygenation levels during cycling after stretching, recent studies showed that stretching can induced temporary ischemia followed by reactive hyperaemia and possibly enhance O_2 delivery at the macro- (Venturelli et al. 2019) and micro- (Trajano et al. 2014) circulatory levels. Therefore, while a reduction in muscle perfusion cannot be completely ruled out, it seems unlikely that oxygen availability in the working muscles would be reduced post stretching. Alternatively, stretching may be associated with a faster oxygen extraction caused by a faster and larger disturbance of intracellular homeostasis. The higher deoxygenation during the fatigued condition may be explained by the greater proportion of glycolytic muscle fibres being involved into exercise. In fact, the glycolytic fibres seem to display higher levels of O_2 extraction compared to the aerobic fibres at the onset of exercise (Ferreira et al. 2006; Koga et al. 2014), possibly due to a local Bohr effect (Jensen 2004). Interestingly, these speculations appear consistent with the finding of a faster projection of VO_2 towards VO_{2max} following stretching.

In conclusion, prolonged stretching caused acute fatigue as indicated by a reduced the maximal power output in isokinetic sprints. Stretching-induced fatigue, in turn, caused augmented levels of muscle excitation at a given work rate when cycling in the severe exercise domain, and a significant reduction in time to exhaustion. No changes were detected between control and stretching in the magnitude of VO_{2sc} “per-se”; however, the time required to reach VO_{2max} was reduced by stretching, suggesting an influence of the increased muscle excitation on the kinetics of the slow component of VO_2. Finally, stretching was associated with an increased metabolic instability/mismatch between O_2 delivery and O_2 utilization during the on-kinetic, increased perception of effort and an increased blood lactate accumulation, all these phenomena are compatible with an increased contribution of the glycolytic energy system to sustain the same absolute intensity.

CHAPTER

5

Bioenergetics of the VO_2 Slow Component Between Exercise Intensity Domains.

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Abstract:

In humans, when exercising at constant work rate in the heavy and severe domains of exercise the VO_2 displays a slow component (VO_{2sc}) that is considered to be associated with increasing energy demand of the muscles over time. While the physiological underpinnings the VO_{2sc} are still debated, recent studies suggested that VO_{2sc} could be attributed to a shift in energetic sources rather than increased energy demand. We tested the hypothesis that the overall cost of cycling is affected by time during metabolic transitions in different intensity domains. Eight active men performed 3 constant work rate trials of 3, 6 and 9 min respectively in the moderate, heavy and severe domains. VO_2, VO_2 of ventilation (VE) and blood lactate accumulation ($[La^-]$) were quantified to calculate the adjusted oxygen cost of exercise ($AdjO_{2Eq}$, i.e. the measured VO_2 minus the VO_2 cost of VE, plus the VO_2 equivalent of $[La^-]$) for the 0-3, 3-6 and 6-9 time segments at the three intensities and compared it by a two-way (time, intensity) RM-ANOVA. After the transient phase, $AdjO_{2Eq}$ was unaffected by time in moderate (ml*3 min^{-1} at 0-3, 0-6, 0-9 min: 2126±939 < 2687±1036, 2731±1035) and heavy (4278±1074 < 5121±1268, 5225±1123 ml*3 min^{-1}). On the contrary, a significant effect of time was identified in severe (5863±1413 < 7061±1516 < 7372±1443 ml*3 min^{-1}). In the moderate and heavy domains, $AdjO_{2Eq}$ was unchanging, suggesting that VO_{2sc} only represents a shift in energetic sources (aerobic and anaerobic). In the severe domain, VO_{2sc} was also associated with increased energy demand.

Introduction:

After the cardio-dynamic phase, oxygen consumption (VO_2) during constant heavy and severe intensity exercise, is better fitted by a two-components rather than a single-component model. Based on this observation, it has been suggested, that a slow component of VO_2 exists (VO_{2sc}), that does not start at exercise onset but rather appears later in exercise (time delay ~ 120-180 sec) (Jones et al. 2011). Furthermore, it has been assumed, but not proven, that in association with VO_{2sc}, the muscle displays an increasing energy demand as a function of time (Jones and Poole 2005).

VO_{2sc} represents an increased O_2 cost of locomotion when exercise is protracted more than 3 min at constant work rate above the lactate threshold (LT) (Jones et al. 2011). Typically, when exercise is performed between the lactate threshold (LT) and the critical power, (i.e. heavy intensity domain) VO_{2sc} tends to steady-state. On the contrary, when effort rises above critical power (i.e. severe exercise domain) a steady state is not achievable and VO_2 increases tending to the maximum oxygen consumption (VO_{2max}) (Jones et al. 2011). The magnitude of VO_{2sc} is considered to be associated with exercise intolerance and fatigue (Grassi et al. 2015). For this reason many researchers during the past forty years have focused their attention on clarifying its physiological mechanisms (Jones et al. 2011).

A key finding from these studies was that roughly 85% of the VO_{2sc} originates from the contracting muscles, while the remaining 15% corresponds to the increased VO_2 cost of ventilation (Poole et al. 1991). Focusing on the muscular component of the VO_{2sc}, subsequent investigations proposed that the recruitment of less efficient type II fibres necessary to maintain a specific power output (PO) (Jones et al. 2011) or metabolic changes occurring within the working fibres (Zoladz et al. 2008; Vanhatalo et al. 2011) could elicit an increased cost of locomotion. Nevertheless, a satisfactory theory explaining the mechanisms underpinning the VO_{2sc} is still missing.

In this context, a recent study (O'Connell et al. 2017) refuted that the energy demand of a constant, high-intensity exercise changes over time. By subtracting the VO_2 cost

of ventilation and accounting for the contribution of the glycolytic energy sources (di Prampero 1986; di Prampero et al. 1999; O'Connell et al. 2017) O'Connell quantified the total, adjusted metabolic cost of muscle exercise over time during a constant work rate trial in the severe domain. The authors concluded that the oxygen cost of locomotion does not increase over time, other than what required by the augmenting cost of ventilation. The apparent VO_2 increases over time would be the result of the shift in metabolic sources after the first three min of exercise (i.e. an increased contribution of the aerobic metabolism to ATP resynthesis, mirrored by a decreased contribution of anaerobic ATP resynthesis over time). In other words, these findings suggest that VO_{2sc} may in fact not represent a loss of efficiency as a function of time but rather a delayed adjustment of the oxidative metabolism. However, while this new hypothesis could explain why a satisfying explanatory theory of VO_{2sc} is still missing, the amount of literature that reported VO_{2sc} in different exercise modalities (i.e. whole-body vs isolated muscles) and models (i.e. humans vs animals) calls for further investigation (Jones et al. 2011). In particular, the intensity domain in which O'Connell measures were obtained is not fully clear; while most studies in the field of VO_2 kinetics define intensity domains based on gas exchange thresholds, O'Connell used lactate-based measures. Yet, the correspondence between the above methods remains a controversial issue (Keir et al. 2015, 2018b; Broxterman et al. 2018). In addition, loss of efficiency, metabolic shift among substrates and excess ventilation manifest and increase differently over time in the three domains of exercise (e.g. no loss of efficiency is present in the moderate domain while an elevated steady state is reached in the heavy yet not in the severe domain) (Poole and Jones 2012). Therefore, O'Connell's findings need to be confirmed and broadened with particular consideration to the three intensity domains of exercise.

Accordingly, by calculating the energy cost of ventilation, the glycolytic contribution to exercise, and directly measuring the aerobic cost of locomotion, we tested the hypothesis that the overall cost of cycling is affected by time during metabolic transitions in different intensity domains. Specifically, we hypothesised that: *i)* the

overall cost of locomotion would progressively increase going from the moderate to the heavy to the severe exercise domain; *ii)* the overall cost of locomotion would not be affected by time during metabolic transitions in the moderate and heavy exercise domains (i.e. no VO_{2sc} exists at these intensities); *iii)* the overall cost of locomotion would be affected by time during metabolic transitions in the severe exercise domain (i.e. VO_{2sc} will manifest at this intensity).

Methods:

Ethical Approval

The study was conducted according to the Declaration of Helsinki and all procedures were approved by the University of Verona Ethics Committee for Research on Human Subjects. Procedures and risks were explained to each subject, and all participants volunteered and gave informed written consent to participate before the start of the study.

Participants

Eight active men were recruited in the study (age 25 ± 2 years, body mass 74 ± 10 kg, height 181 ± 5 cm, VO_{2max} 49.3 ± 3.4 $ml*kg^{-1}*min^{-1}$). Inclusion criteria were male sex and age between 20 and 35 years; exclusion criteria were smoking and any condition that could influence the physiological responses during testing. All participants were instructed to avoid caffeine consumption and physical activity respectively for at least 8 h and 24 h before each testing session. Moreover, to minimise variability of glycogen stores and glucose oxidation, participants followed a standard food intake prescription before all the testing sessions as previously described (Keir et al. 2015).

Experimental Protocol

After medical clearance, participants visited the laboratory on ten occasions within a maximum of four weeks. All subjects completed: i) a preliminary maximal ramp incremental exercise test to exhaustion for the determination of gas exchange threshold (GET), respiratory compensation point (RCP) and the maximum oxygen uptake (VO_{2max}); ii) three constant work rate trials (CWR) respectively of 3, 6 and 9 min in the "moderate" exercise intensity domain iii) three CWR respectively of 3, 6 and 9 min in the "heavy" exercise intensity domain iv) three CWR trials respectively of 3, 6 and 9 min in the "severe" exercise intensity domain. Tests of ii) iii) and iv) aimed at determining VO_2 response and blood lactate ($[La^-]$) accumulation as a function of time in the three exercise intensity domains in order to evaluate the overall energetic contribution of the aerobic and anaerobic metabolisms (di Prampero et al. 1999) to ATP turnover. Moreover, these tests were executed in randomized order with the only exception of the longest CWR in the "severe" exercise domain, that was completed as first to assure that subjects were able to sustain the PO for the required time. All exercise tests were conducted on an electromagnetically braked cycle ergometer (Sport Excalibur, Lode, Groningen, Netherlands), at a similar time of the day in an environmentally controlled laboratory (18°C, 55-65% relative humidity).

Ramp incremental test

The ramp incremental test consisted of a 3-min baseline cycling at 50 W, followed by a 30-W*min^{-1} increase in PO until volitional exhaustion. Participants were asked to pick a self-selected cadence in the range of 70-90 rpm and to maintain it throughout all subsequent tests. Failure to maintain the indicated cadence within 5 rpm (for longer than 5 sec) during testing despite strong verbal encouragement was considered as the criterion for exhaustion. Breath-by-breath pulmonary gas exchange and ventilation were continuously measured using a metabolic cart (Jaeger Oxycon Pro, Viasys Healtcare GmbH, Höchberg, Germany) as previously described (De Roia et al. 2012). Heart rate (HR) was monitored continuously (H7 Sensor, Polar, Kempele, Finland).

Constant work rate trials

After the preliminary ramp incremental test, subjects completed 3 CWR within each exercise intensity domain (i.e. moderate, heavy, and severe) in a randomized order:

i) Moderate: 3 CWR respectively of 3,6 and 9 min at 80% of GET.

ii) Heavy: 3 CWR respectively of 3,6 and 9 min at 50%Δ between GET and RCP.

iii) Severe: 3 CWR respectively of 3,6 and 9 min at 60%Δ between GET and VO_{2max}.

Each CWR was preceded by a 3-min warm-up at 20 W. Throughout the test, subjects kept the same, constant rpm and bike position as selected during the ramp incremental test.

VO_2 and HR data were measured with the same method described for the ramp incremental test. Moreover, Capillary blood samples (65 μl) were drawn from the fingertip in the last 30s of warm-up and at the 1^{st},3^{rd}, 5^{th} and 7^{th} min after each test and were immediately analysed (Radiometer ABL90 FLEX, Radiometer Medical ApS, Brønshøj, Denmark) to measure [La^-]. The highest value was considered as the peak of blood lactate concentration and used for further analysis to calculate anaerobic energetic contribution.

