University of Kent | School of Sport and Exercise Sciences

GROSS EFFICIENCY IN CYCLING: THE EFFECT OF INTENSITY AND DURATION

This thesis is presented for the Degree of Doctor of Philosophy at the University of Kent

February 2021

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Dedication

To my incredible wife Rebecca, thank you for all your love and support through this journey. You are an incredible wife, mother and the love of my life. To my daughter Eileanòir, I wish you could be here to celebrate this success with me. Your brief life and tragic death have given me perspective, and you continually inspire me to engage fully with every moment so that I can be the best version of myself. To my beautiful children Aíbhílin and Aengus, your laughter, smiles and curiosity are a continual source of inspiration. Without you all I would not be the man or father I am today.

Acknowledgements

Sincere thanks to my supervisors Professor James Hopker and Professor Louis Passfield for their incredible support during my PhD. Your expert advice, guidance and support on this journey has been invaluable and greatly appreciated.

Thank you to my peers at the University for their support and help, in particular those who collaborated with me or assisted me on research papers within this PhD. Specifically, thank you to Ciaran O'Grady who I collaborated with on Chapters 5 and 6. I would also like to thank those who volunteered and gave up their time to participate in my research studies.

Finally, a big thank you to my family, friends and work colleagues for their support and encouragement.

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Ebreo, R., Passfield, L. and Hopker, J. (2020). The Reliability of Measuring Gross Efficiency During High-Intensity Cycling Exercise. *International Journal of Sports Physiology and Performance* **15**:126–132.

Conference proceedings

Chapter 3

R., Passfield, L. and Hopker, J. (2020). The Reliability of Measuring Gross Efficiency During High-Intensity Cycling Exercise. *ECSS Dublin, 2018*

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(Egan and Zierath 2013)(Powers and Jackson 2008)

Abbreviations

ANOVA	Analysis of variance
AR5	5-min active recovery
ATP	Adenosine triphosphate
ATT	Adipose tissue thickness
BGE	Back-extrapolated gross efficiency
bpm	Beats per minute
ci-miRNA	Circulating micro ribonucleic acid
CGE	Conventional gross efficiency
CI	Confidence interval
CONT	Constant 40-minute iso-effort interval
CV	Coefficient of variance
CWR	Constant work rate
ΔCt	Threshold cycle change
ΔG	Free energy
EPOC	Excess post-exercise oxygen consumption
FAD	Flavin adenine dinucleotide
GE	Gross efficiency
HHb	Deoxygenated haemoglobin
HIE	High-intensity exercise
HIF-1α	Hypoxia inducible factor alpha
Hr	Hour

INT1	Interval 1
INT2	Interval 2
INT3	Interval 3
INT4	Interval 4
J	Joules
kg	Kilograms
kJ	Kilojoules
LCIE	Low continuous intensity exercise
LCIE_SI	Low continuous intensity exercise with sprint intervals
$L \cdot min^{-1}$	Litres per minute
LI	4 x 5min iso-effort interval
MAP	Maximal aerobic power
miRNA	Micro ribonucleic acid
$mL \cdot kg\text{-}^{1} \cdot min^{-1}$	Millitres of O2, per kilogram of body mass, per minute
$mmol \cdot L^{-1}$	Milimoles per litre
Mb	Myoglobin
NAD	Nicotinamide adenine dinucleotide
NIRS	Near-infrared spectroscopy
P	Significance level
P1	HIE bout at $80\%_{MAP}$
P2	HIE bout at $100\%_{MAP}$
Pcr	Phosphocreatine

PI	Power input
PO	Power output
P/O	Ratio of phosphate incorporated into ATP to oxygen atoms
Pre150	Gross efficiency measured pre-protocol
Post-150	Gross efficiency measured post-protocol
r	Correlation coefficient
RER	Respiratory exchange ratio
ROS	Reactive Oxygen Species
RPE	Rate of perceived exertion
s	Seconds
SD	Standard deviation
SI	Two sets of 10 x 30s interval
SID	Smallest important difference
SIE	Sprint intensity exercise
SIT	Sprint interval training
SOD	Superoxide Dismutase
TSI	Tissue saturation index
UCP3	Uncoupling protein 3
$\dot{V}$CO2	Volume of carbon dioxide
$\dot{V}$E	Minute ventilation
VEGF	Vascular endothelium growth factor
$\dot{V}$O2	Volume of oxygen

$\dot{V}O2_{max}$	Maximum rate of oxygen consumption
W	Watts

1 Introduction

Our understanding of the beneficial adaptations to the physiological stress of exercise has greatly improved over the last four decades (Holloszy and Coyle 1984; Coyle *et al.* 1988; Joyner and Coyle 2008; Perry *et al.* 2010; Serpiello *et al.* 2012; MacInnis and Gibala 2016; McGinley and Bishop 2016). A framework model by Joyner and Coyle (2008) suggests that endurance performance success is dependent on three key variables (see figure 1.1): maximal oxygen uptake ($\dot{V}O_{2max}$), gross efficiency (GE) and lactate threshold (LT) (Joyner and Coyle 2008)