Data Analysis

Ramp Incremental Test:

For the gas exchange variables, aberrant data-points that lay 3 SD from the local mean were removed, and trials were linearly interpolated on a 1-sec basis and then averaged every 5-sec. VO_{2max} was determined as the highest VO_2 obtained over a 10-sec interval (Fontana et al. 2015). GET and RCP were determined with the standard technique from gas exchange variables by three blinded expert reviewers as detailed elsewhere (Fontana et al. 2015). Briefly, GET was determined by visual inspection as the VO_2 at which CO_2 output began to increase out of proportion in relation to VO_2, with a systematic rise in the ventilation (VE)-to-VO_2 relation and end-tidal PO_2 whereas the

ventilatory equivalent of VCO_2 (VE/VCO_2) and end-tidal PCO_2 is stable (Beaver et al. 1986). RCP was determined as the point where end-tidal PCO_2 began to fall after a period of isocapnic buffering (Whipp et al. 1989). This point was confirmed by examining VE/VCO_2 plotted against VO_2 and by identifying the sec breakpoint in the VE-to-VO_2 relation. VO_{2max} was determined as the highest VO_2 obtained over a 10-sec interval. Finally, we determined the constant work rate equivalent to the specific moderate (80% of GET), heavy (50% Δ between GET and RCP) and severe (60%Δ between GET and VO_{2max}) VO_2 targets. To this aim, the VO_2/W relationship identified with the incremental test left-shifted to account for the mean response time (Fontana et al. 2015).

Constant Work Rate Trials:

VO_2, VCO_2, and VE during CTL were sampled breath-by-breath and interpolated using the same procedure described for the ramp incremental test. Interpolated data from different CTL performed at the same exercise intensity were mediated in order to reduce breath-by-breath signals variability (data from 3 tests were mediated in the time segment from 0 to 3 min and data from 2 tests were mediated between 3 and 6 min) (Keir et al. 2014). The sum of the oxygen consumed during each 3-min time segment was then considered as the aerobic energetic contribution to exercise.

VO_{2sc} was calculated as the sum of the amount of oxygen exciding the VO_2 reached at the end of the 0-3 min time segment (Santana et al. 2007).

Work of breathing (WB) was calculated based on VE using the equation by Coast et al.:

$$WB = -0.430 + 0.050 * VE + 0.00161\ VE^2$$

Then, the WB was used to calculate the amount of VO_2 requested by ventilatory muscles (VO_{2VM}):

$$VO_{2VM} = 34.9 + 7.45 * WB$$ (Coast et al. 1993)

Anaerobic (glycolytic) contribution to exercise was calculated from the amount of [La^-] accumulation over time calculated as follows:

- 0-3 min segment [La^-] accumulation = 3-min CWR peak [La^-] – warm-up [La^-]
- 3-6 min segment [La^-] accumulation = 6-min CWR peak [La^-] – 3-min CWR peak [La^-]
- 6-9 min segment [La^-] accumulation = 9-min CWR peak [La^-] – 6-min CWR peak [La^-]

The so obtained values were utilized to estimate the energy contribution from anaerobic glycolysis in each time segment, based on the oxygen equivalent for lactate of Di Prampero (i.e. 1 mmol*L^{-1} [La^-] accumulation = 3.0 ml*kg^{-1} VO_2) (di Prampero et al. 1999).

Overall energetic cost of activity (expressed as ml of oxygen) was calculated as described by O'Connell et al. and defined as "Adjusted Oxygen Equivalent" ($AdjO_{2Eq}$):

$AdjO_{2Eq}$= measured VO_2 (ml O_2 * $3min^{-1}$) - VO_{2VM} (ml O_2 * $3min^{-1}$) + Oxygen Equivalent of Lactate (ml O_2 * $3min^{-1}$).

VO_2 gain (VO_{2gain}, i.e. the amount of oxygen equivalent utilized to sustain each W during cycling) was calculated as the ratio between the amount of $AdjO_{2Eq}$ required to sustain exercise during a specific time segment and the number of W imposed by the test: VO_{2gain}= (3min $AdjO_{2Eq}$ - warm-up $AdjO_{2Eq}$) / (test PO (W) - warm-up PO (W)).

Statistics

After assumptions verification (i.e., normality, homogeneity of variance), the within-subject coefficient of variation and two-way repeated measures ANOVA (trial x intensity domain) were used to evaluate VO_2 data repeatability measured at the end of the third min of exercise of each CWR.

A two-way repeated measures ANOVA was also performed to assess differences over time between different intensities domains (time segment x intensity domain) for VO_2, VO_{2VM}, O_2 equivalent of [La^-], $AdjO_{2Eq}$, VO_{2gain}. Post-hoc analysis was performed using Holm-Sidak method.

Data are presented as means ± SD. All statistical analyses were performed using Sigmaplot version 12 and α was set in advance at the 0.05 level. Statistical significance was accepted when $p < \alpha$.

Results:

Subjects' anthropometrical and functional characteristics obtained during the ramp incremental test are reported in Table 1.

Repeatability of group mean VO_2 data (10-sec averages) is displayed in figure 1. Average measured VO_2 displays a complete overlap among the three durations trials (3, 6, 9 min) at the three intensities (moderate, heavy, severe). Furthermore, mean VO_2 values of the last 10 sec of the third min of exercise for the 9, 6, and 3-min CWR were respectively: 3328 ± 470, 3231 ± 434, 3285 ± 443 $ml*min^{-1}$ (Severe) 2804 ± 408, 2783 ± 492, 2793 ± 470 $ml*min^{-1}$ (Heavy), and 1979 ± 281, 1966 ± 330, 2095 ± 258 $ml*min^{-1}$ (Moderate), with a mean within-subject coefficient of variation of 3.3 ± 2.4 % (Severe), 4.3 ± 1.4 % (Heavy), 3.7 ± 1.4 % (Moderate). As expected, ANOVA on these VO_2 values revealed a significant main effect of the intensity domain at which exercise was performed ($p \leq 0.001$) but no significant main effect among the three trials performed at the same intensity ($p=0.437$).

Mean values of PO and per-min measures of VO_2, VCO_2, VE and HR in the last 30s of each time segment are displayed in Table 2, along with measures of [La^-] at the end of warm-up and at the end of each time segment (peak [La^-]). ANOVA revealed a significant "time segment" x "intensity domain" interaction for VO_2 ($p \leq 0.001$), VCO_2 ($p= 0.014$), VE ($p \leq 0.001$), HR ($p= 0.011$) and [La^-] ($p \leq 0.001$). In detail, HR and [La^-] reached a steady-state within the 3rd min only in the moderate and heavy-intensity

exercise trials, while they increased over time in the severe exercise trials. VO_2, VCO_2 and VE reached a steady-state within the 3rd min in the moderate-intensity and within the 6th min of the heavy-intensity exercise trials. On the contrary, these parameters continued to increase significantly over time in the severe exercise trials.

An overview of the energetic contributors to exercise (i.e. measured VO_2, VO_{2VM}, $[La^-]$ equivalent of O_2, and $AdjO_{2Eq}$) is reported as 10-s averages and over 3-min time segments in figure 2 and in Table 3. For all time segments, the contribution of anaerobic glycolysis (as represented by $[La^-]$ equivalent) was significantly increased going from moderate to heavy to severe intensity trials (significant main effect of intensity, $p<0.001$). Furthermore, for all exercise intensities there was a significant reduction of the contribution of glycolysis over time segments (significant main effect of time segment, $p<0.001$). In particular, a limited and invariant contribution of anaerobic glycolysis was detected for the moderate-intensity domain; Additionally, the contribution of anaerobic glycolysis decreased from 0-3 to 3-6 time segments of the heavy-intensity exercise trials, yet no further after that; finally, contribution of anaerobic glycolysis continued to decrease over time in the severe exercise trials (figure 3).

Table 1, Subjects' anthropometrical and functional characteristics obtained during the ramp incremental test.

Subjects Characteristics				Ramp Incremental Test					
#	**Weight (kg)**	**Height (cm)**	**Age (years)**	**PPO (W)**	**VO_{2max} ($ml*min^{-1}$)**	**VO_{2max} ($ml*min^{-1}*kg$)**	**MRT (sec)**	**GET ($ml*min^{-1}$)**	**RCP ($ml*min^{-1}$)**
8 ♂	74 ± 9	180 ± 5	25 ± 1	376 ± 36	3643 ± 457	49 ± 3	41 ± 12	2418 ± 385	3093 ± 377

Values are means ±SD. PPO: peak PO; VO_{2max}: peak of oxygen consumption; MRT: mean response time; GET: gas exchange threshold; RCP: respiratory compensation point.

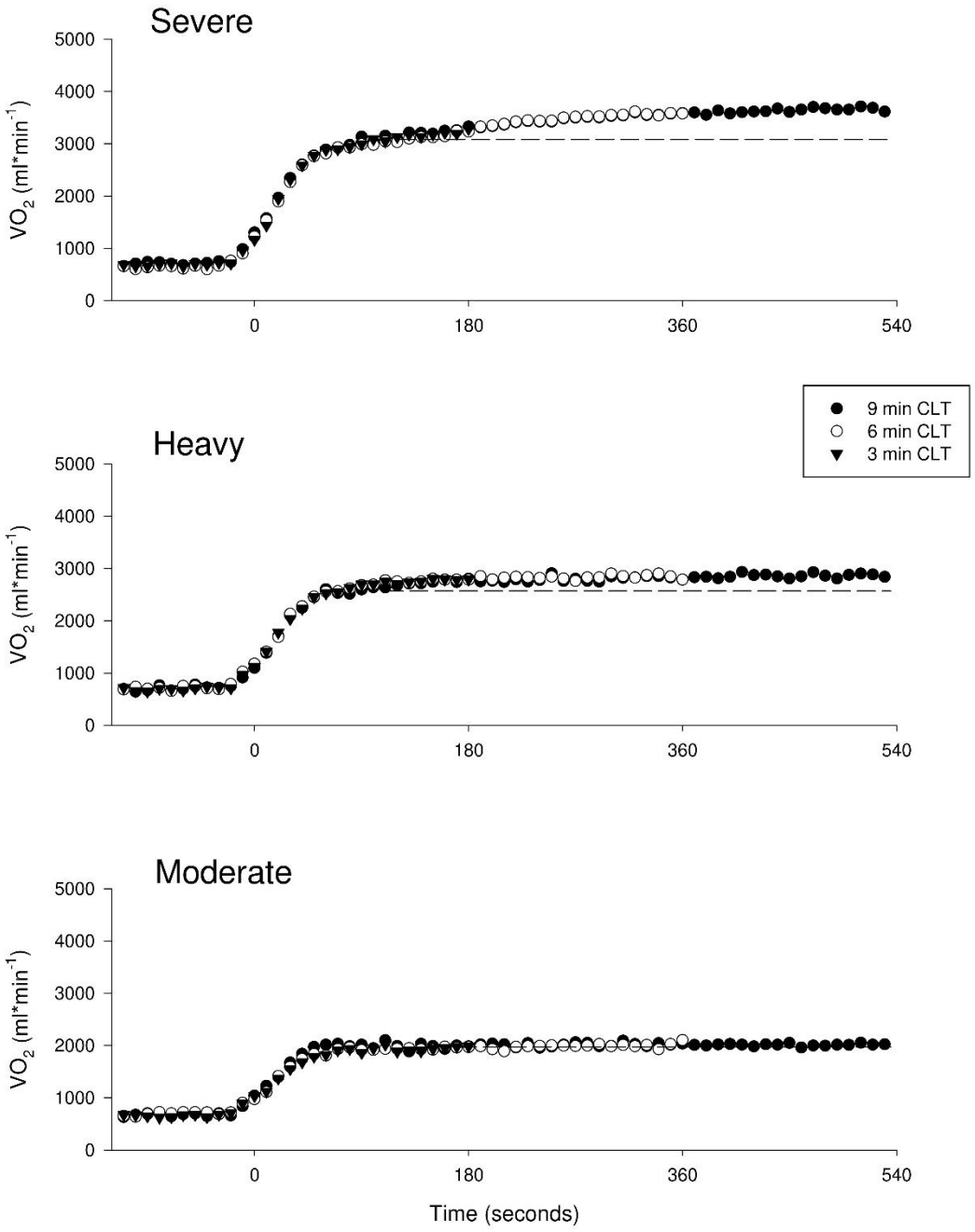

Figure 1, Repeatability of VO_2: group mean VO_2 data are displayed as 10s-averages respectively for the "severe" (top panel), "heavy" (medium panel) and "moderate" (bottom panel) exercise intensity domain. Symbols represent the three duration trials: black dots= 9-min CTL, white dots= 6-min CTL, black triangles= 3-min CTL.

Table 2, Metabolic response in the moderate, heavy and severe exercise domains.