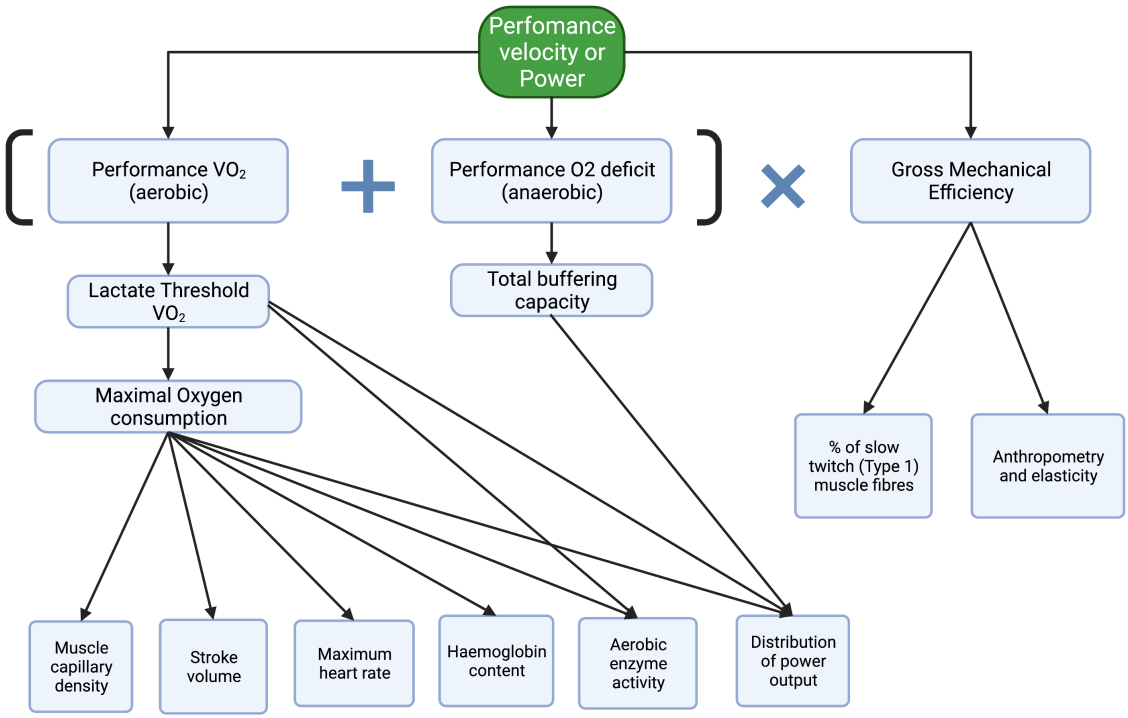

Figure 1.1 *Joyner and Coyle's (2008) model explaining the physiological determinants of endurance performance. Taken from* (Joyner and Coyle 2008)

GE can be defined as the ratio of mechanical power output to the metabolic energy cost expressed as a percentage (Ettema and Lorås 2009) with changes in GE correlating with

changes in cycling performance (Passfield and Doust 2000; Joyner and Coyle 2008; Hopker *et al.* 2009; Jobson, Hopker and Korff 2012; Hopker, O'Grady and Pageaux 2016). Therefore, improvements in GE can either be made by increasing the mechanical power output of an athlete or by overcoming external frictional forces.

GE = PO / PI x100 *Equation 1.1*

Increases in GE could potentially afford a performance advantage. Horowitz, Sidossis and Coyle (1994) demonstrated that a 1.5% increase in GE equated to a significantly higher power output (27W) during a 1-hour cycle time-trial performance. Similarly, using a mathematical modelling approach it is estimated that a 1% increase in GE would result in a 63 second improvement in 40km time-trial time (Jeukendrup, Craig and Hawley 2000).

Until recently, the calculation of GE has assumed that GE determined during exercise tests involving submaximal intensities is representative of GE during both maximal and supramaximal intensities (Koning *et al.* 2013). However, during high-intensity exercise (HIE) with a significant anaerobic energy contribution, $\dot{V}O_2$ measured at the mouth cannot be used to estimate the total energy expenditure. Therefore, conventional measurement of GE (CGE) during submaximal exercise may not be a valid estimate of GE during HIE (Stainbsy *et al.* 1980). A novel approach to estimate GE during HIE has recently been proposed (Koning *et al.* 2013). This back-extrapolation method (BGE) uses linear regression of post-HIE GE values and back extrapolates these values to estimate GE at the end of the HIE-bout (Koning *et al.* 2013) However, the reliability of this method during acute cycling exercise of varying intensity or duration has not been elucidated.