Severe

Time Segment	Work rate (W)	VO_2 ($ml*min^{-1}$)	VCO_2 ($ml*min^{-1}$)	VE ($L*min^{-1}$)	HR ($b*min^{-1}$)	$[La^-]$ (mmol*L)
Warm-up	20.0 ± 0.0	1034.5 ± 51.4	743.8 ± 479.1	17.7 ± 2.2	73.2 ± 9.6	1.7 ± 0.6
0 to 3 min	267.0 ± 37.5	3198.7 ± 435.6	3585.8 ± 599.2	92.9 ± 26.9	157.3 ± 4.7	6.7 ± 1.6
3 to 6 min	267.0 ± 37.5 #	3489.1 ± 498.8 #	3792.3 ± 597.9 #	109.9 ± 31.1 #	169.5 ± 5.5	9.2 ± 2.4 #
6 to 9 min	267.0 ± 37.5 #*	3615.5 ± 521.9 #*	3888.1 ± 634.2 #*	119.1 ± 31.0 #*	175.2 ± 4.8 #	10.7 ± 2.5 #*

Heavy

Time Segment	Work rate (W)	VO_2 ($ml*min^{-1}$)	VCO_2 ($ml*min^{-1}$)	VE ($L*min^{-1}$)	HR ($b*min^{-1}$)	$[La^-]$ (mmol*L)
Warm-up	20.0 ± 0.0	1067.7 ± 57.6	605.8 ± 58.6	18.6 ± 2.6	74.2 ± 8.4	1.8 ± 0.8
0 to 3 min	208.9 ± 28.7	2771.6 ± 429.3	2748.8 ± 438.2	66.5 ± 14.4	141.5 ± 11.2	3.4 ± 1.1
3 to 6 min	208.9 ± 28.7 #	2851.5 ± 425.4 #	2818.3 ± 400.8 #	70.9 ± 13.4 #	146.3 ± 15.3	3.9 ± 1.4
6 to 9 min	208.9 ± 28.7 #	2822.7 ± 395.7 #	2741.1 ± 367.5 #	71.0 ± 13.1 #	151.1 ± 15.2	4.1 ± 1.4

Moderate

Time Segment	Work rate (W)	VO_2 ($ml*min^{-1}$)	VCO_2 ($ml*min^{-1}$)	VE ($L*min^{-1}$)	HR ($b*min^{-1}$)	$[La^-]$ (mmol*L)
Warm-up	20.0 ± 0.0	1026.3 ± 65.3	566.4 ± 60.7	17.1 ± 1.7	73.3 ± 8.5	1.7 ± 0.6
0 to 3 min	128.7 ± 27.0	1951.6 ± 261.7	1731 ± 275.5	42.9 ± 8.1	117.5 ± 23.6	2.4 ± 0.8
3 to 6 min	128.7 ± 27.0	1987.1 ± 277.4	1841.5 ± 275	45.3 ± 7.2	122.0 ± 27.0	1.8 ± 0.6
6 to 9 min	128.7 ± 27.0	1992.3 ± 298.7	1871.7 ± 332.7	47.6 ± 9.8	122.9 ± 29.0	1.3 ± 0.3

Values are means ±SD. Values of PO and per-min measures of VO_2, VCO_2, VE and HR in the last 30s of each time segment are displayed, along with measures of lactate concentration ($[La^-]$) at the end of warm-up and at the end of each time segment. ANOVA revealed a

*significant "intensity" x "time" interaction for VO_2 ($p \leq 0.001$), VCO_2 ($p = 0.014$), VE ($p \leq 0.001$), HR ($p = 0.011$) and $[La^-]$ ($p \leq 0.001$). Multiple comparisons are also displayed: # represents significant statistical difference with 0 to 3 min segment; * represents significant statistical difference with 3 to 6 min segment. For greater clarity were omitted: comparisons vs warm-up (always significantly different, with the only exception of $[La^-]$ in the moderate exercise intensity domain) and between exercise domains (always significantly different, with the only exception of HR between "severe" and "heavy" during the 0-3 segment).*

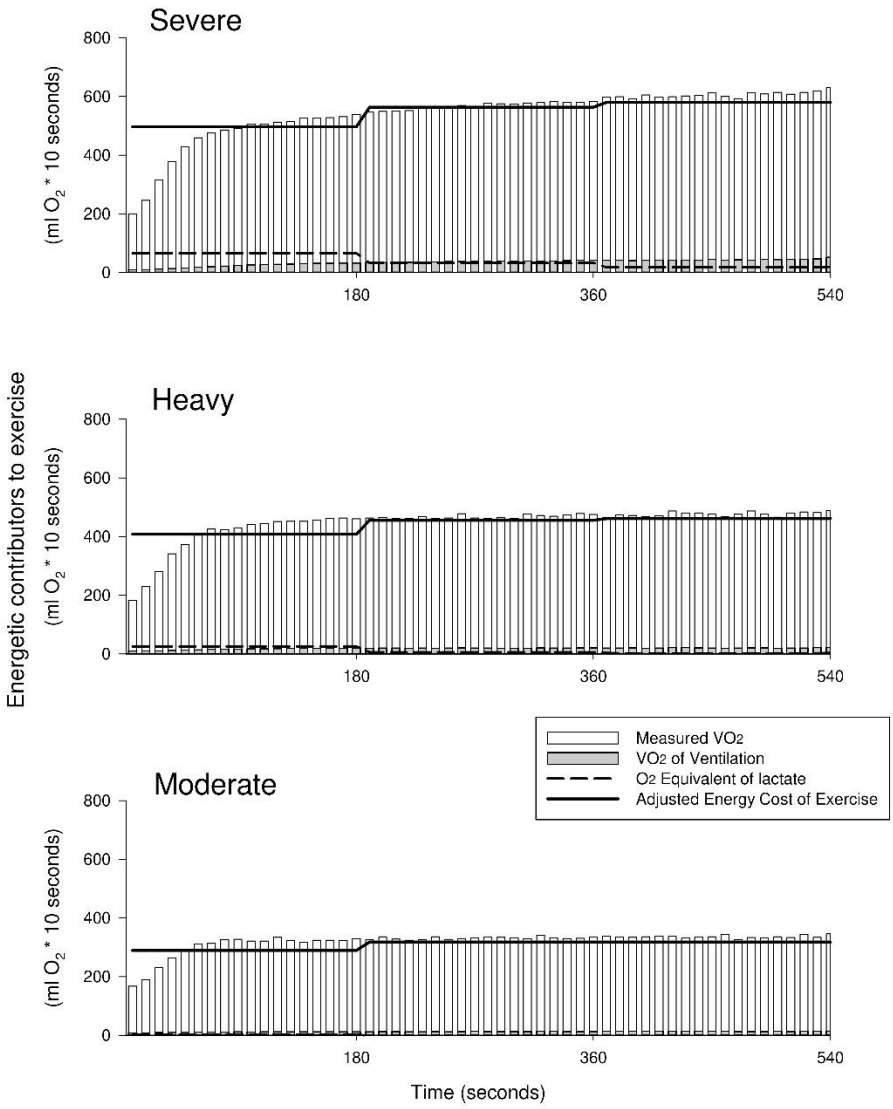

Figure 2, An overview of the energetic contributors to exercise is reported in 10-s averages for the "severe" (top panel), "heavy" (medium panel) and "moderate" (bottom panel) exercise intensity domain. White columns represent directly measured VO_2. Grey columns indicate the O_2 cost requested by ventilation. The black dashed line displays the energy provided by glycolytic sources over 3 min segments. Finally, the black solid line represents the adjusted cost of exercise accounting for both aerobic and glycolytic energy sources. Please note that during all the first 3 min segments, the contribution of immediate energy sources (O_2 and phosphocreatine) was not accounted and was probably the cause of the lower adjusted VO_2 between the first and the sec time segments (di Prampero et al. 1999; Hill 1999).

Table 3, Energetic contributors to exercise in the moderate, heavy and severe exercise domains.

Severe					
Time Segment	**VO_2 (ml O_2*3min^{-1})**	**VO_{2VM} (ml O_2*3min^{-1})**	**[La^-] Equivalent (ml O_2*3min^{-1})**	**$AdjO_{2Eq}$ (ml O_2*3min^{-1})**	**VO_{2gain} (ml O_2*min^{-1} * W)**
0 to 3 min	5037 ± 1155	356 ± 113	1182 ± 492	5863 ± 1413	7.9 ± 1.2
3 to 6 min	7096 ± 1509 #	623 ± 234 #	588 ± 256 #	7061 ± 1516 #	9.5 ± 1.2 #
6 to 9 min	7780 ± 1573 #*	729 ± 270 #*	321 ± 259 #*	7372 ± 1443 #*	9.9 ± 1.1 #
Heavy					
Time Segment	**VO_2 (ml O_2*3min^{-1})**	**VO_{2VM} (ml O_2*3min^{-1})**	**[La^-] Equivalent (ml O_2*3min^{-1})**	**$AdjO_{2Eq}$ (ml O_2*3min^{-1})**	**VO_{2gain} (ml O_2*min^{-1} * W)**
0 to 3 min	4062 ± 1184	216 ± 62	452 ± 254	4278 ± 1074	7.5 ± 1.1
3 to 6 min	5289 ± 1321 #	295 ± 77 #	128 ± 169 #	5121 ± 1268 #	9.0 ± 1.3 #
6 to 9 min	5450 ± 1180 #	304 ± 77 #	79 ± 135 #	5225 ± 1123 #	9.2 ± 1.0 #
Moderate					
Time Segment	**VO_2 (ml O_2*3min^{-1})**	**VO_{2VM} (ml O_2*3min^{-1})**	**[La^-] Equivalent (ml O_2*3min^{-1})**	**$AdjO_{2Eq}$ (ml O_2*3min^{-1})**	**VO_{2gain} (ml O_2*min^{-1} * W)**
0 to 3 min	2200 ± 906	140 ± 25	67 ± 94	2126 ± 939	6.4 ± 1.8
3 to 6 min	2823 ± 1083 #	169 ± 33	34 ± 58	2687 ± 1036 #	8.1 ± 1.7 #
6 to 9 min	2910 ± 1067 #	189 ± 40	0 ± 0	2731 ± 1035 #	8.3 ± 1.8 #

Values are means ±SD. Values are reported over 3-min time segments. VO_2: directly measured VO_2; VO_{2VM}: VO_2 requested by ventilatory muscles; [La^-] equivalent: oxygen equivalent of lactate; $AdjO_{2Eq}$: energy cost of exercise expressed as a sum of aerobic and glycolytic sources (VO_2 + [La^-] equivalent) and subtracted by VO_{2VM}. ANOVA revealed a significant "intensity" x "time" interaction for VO_2 ($p \leq 0.001$), VO_{2VM} ($p \leq 0.001$), [La^-] equivalent ($p \leq 0.001$), and $AdjO_{2Eq}$ ($p \leq 0.001$). Multiple comparisons are displayed in the

*table: # represents significant statistical difference with 0 to 3 min segment; * represents significant statistical difference with 3 to 6 min segment. Please note that values measured during warm-up were subtracted. For greater clarity comparisons between intensity domains were omitted (always significantly different, with the exceptions of i) [La^-] equivalent between "heavy" and "moderate" during the 3-6 and 6-9 time segments and ii) VO_{2gain} between "severe" and "heavy" in the 0-3 segment iii) VO_{2gain} between "severe" and "heavy" and "heavy" and "moderate" in the 3-6 segment).*