Road cycling races are composed of a variety of disciplines such as criteriums, individual or team time trials and longer stage races with varying degrees of both intensity and duration employed both within and between disciplines (Ebert *et al.* 2006). Previous research investigating changes in CGE have focused on steady-state efforts such as time trials (Mulder *et al.* 2015a; Noordhof *et al.* 2015a; Groot *et al.* 2018). However, stage races and criteriums are typically longer than one hour and employ a series of repeated high-intensity efforts above the lactate threshold interspersed with lower or moderate intensity exercise to be

successful (Sanders *et al.* 2016). Prolonged moderate intensity cycling (greater than 2 hours) has been shown to both increase $\dot{V}O_2$ and reduce GE with a subsequent reduction in 5-min all out performance and 30s peak power (Passfield and Doust 2000). A more recent study has found that GE decreases after prolonged low-intensity exercise (4 hours) interspersed with 3 x 30s sprints (Almquist *et al.* 2019). However, the sprints occur at the end of each hour (with no sprints in the last hour) and are calculated using CGE after a period of 4 min (1 min passive rest, 3 min cycling at 100W). The authors concluded that GE decreased as a function of time rather than due to the effects of repeated maximal sprints. GE is likely to be over-estimated using conventionally calculated GE as the true metabolic consequences of repeated sprint exercise are potentially masked by recovery processes. Furthermore, the study design does not mimic a typical road racing format. Therefore, it is unknown how GE is impacted by mixed intensity prolonged cycling that better replicates the physiological demands of a typical cycling road race (Moholdt *et al.* 2011; Weston, Wisløff and Coombes 2014)

CGE has been shown to be higher in trained compared to untrained cyclists (Hopker, Coleman and Wiles 2007). Hopker, Coleman and Passfield (2009) demonstrated that over the course of a competitive season, trained cyclists can increase their GE by as much as 5% with increases correlating with the intensity and duration of training completed. However, it is unclear what acute intensity or duration of exercise incurs the greatest changes in GE or the mechanisms that influence this change.

High-intensity and sprint-intensity exercise (SIE) have been shown to be a potent stimulus for beneficial physiological adaptation (Jacobs *et al.* 2013; Christensen *et al.* 2016) and endurance performance (Burgomaster 2005; Gibala *et al.* 2006; Burgomaster *et al.* 2008; Gibala *et al.* 2012; MacInnis and Gibala 2016). However, the physiological or epigenetic factors that might be influential in the reduction of GE seen following both prolonged submaximal (Hagberg, Mullin and Nagle 1978; Coyle, Sidossis and Horowitz 1992; Hagan, Weis and Raven 1992; Hopker, O'Grady and Pageaux 2016) and high intensity (Noordhof *et al.* 2015b; Vanhatalo *et al.* 2011) bouts of exercise have yet to be investigated.

Various training prescriptions have been shown to increase markers of endurance performance such as $\dot{V}O2_{max}$, lactate threshold and efficiency via the manipulation of the duration, frequency and intensity. However, whilst extensive research been conducted

investigating the efficacy of different training prescriptions (Seiler and Tønnessen 2009; Smith 2003) and the molecular responses that regulate adaptation (Stepto *et al.* 1999) the resultant impact on GE has not been investigated.

The mechanisms underlying reductions in GE after acute exercise remain inconclusive. Decreases in GE have been observed after acute moderate and high-intensity exercise (Passfield and Doust 2000; Bangsbo *et al.* 2001; Krustrup *et al.* 2003; Koning *et al.* 2013). These reductions in efficiency could be caused by changes in muscle-fibre recruitment (Passfield and Doust 2000; Burnley, Doust and Jones 2002; Krustrup *et al.* 2003). Previous research has indicated that during a 3-min all out test, a decrease in efficiency is not caused by the progressive recruitment of muscle fibres (Vanhatalo *et al.* 2011). However, in a study by Noordhof et al. (2015) the authors found that during longer high-intensity sustained power output time-trials there was small increase in both the $\dot{V}O2$ and integrated electromyography (EMG) over the gluteus maximus, vastus medialis, vastus lateralis, and biceps femoris. Whilst, the mean EMG amplitude was not statistically significantly different between time-trial distances there was a significant main effect of time. This suggests that there are different mechanisms underlying GE decreases after sustained intensity exercise or all-out exercise.