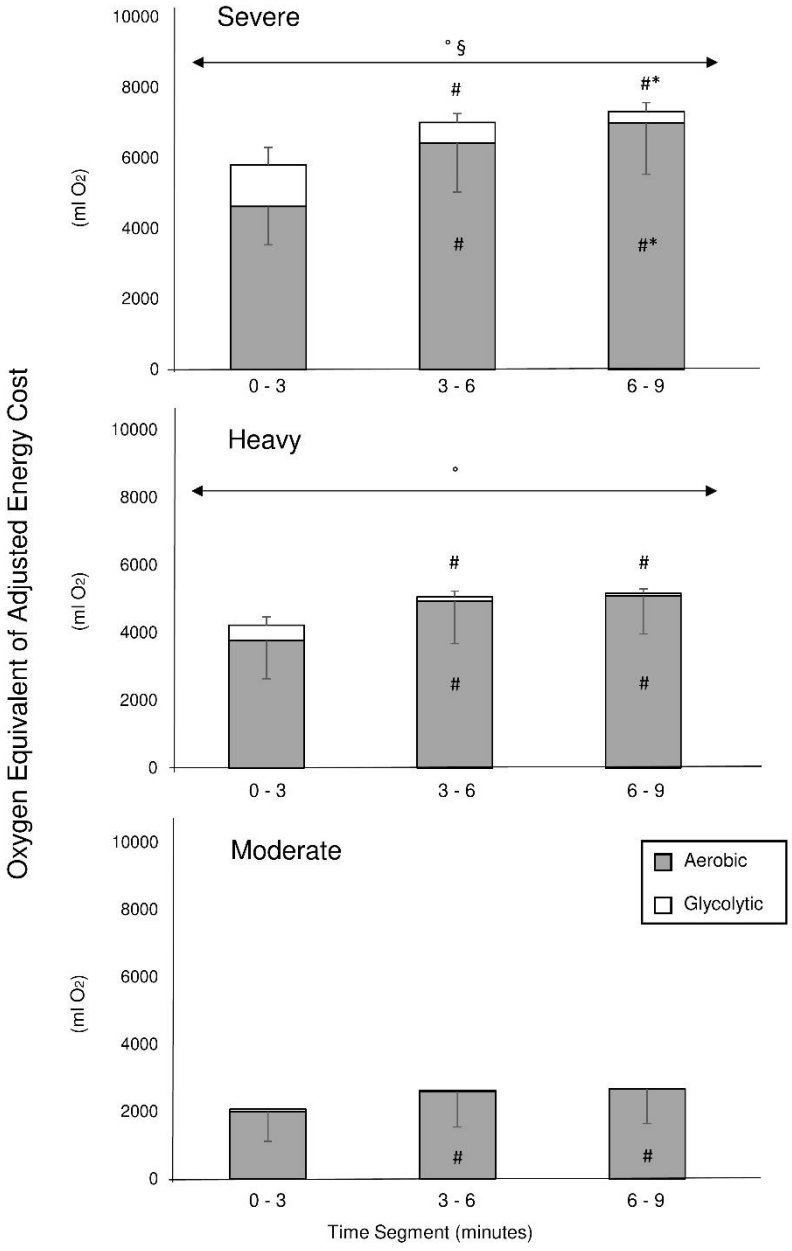

*Figure 3, 3-min mean oxygen equivalents of aerobic (grey, i.e. VO_2 subtracted by VO_{2VM}) and glycolytic (white, i.e. [La^-] equivalent) cost of exercise are represented respectively for the "severe" (top panel), "heavy" (medium panel) and "moderate" (bottom panel) exercise intensity domain. ANOVA revealed a significant "intensity" x "time" interaction both for aerobic ($p \leq 0.001$) and anaerobic ($p \leq 0.001$) cost of exercise. Multiple comparisons are displayed as: # significant statistical difference with 0 to 3 min segment; * significant statistical*

difference with 3 to 6 min segment. ° and § represent respectively statistical difference with the "moderate" and "heavy" exercise intensity domain. Please note that values measured during warm-up were subtracted.

Discussion:

By comprehensively quantifying aerobic and glycolytic energy sources used during exercise, deprived of the VO_2 cost of ventilation (i.e. $AdjO_{2Eq}$), this study tested the hypothesis that the overall cost of cycling is affected by time during metabolic transitions in the moderate, heavy and severe intensity domains. In agreement with our hypothesis, our findings suggest that: i) the overall cost of locomotion does not increase over time during metabolic transitions in the moderate domain; ii) in the heavy intensity domains, the emergence of a VO_2 slow component is associated with a "metabolic shift" between aerobic and anaerobic metabolisms protracted beyond the 3rd min of exercise and to a higher VO_2 cost of ventilation, rather than to an increased cost of locomotion over time; iii) finally, a true loss of efficiency over time may manifest only in the severe intensity domain. This investigation provides new insight into the mechanisms underpinning VO_{2sc} and exercise tolerance.

The fitness level of our sample measured during the ramp incremental tests indicated that the participants of this investigation were active subjects. (VO_{2max}: 49±3 ml*min^{-1}*kg, see Table 1 for the other characteristics) (American College of Sports Medicine 2017). Metabolic responses measured during CWR trials (Table 2) were consistent with values reported by another investigation that tested a similar group of subjects while cycling in comparable exercise intensity domains (Black et al. 2017). In addition, the pulmonary VO_2 kinetics were similar to those previously reported in the literature: no VO_{2sc} (i.e. the % difference between the pulmonary VO_2 at the end and the third min of exercise) was measured in the moderate (0.9±3.51 %) domain; a small VO_{2sc}, evolving to steady state, was present in the heavy domain (4.2±3.4 %); a larger VO_{2sc} and no steady state were detected in the severe domain (11.4±4.9 %) (Santana et al. 2007; O'Connell et al. 2017).

By applying an approach previously proposed by O'Connell et al. (O'Connell et al. 2017), our study quantified the individual contributors to the overall net VO_2 cost of exercise (i.e. $AdjO_{2Eq}$) and their interplay during metabolic transition and steady-state. While O'Connell et al. studied the VO_2 of active muscles, the VO_2 associated with

ventilation and the VO_2 equivalent of [La^-] in the severe domain only, we extended the analysis to the three exercise intensity domains.

As expected, based on the changes in ventilation described above, the VO_2 associated with ventilation increased going from moderate (3±3 % of the VO_{2sc}), heavy (7±3 %), to severe (14±7 %) intensity. In agreement with previous work (Poole et al. 1991), this finding confirms that ~85% of the VO_{2sc} occurs within the working muscles. In addition, our study demonstrated that the VO_2 associated with ventilation increased over exercise time in the severe domain only. Furthermore, for all the exercise domains, the O_2 equivalent of [La^-] accumulation decreased after the transition phase (i.e. 0-3 time segment), compatible with a progressive decrease of the contribution of glycolysis to ATP resynthesis over time (di Prampero et al. 1999). Interestingly, in the domains below RCP, this decreased contribution of glycolysis to the energy provision was "mirrored" by an increased contribution of oxidative metabolism, to satisfy an invariant energetic demand (Table 3 and figure 3). In other words, the VO_{2sc} that is observed in the heavy domain may be related to a "metabolic switch" between anaerobic and aerobic energy sources of ATP resynthesis rather than to an augmented cost of locomotion over time. Such a view is in agreement with the findings and interpretations of previous humans studies, performed in the heavy/severe domain of exercise (Krustrup et al. 2004a; O'Connell et al. 2017) as well as with studies based on computer modeling of the skeletal muscle bioenergetics (Korzeniewski and Zoladz 2015). For exercise in the severe domain however, we found that accounting for the total energetic cost of locomotion did not completely explain VO_{2sc}. We were the first to demonstrate that, in this domain, the $AdjO_{2Eq}$ continued to increase showing an augmented energy requirement to sustain the same PO, compatible with a true loss of efficiency over time.

Relative to the severe intensity domain, the partial discrepancy between our data and O'Connell's may be explained by methodological differences between both studies: *i)* as we did, most studies in the field of VO_2 kinetics define intensity domains based on gas exchange thresholds (De Roia et al. 2012; Poole and Jones 2012); on the contrary,

O'Connell used lactate-based measures. Yet, the correspondence between the above methods remains controversial (Keir et al. 2015, 2018b; Broxterman et al. 2018) and the difference may have led to unmatched intensity domains among our studies. *ii)* O'Connell incremental protocol for VO_{2max} detection was performed after 10 min of recovery from a ~20-min protocol with 3-min steps used for LT determination. This approach could be responsible for an underestimation of peak-PO and consequently of Δ60% and may not have guaranteed an exercise intensity corresponding to the severe domain for all participants. In summary, while a direct comparison between different studies may be difficult, our data provides the first, comprehensive, domain-specific characterization of the contributors to the observed VO_{2sc}.

Traditionally, VO_{2sc} has been attributed to an increased cost of locomotion when exercise is protracted more than 3 min at a constant work rate in the heavy and severe exercise intensity domains, in relation to either fatigue or recruitment of higher order motor units or both (Jones et al. 2011). Interestingly, while a clear distinction between exercises performed above and below RCP is a common concept in exercise physiology (Poole and Jones 2012), VO_{2sc} measured in the heavy and in the severe exercise domains is usually considered as the expression of a single phenomenon (Jones et al. 2011; Grassi et al. 2015). In this context, a recent paper based on mathematical modelling of the muscle bioenergetics, proposed that a metabolic shift between the aerobic and the anaerobic energy systems, caused by a progressive inhibition of the glycolytic ATP supply by cytosol acidification, may also contribute to VO_{2sc} (Korzeniewski and Zoladz 2015). The authors also suggest that the size of the VO_{2sc} can increase when the contribution of glycolysis to ATP resynthesis (and in turn proton accumulation) is higher; a lower VO_2 in the initial stages of exercise rather than an increased VO_2 after 3-6 min into exercise may explain the larger VO_{2sc} at the higher exercise intensities (Korzeniewski and Zoladz 2015).

To our knowledge, this is the first experimental study to determine the contributors of VO_{2sc} in the three intensity domains of exercise. Our findings (i.e. a delayed metabolic shift in the heavy domain and a true loss of efficiency over time in the severe intensity

domain only) appear to support the theory proposed by Korzeniewski & Zoladz, 2015. We speculate that, with increasing exercise intensity, the recruitment of bigger, preferentially glycolytic muscle fibres could explain a higher contribution of glycolysis to ATP resynthesis at exercise onset and therefore a delayed VO_2 steady state. Furthermore, the recruitment of these intrinsically less efficient and fatigable type II fibres could explain the true loss of efficiency that appears over time in the severe domain of exercise (Jones et al. 2011; Grassi et al. 2015; Colosio et al. 2019). While direct measures of muscle activation were not performed in this investigation, the appearance of a loss of efficiency as a function of exercise intensity/PO seems compatible with this hypothesis. Future researches should focus on quantifying and separating the contribution of ventilation and anaerobic glycolysis to the overall cost of exercise while contextually measuring muscle excitement.

In conclusion, the innovative methodological approach applied in this study allowed to discriminate three contributors to the VO_{2sc}: increased VO_2 cost of ventilation, delayed shifting between aerobic and glycolytic metabolism, and loss of efficiency over time. How these mechanisms contribute to the VO_{2sc} depends on relative exercise intensity, with a true loss of efficiency over time occurring only at intensities above RCP.

Limitations:

It should be acknowledged that data interpretation in this investigation depends upon estimates of the energetic yield of lactate accumulation and of the VO_2 cost of ventilation and ignores the contribution of oxygen stores and anaerobic alactic mechanism of ATP resynthesis.

The di Prampero equation (di Prampero et al. 1999), was used to obtain the energy equivalent of blood lactate accumulation. This equation was developed using the following approach: first the O_2 cost of a supramaximal exercise was estimated by extrapolating the sub-maximal VO_2/PO relationship; then, the difference between the estimated and the actual VO_2 of exercise was calculated; finally, this difference was

divided by the lactate accumulated over the time of the measure in order to calculate the O_2 equivalent of lactate accumulation.

The above described method relies on the following assumptions: *i)* fingertip capillary lactate reflects whole-body lactate accumulation; *ii)* blood lactate accumulation in *whole-body, in vivo conditions* reflects the net result of whole-body lactate production and clearance and therefore it is an indicator of the net glycolytic contribution to ATP resynthesis. This approach has been equally criticized and endorsed and its validity is still debated. Clearly, using the same fixed value of O_2 equivalent of lactate accumulation for all the individuals may impact the accuracy of the estimate of the glycolytic contribution to exercise at the individual level. However, this systematic bias would have a similar impact across domains and across time, and therefore should not preclude our ability to estimate the contribution of anaerobic metabolism to the overall cost of exercises in our experimental setup. An extensive explanation of the advantages and the limitations of this method is reported elsewhere (di Prampero et al. 1999).

Referring the VO_2 cost of ventilation, different predictive equations were proposed for calculating the work of ventilation and its translation in VO_2 cost (Aaron et al. 1992b, 1992a; Coast et al. 1993) and a variability across individuals of around ±10% has been described (Aaron et al. 1992a, 1992b). With the aim to quantify the effect of using different predictive equations, we compared the equations from Aaron et al. (characterized by a higher cost of ventilation) and the equations of Coast et al. (used in our study) at different levels of ventilation. Using Aaron's compared to Coast's equation would cause an increase in the estimated cost of ventilation from a minimum of 19% in the moderate to a maximum of 25% in the severe domain of exercise. The effect of using the less conservative Aaron's equation would be a larger contribution of ventilation to the VO_{2sc}, implying a smaller loss of efficiency over time for all exercise intensities. Finally, as also discussed in relation to the O_2 equivalent of lactate, the use of an identical equation for different individuals may have affected the accuracy of our calculations; however, this systematic bias would have a similar impact across domains and across time, and therefore should not preclude our ability to estimate the

contribution of the cost of ventilation to the overall cost of exercises in our experimental setup. As a final consideration, a possible effect of time or fatigue on the VO_2 cost of ventilation should also be considered. However, to our knowledge the existence of this phenomenon, its temporal appearance and its magnitude have not yet been described. A reduction of the VO_2 cost of ventilation over time during a constant work rate exercise appears very unlikely. On the contrary, similarly to the loss of efficiency of locomotor muscles, a loss of efficiency of ventilator muscles might manifest when exercise is protracted. Also in this case, the effect of an unaccounted loss of efficiency of ventilation over time on our results would imply an overestimation of the locomotor loss of efficiency.