During prolonged (>60 min) fixed intensity exercise previous studies have found increases in the oxygen cost of exercise ($\dot{V}O2$ slow component) with simultaneous decreases in GE (Passfield and Doust 2000; Hopker, O'Grady and Pageaux 2016; Almquist *et al.* 2019) which cannot be accounted for by increases in ventilation, lipid or lactate metabolism or body temperature (Hagberg, Mullin and Nagle 1978; Hagan, Weis and Raven 1992). Hopker, O'Grady and Pageaux (2016) speculate that this decrease in GE after prolonged moderate intensity cycling exercise could be due to decreases in the efficiency of oxidative phosphorylation. Specifically, the back leak of protons across the mitochondrial inner membrane without driving ATP-synthase would decrease the ratio between ADP phosphorylation and oxygen consumption (P/O ratio) in a process called mitochondrial uncoupling (Mogensen *et al.* 2006; Brand *et al.* 2005).

Uncoupling proteins (UCP) have been shown to mediate the increase in mitochondrial membrane permeability. Jiang *et al.* (2009) demonstrated that during a prolonged bout of

exercise ROS production increased progressively with increased duration but decreased after 150 min when UCP3 increased to its highest level. The authors suggest that mitochondrial uncoupling driven by UCP3 during prolonged exercise may reduce ROS production and thus protect mitochondria from oxidative stress (Jiang *et al.* 2009). This increase in uncoupling could also be utilized to reduce the cellular oxidative damage caused by high levels of reactive oxygen species (ROS) (Sahlin *et al.* 2010) during both aerobic and anaerobic exercise (Mastaloudis, Leonard and Traber 2001; Radak *et al.* 2013) with subsequent decreases in GE after acute exercise.

The production of ROS after both aerobic and anaerobic exercise has also been shown to be an important factor for muscle fibre adaptation (Gomez-Cabrera *et al.* 2005; Gomez-Cabrera, Domenech and Viña 2008) and muscle force production (Radak *et al.* 2008; Powers and Jackson 2008). Intermittent exercise provides a metabolic and oxidative challenge, which after a rest period, allows for the upregulation of antioxidant enzymes (Radak *et al.* 2001; Gomez-Cabrera, Domenech and Viña 2008) and may play a part in increases in GE seen after training (Hopker, Coleman and Wiles 2007; Hopker, Coleman and Passfield 2009).

Changes in GE appear to be driven by progressive reductions in mitochondrial/contractile efficiency of the working muscle (Hopker, O'Grady and Pageaux 2016). Endurance exercise training elicits cellular stress signals, initiating a biochemical cascade leading to both whole body and skeletal muscle adaptation. Progressive and repeated activation and/or repression of specific cell signalling pathways in response to physiological stress of exercise regulate the transcription and translation of mRNAs that facilitate the synthesis of their respective proteins, enzymes and genes (see figure 1.2) that have been linked to beneficial exercise adaptation through structural remodelling and functional adjustments (Pilegaard, Saltin and Neufer 2003; Perry *et al.* 2010; Hoppeler *et al.* 2011). These beneficial adaptations include muscle growth, differentiation and regeneration, enhanced substrate metabolism (Hawley, Hargreaves and Zierath 2006), mitochondrial biogenesis (Egan *et al.* 2013) and angiogenesis (Russell *et al.* 2003; Mogensen *et al.* 2006). The efficiency of an isolated muscle can be seen as the mathematical product of metabolic efficiency (phosphorylative coupling – ATP synthesis / energy liberation from the decomposition) and mechanical efficiency (mechanical coupling – contractile exercise / energy liberation during ATP-splitting) (Whipp and Wasserman 1969; Gaesser and Brooks 1975).

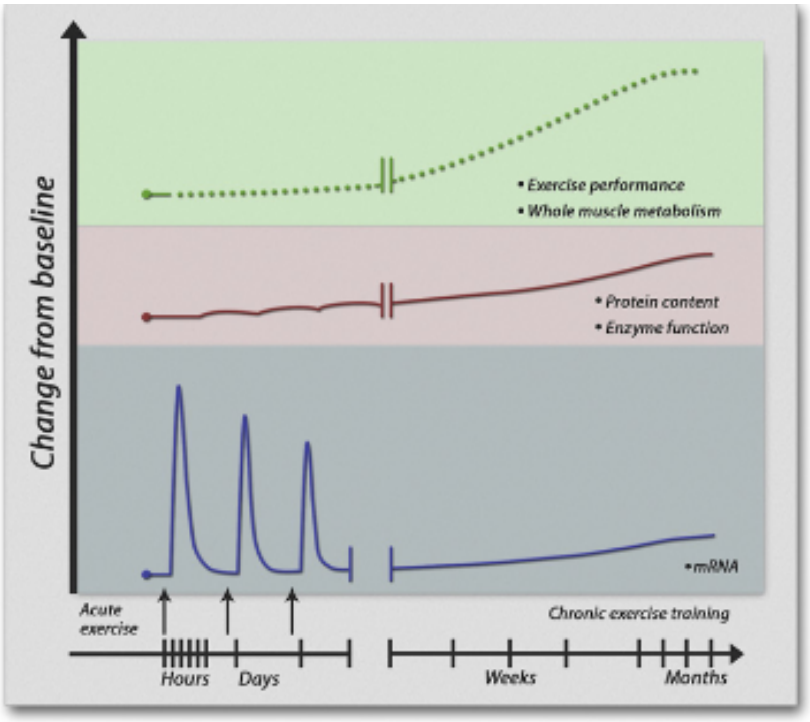

Figure 1.2 *Molecular basis of adaptation to Exercise. Taken from Exercise Metabolism and the Molecular Regulation of Skeletal Muscle Adaptation* (Egan and Zierath 2013)

Traditionally, the measurements of skeletal muscle mRNAs, proteins and mitochondria involve invasive muscle biopsies. Recently it has been found that small non-coding circulating microRNAs (ci-miRNAs) regulate mRNAs and the activation or repression of downstream protein targets (Bartel 2004; Sharma 2014). By characterizing the acute response of ci-miRNAs involved in the transcriptional regulation of skeletal muscle angiogenesis, mitochondrial and cardiovascular function (Poliseno *et al.* 2006; Thum *et al.* 2008; Liu *et al.* 2015; Liu *et al.* 2011; Zhang *et al.* 2012a; Chistiakov *et al.* 2015) to different exercise protocols, greater insights into the mechanisms that may affect gross efficiency can be elucidated. Furthermore, the ci-miRNA response to different exercise protocolss and their relationship to traditional physiological performance markers such as $\dot{V}O_{2max}$ and GE may reveal a viable molecular biomarker to both predict and individualise exercise training.

The goals of this thesis are to test the reliability of BGE, to investigate how higher intensities and longer duration of exercise affect GE and, explore the potential physiological and epigenetic mechanisms underlying these changes.

2 Literature review

2.1 The biochemical basis for metabolic energy production

For GE and the production of mechanical power output, the liberation and utilisation of energy is central to athletic performance. Adenosine Triphosphate (ATP) is a critical component of skeletal muscle contractility and its availability is essential for skeletal muscle activation across the spectrum of intensity and duration of exercise. Multiple metabolic pathways generate free energy within skeletal muscle by hydrolysing both adenosine triphosphate (ATP) and phosphocreatine (PCr) to adenosine diphosphate (ADP), inorganic phosphate (P) and free energy (see figure 2.1)

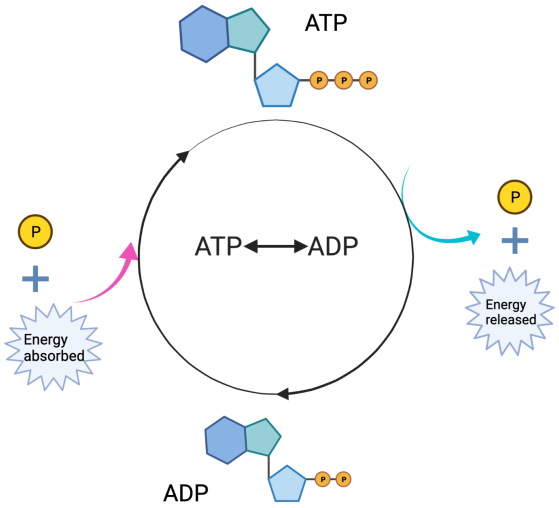

Figure 2.1 *Breakdown and synthesis of ATP (created with Biorender*)

For ATP to be regenerated, a molecule of ADP needs to be re-phosphorylated using energy from one of three different pathways 1) the hydrolysis of phosphocreatine (the phosphagen pathway) 2) anaerobic glycolysis (the glycolytic pathway) 3) the oxidation of glycogen /glucose and fatty acids (the oxidative pathway). These pathways supply immediate, short-term and long-term sources of energy respectively and their relative contribution is primarily dependent on the intensity and duration of exercise.

2.2 Anaerobic energy metabolism

Cycle races contain multiple maximal and supramaximal intensity sprints (Ebert *et al.* 2006). Sprints by their nature require an immediate supply of energy and are thus best served by the anaerobic phosphagen or glycolytic pathways depending on the duration of activity (Medbo and Tabata 1989). However, anaerobic energy production is also important at the start the submaximal exercise as the kinetics of aerobic energy production are relatively slow in comparison. Therefore, both anaerobic pathways are important prior to $\dot{V}O2$ reaching a steady state where external respiration meets the demands of muscle respiration.