Finally, during the first three minutes of exercise, the contribution of the immediate energy sources (i.e. the O_2 stores and phosphocreatine contribution) to ATP resynthesis were not quantified. Ignoring this contribution, quantifiable around 680±90 ml of O_2 (di Prampero et al. 1999; Hill 1999), has clearly caused an underestimation of the energy cost of exercise during the onset phase, but should not have altered the cost quantification of the following part of exercise, and therefore the interpretation of our findings in the relation to the VO_{2sc}

CHAPTER

6

Mechanisms of the VO_2 Slow Component Between Exercise Intensity Domains.

Alessandro L Colosio, Kevin Caen, Jan G. Bourgois, Jan Boone, Silvia Pogliaghi.

Abstract:

This investigation was designed to focus on the steady-state phase of exercise by evaluating the relative contribution of metabolic instability and muscle activation to the oxygen consumption (VO_2) slow component (VO_{2sc}). We tested the following hypotheses: i) after the initial 3 min, VO_2, metabolic instability and muscle activation display a different tendency to increase over time depending on the relative exercise intensity (i.e. no changes occur in the moderate domain and increasing dynamics are observed in the heavy and severe intensity domains); ii) the increase in VO_{2sc} is explained by a combination of metabolic instability and muscle activation. Eight active men performed 3 constant work rate trials of 9 min respectively in the moderate, heavy and severe domains. VO_2, root mean square by EMG (RMS), deoxyhaemoglobin by NIRS ([HHb]) and hematic values of metabolic stability (i.e. [La^-], pH, HCO_3^-) were measured. The physiological responses during exercise in the three intensities domains were compared by a two-way (time, intensity) RM-ANOVA. Moreover, the relationships between the increases after the third min of NIRS and EMG with VO_2 were compared with simples and a multiple linear regressions. We found a domain-dependent dynamic over time for VO_2, [HHb] (taken as an index of metabolic instability), RMS (taken as an index of muscle excitation) and the whole-body hematic markers of metabolic instability. Both [HHb] and RMS showed a medium to high correlation with VO_2 ([HHb] $r = 0.68$, $p < 0.001$; RMS $r = 0.59$, $p = 0.002$). Moreover, the multiple linear regression showed that both metabolic instability as measured by NIRS and muscle excitation detected by EMG concurred to VO_2 ($r = 0.75$, [HHb] $p = 0.005$, RMS $p = 0.042$). About 75% of the variability of the VO_{2sc} was explained by a combination of the dynamics of [HHb] and RMS.

Introduction:

During constant workrate exercise (CWR), oxygen consumption (VO_2) response is linearly related to work rate with a ratio of around ~ 10 ml*min^{-1}*W (Poole and Jones 2012). However, as exercise intensity increases, and particularly at intensities above the gas exchange threshold (GET), this relationship is lost and a further, theoretically unexpected, increase in VO_2 is detectable. This increase in VO_2, defined as the "slow component" of VO_2 (VO_{2sc}), is usually interpreted as an increased O_2 cost of locomotion and manifest ~3 min after exercise onset (Jones et al. 2011). Typically, when exercise is performed in the heavy intensity domain, between GET and the respiratory compensation point (RCP, or the critical power), VO_{2sc} tends to steady-state. On the contrary, when effort rises above RCP (i.e. severe exercise domain) a steady-state is not achievable and VO_2 tends to the maximal oxygen consumption (VO_{2max}) (Jones et al. 2011).

Of the VO_{2sc} recorded at the mouth, around 85% originates from the contracting muscles, while the remaining 15% is caused by the increased cost of ventilation (Poole et al. 1991). Pointing the muscular component of the VO_{2sc}, it was proposed that either the recruitment of less efficient type II motor units necessary to maintain a specific power output (PO) (Jones et al. 2011; Colosio et al. 2019) or metabolic instability occurring within the working fibres (Zoladz et al. 2008; Vanhatalo et al. 2011) could represent the main physiological underpinnings. In both these scenarios, muscle contractions become less efficient, therefore requiring a higher energetic demand in order to maintain the same external power output. Moreover, Grassi et at. have proposed that as exercise is protracted these two phenomena may mutually influence each other in a vicious circle in which the changes in homeostasis of the working muscle lead to a loss of efficiency, that in turn may cause the recruitment of larger and less efficient motor units, therefore further affecting metabolic stability (Grassi et al. 2015).

Adding complexity, recent studies (O'Connell 2017, Study 3), by applying a method that accounts for the VO_2 cost of ventilation and the VO_2 equivalent of lactate

accumulation, questioned the very existence of a VO_{2sc}. Analysing the response between different intensity domains, the authors (Colosio et al. 2020) suggested that in the heavy domain the observed increase in VO_2 over time could be the result of a delayed adjustment of VO_2, while in severe also a "true" loss of efficiency manifest. It was speculated that the recruitment of intrinsically less efficient and fatigable type II fibres could explain the loss of efficiency reported in the severe intensity domain (Jones et al. 2011; Grassi et al. 2015; Colosio et al. 2019).

Given that both metabolic instability and muscle recruitment can change according to the domain in which exercise is performed (Poole and Jones 2012), and because the VO_{2sc} may not entail the same physiological underpinnings in heavy and in severe (Study 3), a study aimed at testing the muscle contributors to the VO_{2sc} in different domains would help in clarifying the origins of this phenomenon. However, the implementation of methods designed to quantify muscle metabolism and muscle excitation are not often performed simultaneously due to complexity or economic reasons.

In this context, near-infrared spectroscopy (NIRS) provides a non-invasive index of oxygen extraction that reflects the imbalance between delivery and utilization within the working muscle (Grassi et al. 2003; Grassi and Quaresima 2016) and in turn may be associated with metabolic instability (Grassi et al. 2015). Furthermore, electromyography (EMG), allows an indirect estimate of motor units activation during muscle contraction. Interestingly, while the use of these techniques has increased exponentially during the past two decades, no study has applied them in unison to gain insight into the origins of the VO_{2sc}.

Therefore, this investigation was designed to focus on the steady-state phase of exercise by evaluating the relative contribution of metabolic instability (measured with NIRS and the hematic changes in metabolic balance/homeostasis) and muscle excitation to the VO_{2sc}. We tested the following hypotheses: i) after the initial 3 min, VO_2, metabolic instability and muscle excitation display a different tendency to increase over time depending on the relative exercise intensity (i.e. no changes occur in the moderate

domain and increasing dynamics are observed in the heavy and severe intensity domains); ii) the increase in VO_{2sc} is explained by a combination of metabolic instability and muscle excitation.

Methods:

Ethical Approval

The study was conducted according to the Declaration of Helsinki and all procedures were approved by the University of Verona Ethics Committee for Research on Human Subjects. Procedures and risks were explained to each subject, and all participants volunteered and gave informed written consent to participate before the start of the study.

Participants

Eight active men were recruited in the study (age 25 ± 2 years, body mass 74 ± 10 kg, height 181 ± 5 cm, VO_{2max}: 3643±457 ml*min^{-1}, 49±3 ml*min^{-1}*kg). Inclusion criteria were male sex and age between 20 and 35 years; exclusion criteria were smoking and any condition that could influence the physiological responses during testing. All participants were instructed to avoid caffeine consumption and physical activity respectively for at least 8 h and 24 h before each testing session. Moreover, to minimise variability of glycogen stores and glucose oxidation, participants followed a standard food intake prescription before all the testing sessions as previously described (Keir et al. 2015).

Experimental Protocol

VO_2, EMG and NIRS data for this investigation were collected during the 9-min trials of a previous investigation (Study 3) in which participants performed 3 tests

(respectively of 3, 6, and 9 min) in every exercise intensity domain within a maximum of four weeks. Briefly, all subjects completed: *i)* a preliminary maximal ramp incremental exercise test to exhaustion for the determination of gas exchange threshold (GET), respiratory compensation point (RCP) and the maximum oxygen uptake (VO_{2max}); *ii)* three constant workrate (CWR) trials in the "*moderate"* exercise intensity domain *iii)* three CWR in the "*heavy"* exercise intensity domain *iv)* three CWR trials in the "*severe"* exercise intensity domain. (di Prampero et al. 1999). Moreover, the hematic response to exercise (i.e. blood lactate ($[La^-]$) accumulation, pH, bicarbonate (HCO_3^-)) was characterized at baseline and every 3 min, by taking blood samples at the 1^{st},3^{rd}, 5^{th} and 7^{th} min after exercise stop of the 3, 6 and 9 min CWR respectively. Tests were executed in randomized order with the only exception of the longest CWR in the "severe" exercise domain, that was completed as first to assure that subjects were able to sustain the PO for the required time. All exercise tests were conducted on an electromagnetically braked cycle ergometer (Sport Excalibur, Lode, Groningen, Netherlands), at a similar time of the day in an environmentally controlled laboratory (18°C, 55-65% relative humidity).

Ramp incremental test:

The ramp incremental test consisted of a 3-min baseline cycling at 50 W, followed by a 30-$W*min^{-1}$ increase in PO until volitional exhaustion. Participants were asked to pick a self-selected cadence in the range of 70-90 rpm and to maintain it throughout all subsequent tests. Failure to maintain the indicated cadence within 5 rpm (for longer than 5 sec) during testing despite strong verbal encouragement was considered as the criterion for exhaustion. Breath-by-breath pulmonary gas exchange and ventilation were continuously measured using a metabolic cart (Jaeger Oxycon Pro, Viasys Healtcare GmbH, Höchberg, Germany) as previously described (De Roia et al. 2012). Heart rate (HR) was monitored continuously (H7 Sensor, Polar, Kempele, Finland).

Constant workrate trials:

After the preliminary ramp incremental test, subjects completed 3 CWR within each exercise intensity domain (*i.e. moderate* at 80% of GET, *heavy* at 50%Δ between GET and RCP, and *severe* at 60%Δ between GET and VO_{2max}) in a randomized order.

Each CWR was preceded by a 3-min warm-up at 20 W. Throughout the test, subjects kept the same, constant rpm and bike position as selected during the ramp incremental test.

VO_2 and HR data were measured with the same method described for the ramp incremental test.

Capillary blood samples (65 µl) were drawn from the fingertip in the last 30s of warm-up and at the 1^{st},3^{rd}, 5^{th} and 7^{th} min after each test and were immediately analysed using a benchtop blood analyser (Radiometer ABL90 FLEX, Radiometer Medical ApS, Brønshøj, Denmark) to measure [La^-], pH and HCO_3^-. The highest value of [La^-] was considered as the peak of blood lactate concentration and used for further analysis. The blood sample with the highest [La^-] was also used to define pH and HCO_3^- at a given time point.

Deoxygenation of the left *vastus lateralis* was evaluated in microcirculation using a quantitative near-infrared spectroscopy system (Oxiplex TSTM, ISS, Champaign, USA) that provided continuous measurement (sampling frequency 1 Hz) of the absolute concentrations (µM) of deoxyhaemoglobin ([HHb]). After shaving, cleaning and drying of the skin area, the NIRS probe was positioned longitudinally on the belly of the vastus lateralis muscle ~15 cm above the patella, attached to the skin with a bi-adhesive tape and secured with elastic bandages around the thigh. The device was calibrated before each test after a warm-up of at least 30 minutes as per manufacturer recommendations.

Surface EMG of the right *vastus lateralis* muscle was continuously recorded by means of a wireless system (1000 Hz; ZeroWire, Noraxon, Scottsdale, AZ, USA). A pair of surface Ag/AgCl electrodes (Blue sensor, Ambu®, Ballerup, Denmark) was attached to the skin with a 2-cm inter-electrode distance. The electrodes were placed

longitudinally with respect to the underlying muscle fibres arrangement, according to the recommendations by Surface EMG for Non-Invasive Assessment of Muscles (Hermens et al. 2000). Before electrode application, the skin was shaved, scratched with sand-paper and cleaned with alcohol in order to minimize impedance. Semi-permanent ink marks allowed consistent re-positioning of the electrodes between sessions. The EMG transmitter connected to the electrodes was well secured with adhesive tape to avoid movement-induced artefacts.

Data Analysis

Ramp Incremental Test:

For the gas exchange variables, aberrant data-points that lay 3 SD from the local mean were removed, and trials were linearly interpolated on a 1-sec basis and then averaged every 5-sec. VO_{2max} was determined as the highest VO_2 obtained over a 10-sec interval (Fontana et al. 2015). GET and RCP were determined with the standard technique from gas exchange variables by three blinded expert reviewers as detailed elsewhere (Fontana et al. 2015). Briefly, GET was determined by visual inspection as the VO_2 at which CO_2 output began to increase out of proportion in relation to VO_2, with a systematic rise in the ventilation (VE)-to-VO_2 relation and end-tidal PO_2 whereas the ventilatory equivalent of VCO_2 (VE/VCO_2) and end-tidal PCO_2 is stable (Beaver et al. 1986). RCP was determined as the point where end-tidal PCO_2 began to fall after a period of isocapnic buffering (Whipp et al. 1989). This point was confirmed by examining VE/VCO_2 plotted against VO_2 and by identifying the second breakpoint in the VE-to-VO_2 relation. Finally, we determined the constant work rate equivalent to the specific moderate (80% of GET), heavy (50% Δ between GET and RCP) and severe (60%Δ between GET and VO_{2max}) VO_2 targets. To this aim, the VO_2/W relationship identified with the incremental test left-shifted to account for the mean response time (Fontana et al. 2015).