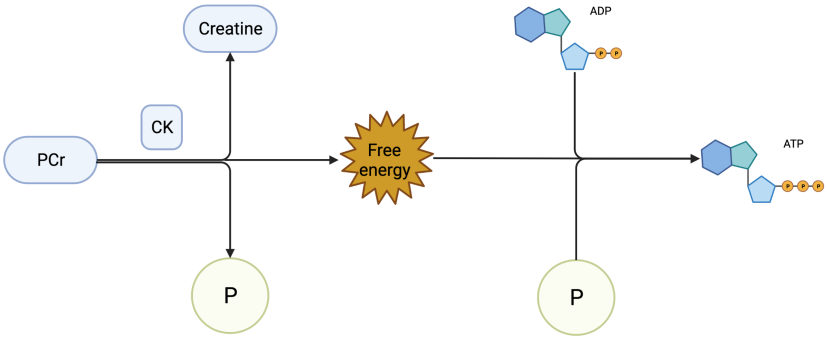

Figure 2.2 PCr hydrolysis and the production of ATP. PCr = Phosphocreatine; CK = creatine kinase; P = inorganic phosphate (created with Biorender)

PCr hydrolysis is the initial primary source of ATP during the first few seconds of maximal or supramaximal intensity exercise (McArdle, Katch and Katch 2009). PCr consists of creatine joined to a single phosphate group with a "high-energy bond". When PCr is hydrolysed, the free energy from this process is utilised to re-phosphorylate ADP to ATP (see figure 2.2). PCr hydrolysis is a highly efficient reaction catalysed by the enzyme creatine kinase with the maximum rate of ATP resynthesis being four to eight times higher than aerobic energy metabolism (McArdle, Katch and Katch 2009). This system provides an important energy buffer during explosive supramaximal efforts lasting around 2 seconds or less. Like ATP, PCr

can be re-phosphorylated using energy from the oxidation of glucose/glycogen and fatty acids in a reaction catalysed by creatine kinase (see figure 2.3) .

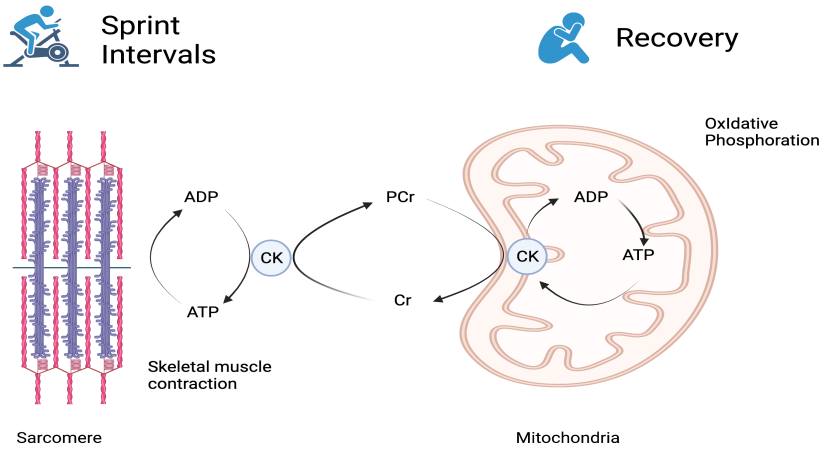

Figure 2.3 *The resynthesis of ATP from ADP catalysed by creatine kinase (CK) in the sarcoplasm and the rephosphorylation of phosphocreatine (PCr) via oxidation in the mitochondria (created with Biorender)*

Cycle road races typically have sprint intervals of greater than 10 seconds with multiple "attacks" of maximal or supramaximal intensity efforts followed by periods of submaximal cycling recovery (Ebert *et al.* 2006). However, the complete restoration of PCr from single sprint interval could take 3 – 5 minutes of rested recovery (MacDougall and Sale 2014). PCr recovery is impacted when sprint-intervals occur in rapid succession or are combined with sustained high-intensity cycling (MacDougall and Sale 2014). Alternatively, creatine kinase activity may be reduced secondary to an increase in hydrogen ions generated by anaerobic glycolysis (Meyer and Foley 2013). Therefore, the PCr re-phosphorylation "recovery" curve may be delayed and full PCr resynthesis would take longer to occur when exercise intensity remains moderate to high.

Beyond the initial few seconds of maximal or supramaximal exercise, glycolysis is an important source of anaerobic energy (Parolin *et al.* 1999). The glycolytic pathway (see figure 2.4) involves the stepwise breakdown of either muscle glycogen (glycogenolysis) or free glucose to lactate. This process requires 11 chemical reactions, with each reaction catalysed by a specific enzyme. Anaerobic glycolysis results in the formation of 2 pyruvate molecules

and a net gain of 2 ATP with the breakdown of glucose and a net gain of 3 ATP for the breakdown of glycogen.