Constant workrate trials:

VO_2 during CWR was sampled breath-by-breath, interpolated using the same procedure described for the ramp incremental test, and time-aligned with the onset of exercise. To isolate O_2 contributing to locomotion (VO_{2m}), the VO_2 requested by ventilation was subtracted from the VO_2 measured at the mouth level as broadly described elsewhere (study 3). In brief, the work of breathing was calculated based on VE using the equation by Coast et al.:

Work of breathing = $-0.430 + 0.050 * VE + 0.00161\ VE^2$

Then, the work of breathing was used to calculate the amount of VO_2 requested by ventilatory muscles:

VO_2 requested by ventilation = 34.9 + 7.45 * work of breathing (Coast et al. 1993)

NIRS derived [HHb] response was time-aligned with the onset of exercise transition.

Raw EMG signal was rectified and smoothed using a fourth-order band-pass Butterworth digital filter with a frequency range set between 20 and 500 Hz. Root mean square (RMS) was calculated every second from the raw signal and was used as an index of the total muscle excitation for *vastus lateralis* (Moritani et al., 1986; Ryan & Gregor, 1992). Thereafter, the RMS recorded during the last 2 minutes of 20 W baseline for each test was used to normalize the CWR and expressed as multiples of baseline.

Then, the slopes of increase of each signal were calculated (i.e. VO_{2m}, [HHb], RMS, $[La^-]$, pH and HCO_3^-) in the time window between the 180 and 540 sec after exercise start. Based on the prediction of the time to steady-state of this sample of subjects (calculated as the time delay (25±2 sec) + 4 * tau (30±3 sec)), 180 sec was chosen as the minimum time to achieve a steady-state in all the intensity domains.

These slopes were used to calculate the % increases after the values reached after the first 180 sec of exercise, as follow:

$Y_{(t)} = Y_{180} + (Slope_Y * t) / (Y_{180} / 100)$

Where $Y_{(t)}$ represents the increase of a given signal at the time point corresponding to 180 sec after exercise onset, Y_{180} is the mean value obtained from data between 170 and 180 sec after exercise onset, $Slope_Y$ is the slope of increase of a given signal in the time window between 180 and 540 sec, t is the time coordinate.

Statistics

After assumptions verification (*i.e.*, normality, homogeneity of variance), a two-way repeated-measures ANOVA was performed to assess differences over time in the 180-540 sec time window, and between different intensities domains (time segment x intensity domain) for VO_{2m}, [HHb], RMS, $[La^-]$, pH, and HCO_3^-. Post-hoc analysis was performed using Holm-Sidak method.

The linear relationships between the slope of the % change of VO_{2m} and the % change of RMS, and [HHb] were modelled, and Pearson's product moment correlation coefficients were calculated. Finally, a multiple linear regression was run incorporating both RMS and [HHb] to test the combined muscle recruitment/metabolic instability contribution in predicting the slope of VO_{2m} (i.e. VO_{2sc}).

Data are presented as means ± SD. All statistical analyses were performed using Sigmaplot version 12 and α was set in advance at the 0.05 level. Statistical significance was accepted when $p < \alpha$.

Results:

GET and RCP were identified at a VO_2 respectively of 2418±385 and 3094±377 ml*min^{-1}, while the VO_2 targets in the moderate, heavy and severe exercise domains were identified at: 1935±308 ml*min^{-1}, 2743±348 ml*min^{-1} and 3154±408 ml*min^{-1}. VO_2 values recorded in the last 30 sec of exercise were: 1973±478 ml*min^{-1} for moderate, 3013±365 ml*min^{-1} for heavy and 3640±514 ml*min^{-1} for severe, highlighting a clear contribution of the VO_2 slow component in the rise of VO_2 over time both in the heavy and severe domains. The mean VO_2 profile, and the other physiological variables, are represented in the three exercise intensity domains in figure 1.

In figure 2, the dynamic changes in these variables after 180 sec of exercise are presented. ANOVAs revealed a significant "time" x "intensity domain" interaction for VO_{2m} ($p<0.001$), [HHb] ($p<0.001$), RMS ($p<0.001$), [La^-] ($p<0.001$), and HCO_3^- ($p<0.001$), but not for pH, in which there was a tendency to significance ($p=0.068$) and a significant main effect of the intensity domain in which exercise was performed ($p=0.009$). All the multiple comparisons between domains at different time-points resulting from the post-doc analysis are presented in figure 2. In brief, VO_{2m} and muscle [HHb] continued to increase over time after the first 180 sec of exercise in severe (% difference between 540 and 180 sec: VO_{2m} +9.4±4.7%, $p<0.001$; [HHb] +9.4±5.4%, $p<0.001$) and in heavy (% difference between 540 and 180 sec: VO_{2m} +2.7±2.7%, $p<0.001$; [HHb] +5.8±4.9%, $p<0.001$), but not in moderate (VO_{2m} -2.4±2.1%, $p= 0.001$; [HHb] +0.3±2.7%, $p=1.000$). Muscle excitation increased over time only in severe (% difference between 540 and 180 sec in RMS: severe +13.1±13.2%, $p<0.001$, heavy +5.4±8.5%, $p=0.110$, moderate +2.5±8.5%, $p=1.000$). The comparison between different domains revealed that in severe, both VO_{2m}, [HHb] and RMS were different from heavy and moderate; while in heavy VO_{2m} and [HHb] were the only signals to reach statistical significance versus moderate (figure 2). Regarding the hematic values, blood lactate concentration increased over time in severe (% difference between 540 and 180 sec: +63.4±39.1%, $p<0.001$), remained stable in

heavy (% difference between 540 and 180 sec: +22.3±33.7%, p=0.093), and decreased in moderate (% difference between 540 and 180 sec: -35.5±18.5%, p=0.004); while both pH and HCO_3^- decreased over time in the severe domain only (% difference between 540 and 180 sec in severe: pH -0.6±0.6%, p<0.001, HCO_3^- -16.1±5.8%, p<0.001; heavy: pH +0.1±0.3%, p=0.646, HCO_3^- -1.6±5.7%, p=0.418; moderate: pH +0.2±0.4%, p=0.438, HCO_3^- +2.5±3.8%, p=0.463).

Finally, the relationships of [HHb] and RMS with VO_{2m} are presented in figure 3. Both the variables showed a medium to high correlation with VO_{2m} ([HHb] r =0.68, p<0.001; RMS r=0.59, p=0.002). Moreover, the multiple linear regression showed that both metabolic instability as measured by NIRS and muscle excitation detected by EMG concurred to VO_{2m} (r =0.75, [HHb] p=0.005, RMS p=0.042), as described by the following equation:

$$VO_{2sc} = -0.00289 + (0.520 * [HHb]) + (0.193 * RMS)$$

Where: VO_{2sc} represents the slope of % increase in VO_{2m} after the third minute of exercise; [HHb] represents the slope of % increase in deoxyhaemoglobin after the third minute of exercise; and RMS represents the slope of % increase in root mean square after the third minute of exercise.

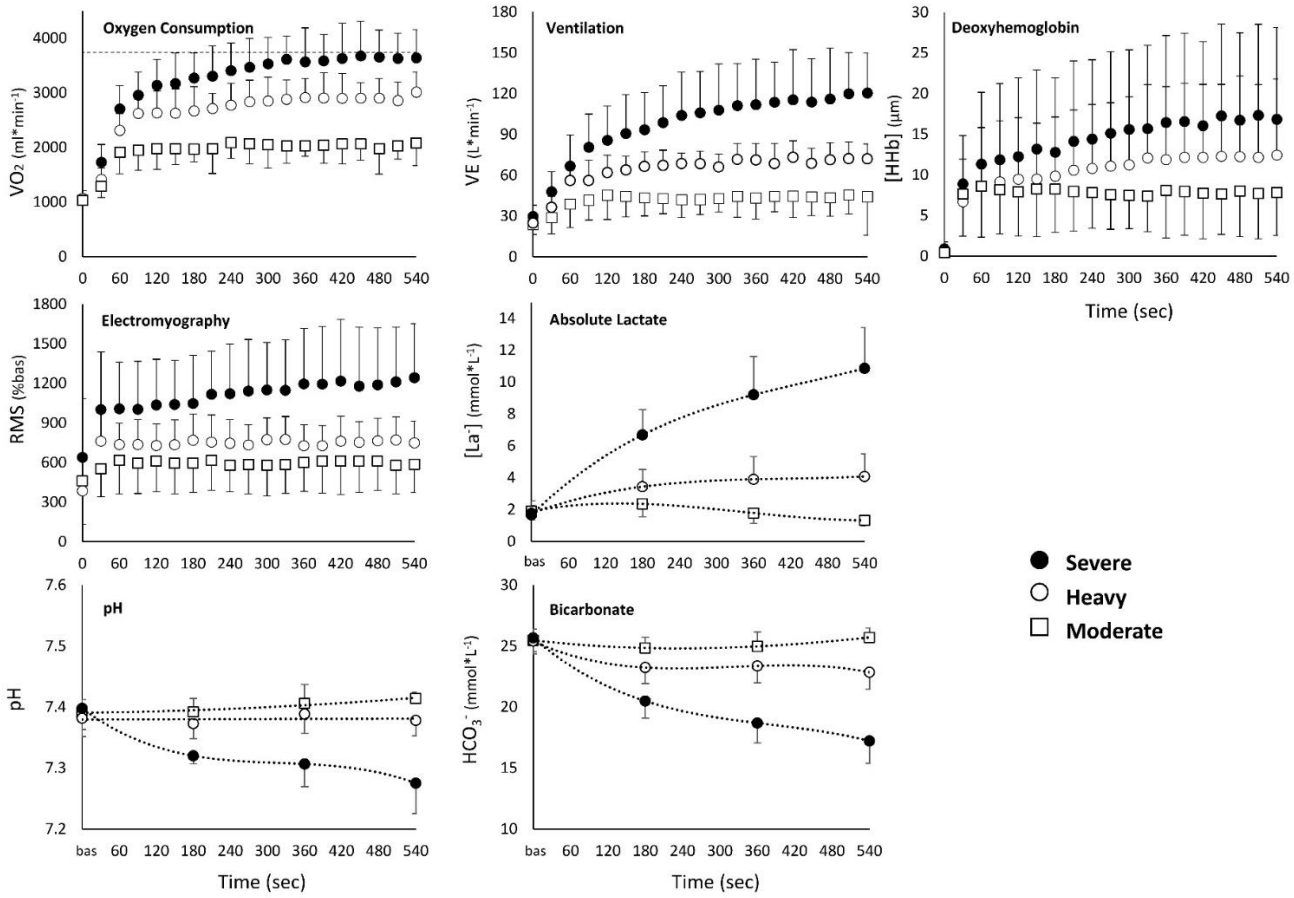

Figure 1, the physiological responses during cycling in different exercise domains are presented in 30 sec means ±SD. Symbols represent: white square: moderate exercise domain, white circle: heavy exercise domain, black circle: severe exercise domain.