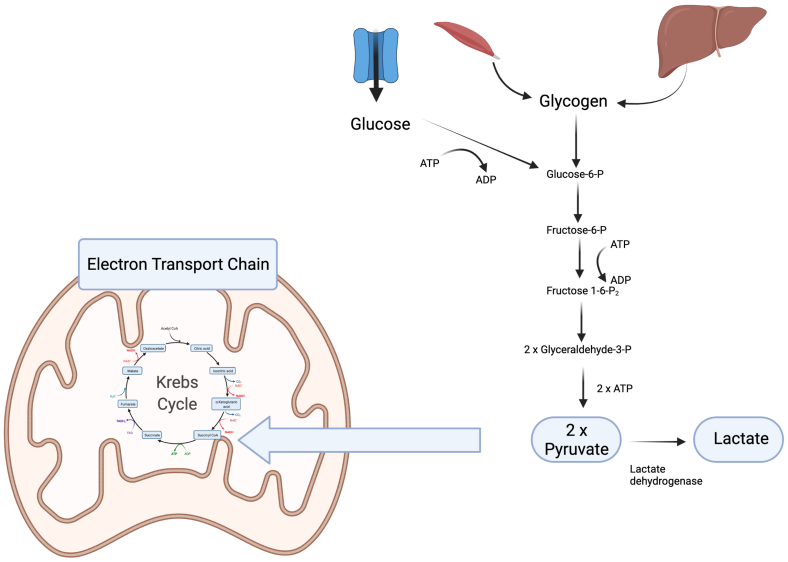

Figure 2.4 *Anaerobic glycolysis from the breakdown of glycogen and glucose (created with Biorender)*

The rate and activation of glycolysis are controlled by two allosteric, or "rate limiting" enzymes which allow ATP to increase or decrease in proportion to exercise intensity. Glycogen phosphorylase and phosphofructokinase determine the maximal rate of glycogenolysis and are stimulated by the appearance of ADP, adenine monophosphate (AMP), P_i and calcium ions (McGilvery and Goldstein 1983). NAD^+ needs to be constantly regenerated from NADH if anaerobic glycolysis is to keep up with metabolic energy demand.

As exercise intensity and subsequently ATP utilization increase, a greater proportion of pyruvate is converted to lactate by the enzyme lactate dehydrogenase. Lactate readily dissociates its hydrogen ions into the muscle intracellular fluid or the bloodstream. During repeated sprint-intensity intervals this may become a major limitation as there is not enough recovery time between sprints for lactate to be removed resulting in a steady elevation of hydrogen ions with each subsequent sprint interval. Phosphofructokinase is known to be inhibited or downregulated repeated short bursts of sprint-intensity exercise or when sprint-intensity exercise is prolonged due to the inhibitory effects of a reduction of pH caused by the production of hydrogen ions generated by lactic acid (Sahlin 1978; Spriet 2014).

The anaerobic pathways have a higher rate of ATP production, but a small net total ATP produced. The aerobic pathways (glucose and fatty acid oxidation) are better suited to supplying energy during prolonged endurance exercise as they have a lower rate but larger total net production of ATP.

2.3 Aerobic energy metabolism

Beyond ~1-2 minutes, aerobic energy metabolism becomes the predominant ATP producing system (MacDougall and Sale 2014). The major substrates of aerobic energy metabolism are derived from ingested carbohydrate absorbed from the gut, muscle glycogen, liver glycogenolysis and gluconeogenesis and fatty acids (intramuscular triglyceride and adipose triglyceride)(Hargreaves and Spriet 2020). The relative contribution of these substrates is both intensity and duration dependant (Romijn *et al.* 1993; Loon *et al.* 2001).