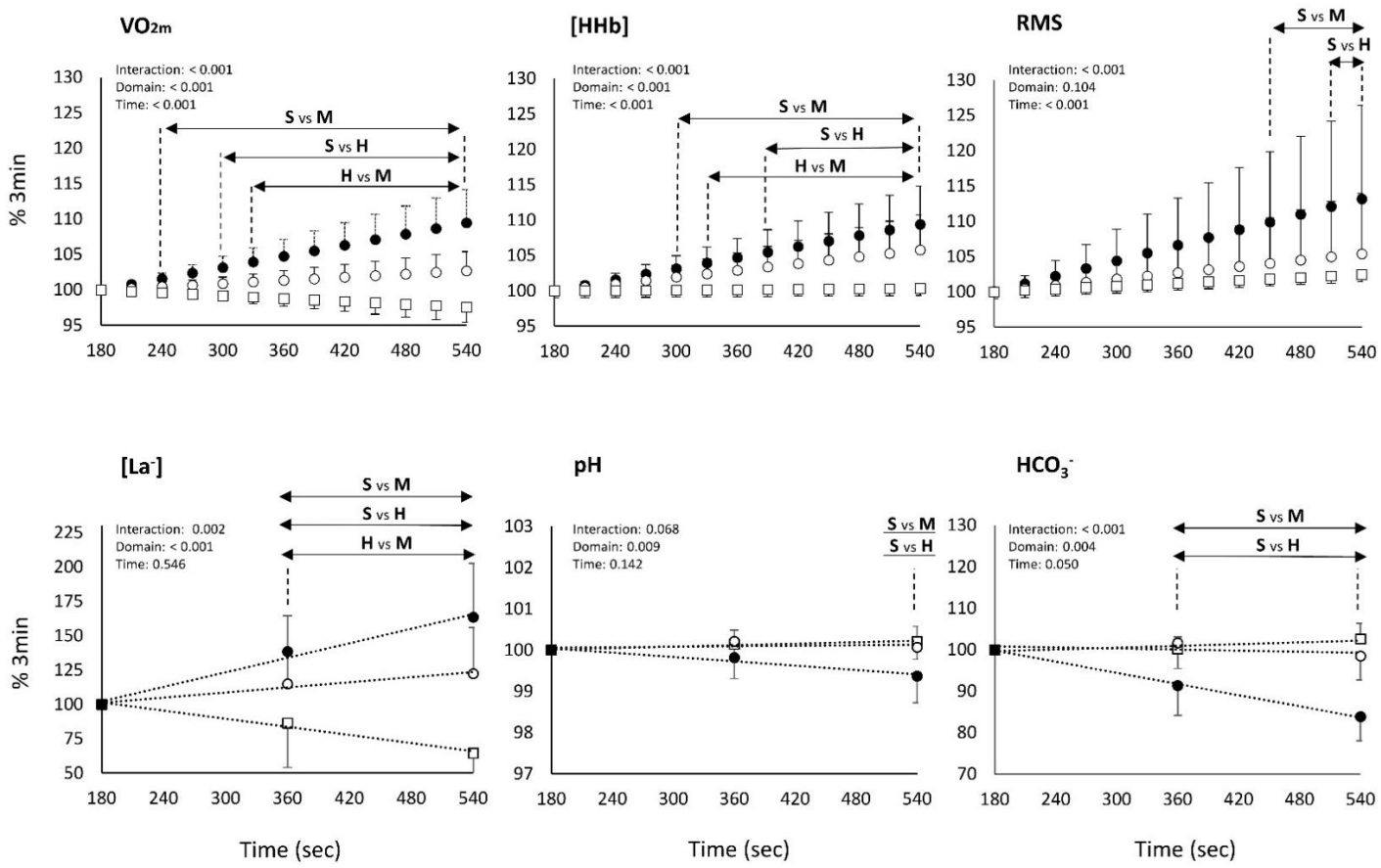

Figure 2, the dynamic changes of VO_2, [HHb], and RMS after the third minute of exercise are represented in the top panels (as the % increase after the value reached the third minute of exercise). In the bottom panels the changes of the hematic values of metabolic stability are displayed. Symbols represent: white square: moderate exercise domain, white circle: heavy exercise domain, black circle: severe exercise domain. Main effects resulting from ANOVA are represented in each panel, and the statistical differences resulting from the posthoc analysis are expressed by the letters (S vs M: severe vs moderate; S vs H: severe vs heavy; H vs M: heavy vs moderate)

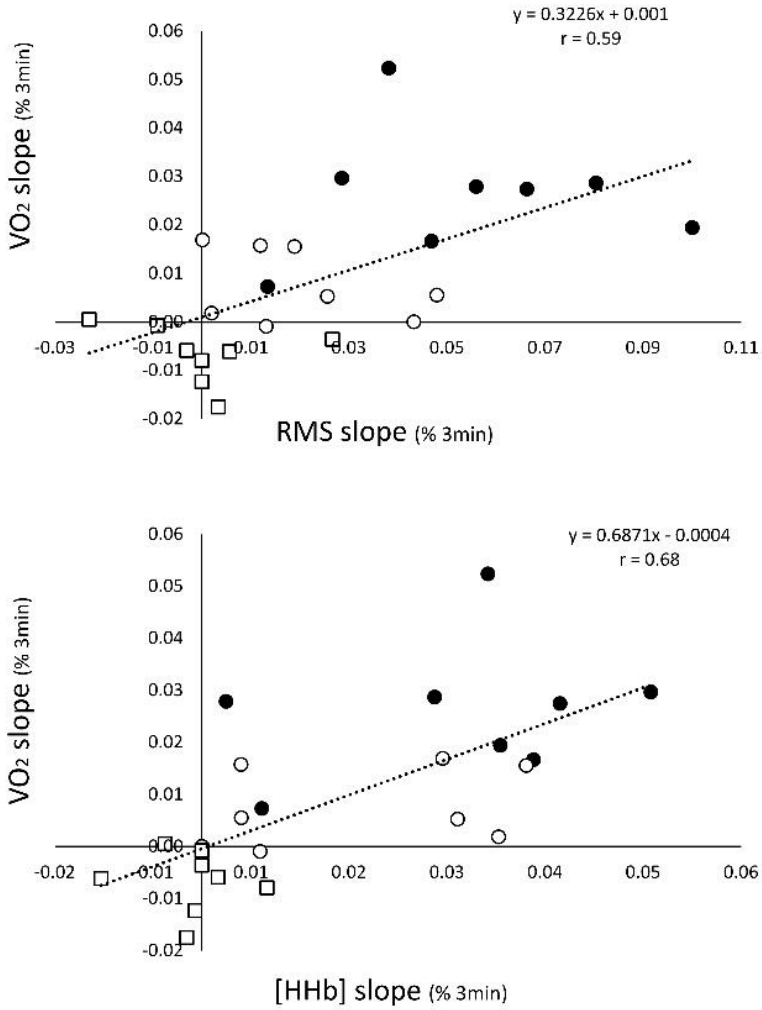

Figure 3, the correlations between the increases in RMS and [HHb] with VO_{2m} are presented. Symbols represent: white square: moderate exercise domain, white circle: heavy exercise domain, black circle: severe exercise domain.

Discussion:

By implementing measures of peripheral metabolism and muscle excitation, this study investigated the contribution of metabolic instability (i.e. [HHb]) and increased muscle excitation (i.e. RMS) to the VO_{2sc} in the moderate, heavy and severe exercise intensity domains. According to our hypothesis, our results showed that after the third min of exercise: i) in the moderate domain, neither VO_{2m}, nor [HHb] or RMS increased over time; in heavy, an increase in VO_{2sc} occurred, in association with an increase in [HHb] but not in RMS; in severe, VO_{2sc}, [HHb] and RMS were all increasing over time and the amplitude of these responses significantly differed from both moderate and heavy; ii) the increases in [HHb] and RMS were significantly correlated with the increase in VO_{2sc}. Overall, our findings are consistent with the hypothesis that the appearance of a loss of efficiency of locomotion over time may be caused by a combination of metabolic instability within the working fibres and the increased muscle excitation.

In 2015, Grassi et al. extensively reviewed the complex interplay of mechanisms underpinning the VO_{2sc} (Grassi et al. 2015), but until today no study has focused on directly testing the changes and interactions of these mechanisms in different intensity domains. Therefore, this investigation contributed to fill this gap.

As expected, our data confirm no further increases in VO_{2m} (-2.4±2.1% p=0.001), [HHb] (+0.3±2.7%, p=1.000), and RMS (+2.5±8.5%, p=1.000) after the third minute of exercise during moderate intensity transitions, indicating that a steady-state was achieved in all these variables within 180s.

In the heavy domain, a slow component of VO_{2m} (+2.7±2.7%, $p<0.001$) ensued after the third minute and was accompanied by increased [HHb] (+5.8±4.9%, $p<0.001$) but only a small, non-significant increase in RMS (+5.4±8.5%, p=0.110). In this domain, the VO_{2sc} is typically described as a loss of efficiency over time; however, it was recently proposed that VO_{2sc} may, in fact, represent a delayed "metabolic shift" between anaerobic and aerobic sources for ATP resynthesis that occurs in coincidence with an increased cost of locomotion in the heavy domain of exercise (i.e. loss of

efficiency vs moderate but not developing over time) (Study 3). Indeed, performing exercise in the heavy exercise domain entails the recruitment of bigger, intrinsically more glycolytic and less efficient moto units (Poole and Jones 2012); this, in turn, may lead to slower VO_2 kinetics (Barstow et al. 1996) and larger VO_2 gain (i.e. loss of efficiency of locomotion) (Jones et al. 2011) even without the recruitment of new muscle fibres over time. A possible physiological explanation for this phenomenon was proposed in a recent paper by Korzeniewski and Zoladz (Korzeniewski and Zoladz 2015). The authors suggested that a larger contribution of glycolysis to ATP resynthesis (as during exercise above GET due to the recruitment of higher order muscle fibres) would slow the on-set VO_2 kinetics and justify the appearance of a delayed steady-state or VO_{2sc}. This possibility was also corroborated by a study showing an increase in the amplitude of the primary component of VO_2, and in turn a reduction of the amplitude of the VO_{2sc}, when heavy exercise was performed in a condition of glycogen depletion (Carter et al. 2004). The possibility that the VO_{2sc} may in fact represent a delayed adjustment of VO_2 seems therefore possible, even if further studies will need to confirm this hypothesis. In support of this view, our data indicate increased levels of [HHb], compatible with increased metabolic instability within the working muscle or increased O_2 cost, without augmented muscle excitation (i.e. EMG) over time (figure 1 and 2). Moreover, [HHb] showed a slow component until the ~6^{th} min of exercise, reaching a steady-state thereafter (figure 1). It is reasonable to speculate that if a loss of efficiency over time would have occurred within the working motor units, its effects would have been protracted until the end of exercise. In fact, the progressive accumulation of metabolites (P_i, IMP, ADP, H^+, K^+) (Grassi et al. 2015) would have probably led to a decreased efficiency/fatigue of the active fibres, to the recruitment new fibres, and further moving away from homeostasis.

Finally, in the severe domain, VO_{2m}, [HHb] and RMS continuously increased over time of ~9-13% after the third minute of exercise (figure 2). This is an expected finding for exercise performed at an intensity in which a metabolic steady-state is not achievable, an increasing level of metabolic perturbation occurs and the recruitment of higher-order

motor units is necessary to protract exercise (Poole and Jones 2012). In fact, the aerobic energetic system alone is unable to sustain the required levels of ATP resynthesis and increased dependence on anaerobic metabolism is needed. This leads to release of H^+ and other metabolites (Grassi et al. 2015) that impair muscle contraction and require the further contributions of larger, less efficient motor units to sustain exercise.

A different development of metabolic instability over time in the three domains was also confirmed by the hematic markers of metabolic acidosis (figure 1 and 2). As expected, increasingly larger amounts of [La^-] accumulated in the initial 3 min of exercise (early lactate) in the three domains, along with increasing reductions of blood pH and bicarbonate concentration. Thereafter, a steady-state of these variables was maintained in the 3 to 9 min time-window for both the moderate and heavy exercise domains. In the severe domain only, [La^-] significantly increased (+63.4±39.1%, $p<0.001$), and both pH (-0.6±0.6%, $p<0.001$) and HCO_3^- -16.1±5.8%, $p<0.001$) decreased over time, indicating a mismatch between blood lactate production and removal and an increasing metabolic acidosis. While changes in [La^-], and acidosis between domains were recently shown by others (Vanhatalo et al. 2016; Black et al. 2017), this is the first study that concurrently performed non-invasive and time-resolved measures of VO_{2m}, local muscle metabolism, muscle excitation and blood markers of metabolic instability.

The final aim of this investigation was to gain insight into the relative contribution of the two main putative physiological underpinnings of the VO_{2sc}, metabolic instability and increased muscle recruitment. Our data indicate that [HHb] and RMS, both individually (figure 3) and combined in a multiple linear equation, had a significant impact on the prediction of the slope of the slow component. Moreover, the multiple linear equation indicated that the contribution to the VO_{2sc} was ~2.7 times larger for [HHb] than RMS, even if this quantitative indication should be taken carefully due to the different signal-to-noise ratio of the variables (higher for EMG) and to the presence of a slow component of RMS only in the severe domain (figure 1 and 2). To our knowledge, only two other studies provided data of simultaneous measurement of

metabolic instability and muscle excitation during constant work rate exercises in different intensity domains (Keir et al. 2016; Black et al. 2017). In one of these studies, Keir et al. (Keir et al. 2016) found that the development of the VO_{2sc} in the severe domain was accompanied by peripheral muscle fatigue (i.e. reduced maximal isometric contraction), without increases in the EMG signal, concluding that metabolic instability was the only probable cause of the VO_{2sc}. However, the lack of a slow component in the EMG response compared to our results may be explained by the different exercise intensities that were used in our studies. In Keir et al. 2016 the average VO_2 reached at the 9^{th} minute into exercise corresponded to ~+5% of the VO_2 at RCP, i.e. in close proximity with the heavy-severe boundary (Thomas et al. 2016), while in our study it represented ~ +17% of RCP. Pertinently with this speculation, recent findings showed that the muscle excitation might change according to the relative intensity *within* the severe domain, with larger activation occurring at the higher intensities (Iannetta et al. 2019b). The relatively small dynamics of the VO_{2sc} reported in Keir's study is also compatible with an intensity only slightly above the maximal lactate steady-state (Iannetta et al. 2018) or the critical power (Billat et al. 1995; Carter et al. 2002; Sawyer et al. 2012). The only other investigation that examined contemporarily neuromuscular and metabolic changes over prolonged exercise sessions reported a clear difference in the neuromuscular response during exercise in the severe vs heavy and moderate domains: fatigue/metabolic instability develops intensively in the severe domain (due to a large metabolic imbalance) and extensively in the heavy and moderate domains (due to substrates depletion and accumulation of fatigue-related metabolites) (Black et al. 2017). While their study is not easily comparable with ours due to difference in the design, Black's data support a significant accumulating metabolic imbalance and muscle excitation in the initial 10 min of exercise only for the severe intensity domain.

In conclusion, this investigation tested the "instability-recruitment" theory proposed by Grassi et al. in 2015 (Grassi et al. 2015) to explain the origins of the VO_{2sc}. In the three exercise intensity domains, our study demonstrated a domain-dependent dynamic over time for VO_2, [HHb] (taken as an index of metabolic instability), RMS (taken as an

index of muscle excitation) and the whole-body hematic markers of metabolic instability. About 75% of the variability of the VO_{2sc} was explained by a combination of the dynamics of [HHb] and RMS.

CHAPTER

7

General Discussion

The main purpose of this thesis was to investigate the physiological underpinnings of the so-called oxygen consumption (VO_2) "slow component" (VO_{2sc}). In particular, the study focused on the two main putative mechanisms, i.e. metabolic instability and muscle recruitment, behind the loss of efficiency of human locomotion at intensities above the gas exchange threshold (GET). The first part of the thesis, composed by two studies, tested the hypothesis that acute non-metabolic fatiguing interventions, by reducing the maximal force of the lower limbs and thus possibly increasing the muscle excitation for a given work rate, would increase the VO_{2sc}. In the second part of the thesis, the physiological response to exercise was investigated across the different exercise intensity domains by i) evaluating the bioenergetic contributors to exercise and by ii) applying non-invasive measures of muscle metabolism and activation in order to test the contribution of metabolic instability and muscle recruitment to the loss of efficiency. This section will briefly summarise the findings of these studies.

Muscle recruitment contribution to the VO_{2sc}:

Typically, two main mechanisms are considered responsible for the rise of the VO_{2sc}: the recruitment of less efficient, bigger muscles fibres; or the loss of metabolic stability within the already working fibres, described as a loss of the capacity to produce a specific mechanical output for a given energetic input (i.e. increased O_2 cost of ATP resynthesis and/or increased ATP cost of contraction (Jones et al. 2011; Vanhatalo et al. 2011; Grassi et al. 2015)). While a more recent theory considers these two phenomena interdependent (Grassi et al. 2015), it is still not clear if and to what extent one of these two may prevail and/or occur first. One of the main difficulties in this sense is the difficulty to selectively affect either metabolic stability or fibres recruitment in human models. The different manipulations used in interventional studies (e.g. speed of movement, intensity modulation, aerobic training, priming exercise, nutritional interventions) affect, to some extent, both metabolic stability and fibres recruitment.

Considering the possible role of increased muscular activation (necessary to maintain a given absolute work rate) as the explanatory theory of the VO_{2sc}, in study 1 and 2 it was observed that acutely affecting the maximal force can indeed modify the VO_2 response. In study 1, non-metabolic fatigue augmented the level of muscle excitation for a given absolute work rate during ramp incremental cycling, reduced the attainable level of maximum VO_2 (VO_{2max}), and reduced the absolute work rate corresponding to GET and the respiratory compensation point (RCP). Moreover, acute fatigue was associated with a higher VO_2 at an identical submaximal work rate, suggesting a possible link between loss of efficiency with the observed decreased ability to produce force and the augmented muscle activation. In study 2, non-metabolic fatigue during severe constant workrate cycling led to a similar physiological response. The reduction in maximal force increased the level of muscle excitation, induced early achievement of VO_{2max}, and impaired performance. These results suggest an influence of the increased muscle excitation on the kinetics of the VO_{2sc}.

While limitations of these studies should be taken into accounts, such as the indirect, non-invasive nature of our measures and the confunding effect of a small yet possible metabolic perturbation induced by the fatiguing interventions, these results seem to confirm a key role of muscle recruitment in the origin of the $VO_{2sc.}$ From a practical standpoint, these studies suggest adopting strategies to delay the involvement of bigger muscle fibres during aerobic exercise in order to improve exercise tolerance.

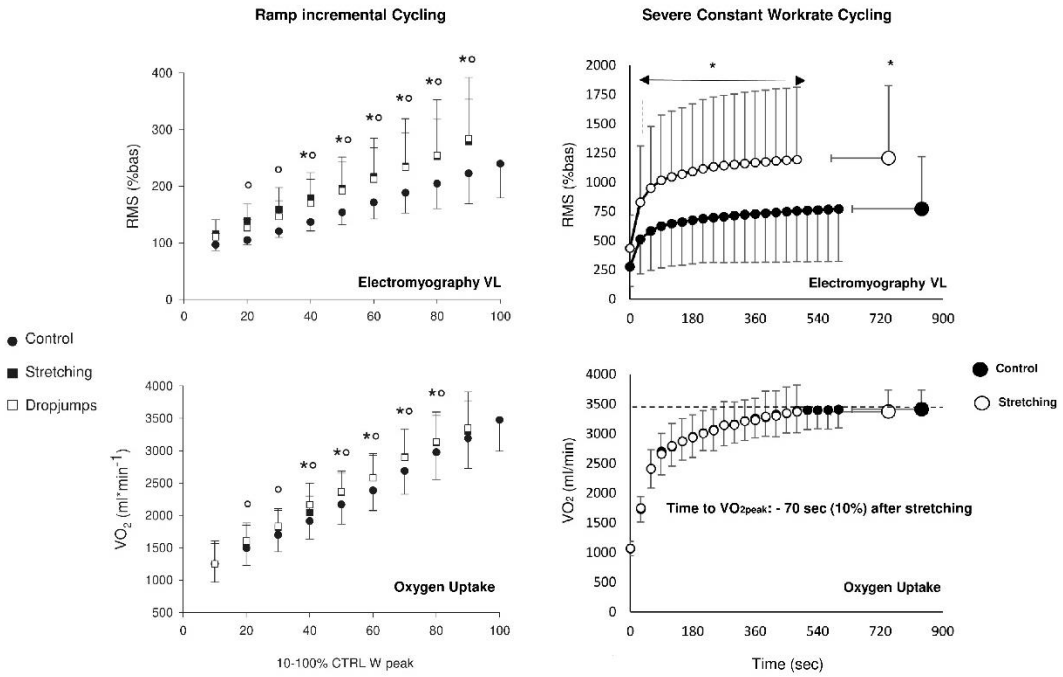

Figure 1 (readapted from study 1 & 2). left panels: : root mean square (top) and VO_2 (bottom) values are presented every 10-100% of the three ramp incremental tests performed in control condition or after the fatiguing interventions (stretching and dropjumps); right panels: root mean square (top) and VO_2 (bottom) during severe cycling in control condition or after the fatiguing intervention (stretching).

Bioenergetics of the VO_{2sc} in different exercise domains:

This study was inspired by the publication of an article from O'Connell et al. (O'Connell et al. 2017) who, contrary to the very existence of a VO_{2sc}, proposed that the energy demand of a constant high-intensity exercise does in fact not change over time. These authors used an innovative approach to investigate the VO_{2sc} by subtracting the VO_2 cost of ventilation and accounting for the contribution of the glycolytic energy sources in order to quantify the total metabolic cost of exercise (di Prampero 1986; di

Prampero et al. 1999; O'Connell et al. 2017). Using the same approach, we quantified the energetic contributors to locomotion in the different exercise intensity domains, finding that every exercise domain is characterised by a different bioenergetic response. In specific, we found that: in moderate the cost of locomotion does not increase over time during metabolic transitions; in the heavy intensity domain, the emergence of a VO_{2sc}, is explained by a "metabolic shift" between aerobic and anaerobic metabolisms protracted beyond the 3rd min of exercise (i.e. indicative of a "delayed adjustment of VO_2") and to a higher VO_2 cost of ventilation, rather than to an increased cost of locomotion over time; finally, the severe was the only exercise domain in which an actual loss of efficiency manifests over time. If confirmed by future studies, the main implications of these results would be that:

- The VO_{2sc} in heavy indeed represents a loss of efficiency of locomotion compared with exercise performed in moderate domain (i.e. higher O_2 cost * W); however, this increased cost of locomotion is present at the very onset of exercise rather than developing over time.
- In this domain, a steady-state in VO_2 is not reached within ~3 min but requires a more extended amount of time (i.e. delayed adjustment).
- The VO_{2sc} in heavy and in the severe domains of exercise may be explained by different physiological mechanisms and should not be studied interchangeably.

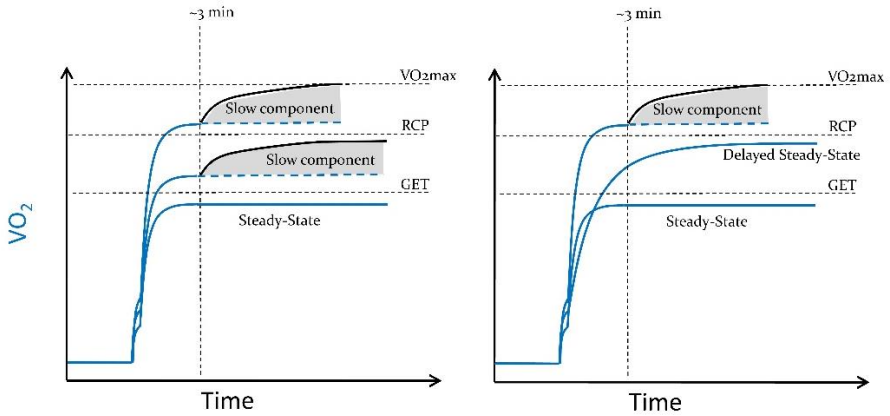

Figure 2 left panel: the classical model of VO_2 kinetics in response to different work-rate intensities; right panel: the new, hypothetical model with the delayed steady-state in the heavy domain.

Muscle metabolic instability *vs* muscle recruitment:

Study 4 was designed to test the "instability-recruitment" proposed by Grassi et al. in 2015 (Grassi et al. 2015), and the contribution of muscle metabolic instability and/or fibres recruitment to the rise of the VO_{2sc} between different intensity domains. To do this, we implemented:

- near-infrared spectroscopy, to gain insight of muscles oxygen extraction (by means of deoxyhaemoglobin, [HHb]), reflective of the imbalance between O_2 delivery/utilization, that in turn may be associated with intracellular metabolic instability (Grassi and Quaresima 2016).
- Electromyography, to indirectly estimate the activation of motor units during muscle contraction (quantified with the root mean square, RMS).
- Blood markers of whole-body metabolic instability (i.e. $[La^-]$, pH, HCO_3^-).

Results of this investigation demonstrated a domain-dependent dynamic over time for VO_2, [HHb], RMS, and the whole-body hematic markers of metabolic instability. About 75% of the variability of the VO_{2sc} was explained by a combination of the dynamics of [HHb] and RMS. These findings were consistent with the hypothesis that the appearance of a loss of efficiency of locomotion over time may be caused by a combination of metabolic instability within the working fibres and the increased muscle activity.

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Il dottorato è stato il mio modo di vivere le avventure che volevo quando ero piccolo. Può sembrare strano se si pensa alle ore trascorse in laboratorio o davanti ad un monitor, ma non è così difficile se si va un pochino oltre: ho potuto viaggiare, conoscere e incontrare. Ho imparato come cavarmela parlano altre lingue, come ambientarmi in nuovi posti e con nuove genti, ho imparato a pensare. Ho forse anche bevuto un po' troppa birra, ma meglio non scendere nei dettagli.

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"Many a law, many a commandment have I broken, but my word never" – Sir Walter Scott

"Vivere ardendo e non bruciarsi mai" – Gabriele D'Annunzio

"What's normal anyways?" – Forrest Gump

Ale