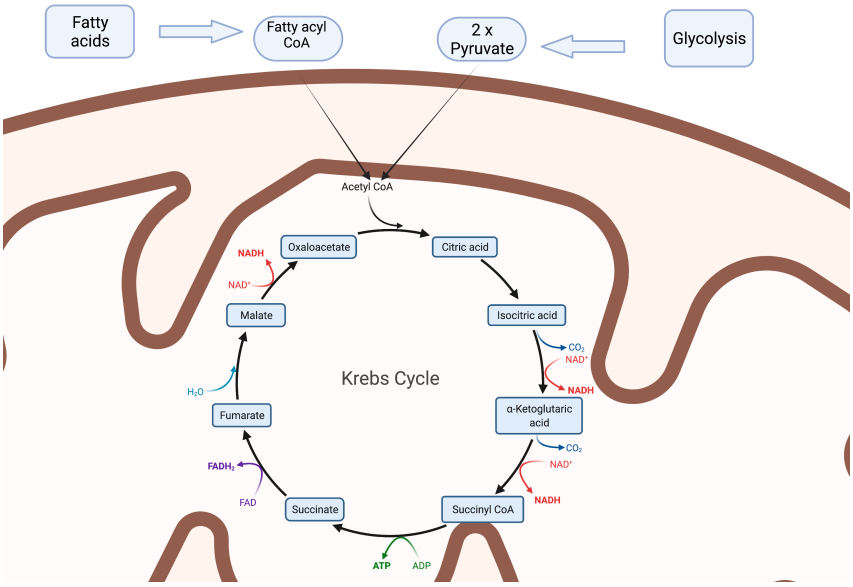

Figure 2.5 *Aerobic energy metabolism via the Krebs cycle in the mitochondrial matrix (created with Biorender)*

Both pyruvate and fatty acids are catalysed by pyruvate dehydrogenase into acetyl-CoA in the mitochondrial matrix where it enters the Krebs cycles for aerobic energy metabolism. As two molecules of acetyl-CoA are produced per molecule of glucose or glycogen, the Krebs cycle rotates twice per molecule of glucose or glycogen broken down. This gives rise to the following products at this point: 4 molecules of CO_2, 2 molecules of ATP and eight pairs of

hydrogen atoms. A further 2 molecules of ATP and 4 pairs of hydrogen atoms can be added to this total (two pairs from glycolysis and two pairs from the transformation of pyruvate to acetyl-CoA).

The hydrogen atoms are then transported to the inner membrane of the mitochondrion by the reduced carrier coenzymes nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD) to undergo oxidative phosphorylation in a stepwise process known as the electron transport chain (ETC). In a series of reactions, the electrons are separated from the hydrogen atoms. The electrons are alternately reduced and oxidised (redox reactions) as they are transferred through a series of carriers known as cytochromes. Three of these carriers span the mitochondrial membrane and act as proton pumps that create a proton gradient that releases free energy for phosphorylating ADP to ATP. In the final step, the protons and electrons involved in these redox reactions are accepted by oxygen to form water. Therefore, this pathway can only exist in the presence of oxygen and the maximal rate of ATP generation is dependent on the maximal rate of oxygen availability to the mitochondria. During mild- to moderate-intensity exercise, the Krebs cycle (or tricarboxylic acid (TCA) or citric acid cycle), ETC and oxidative phosphorylation will yield a net gain of 32 molecules of ATP per molecule of glucose.

2.4 Measuring metabolic energy production

2.4.1 Direct calorimetry

The understanding of cellular metabolism and the measurement of energy expenditure was greatly advanced in the late Nineteenth century with the introduction of the first direct calorimeter (Atwater and Rosa 1899). Direct calorimetry is based on the law of conservation of energy, which states that the total energy of an isolated system remains constant with the fuel (energy) consumed equivalent to the heat (energy) produced (Atwater and Rosa 1899).

Direct calorimetry requires a participant to live within a large, insulated chamber (i.e. a whole room calorimeter), which is surrounded by pipes with a known volume of water that absorb heat radiated from the participant. The airflow in and out of the chamber is tightly

regulated for both temperature and volume. All food, drink and outgoing of excreta are also monitored. (Atwater and Rosa 1899). Direct calorimeters are large and expensive and are not practical during activities such as cycling, where the rate of energy production is dynamically changing.

2.4.2 Indirect calorimetry

The development of indirect calorimeters can be traced back to the 1800's when Regnault and Reiset devised a system for measuring oxygen (O_2) consumption. The closed-circuit system demonstrated that the type of food ingested effected the ratio of carbon dioxide (CO_2) expelled and the O_2 consumed (Poncet and Dahlberg 2011). Subsequently it has been demonstrated that metabolism could be interpreted in relation to the the oxidation of protein, fat and carbohydrate (Kenny, Notley and Gagnon 2017) and that the respiratory analysis and estimate of heat production via indirect calorimetry consistently and closely agrees with direct calorimetry by ~1% (Medbo and Tabata 1989).

It should be acknowledged that despite this agreement, indirect calorimetry is based on the following assumptions (Porter and Cohen 1996; Mtaweh *et al.* 2018):

1. Any consumed fuel has an intrinsic content that upon metabolic modifications within a living organism will result in the production of heat or energy
2. The combustion of protein, fat and carbohydrate is the end result of all biochemical reactions within a living organism
3. The oxidation of protein, fat and carbohydrate results in a substrate specific fixed ratio between the amount of O_2 consumed and CO_2 expelled
4. Loss of substrates in faeces and urine is negligible

Gas exchange in this thesis was measured by indirect calorimetry using open circuit spirometry where the participant breathes from the atmosphere and expires into a separate outlet. The expired gases are then sampled continuously for analysis.

2.4.3 Measurement of GE

Energy expenditure by open circuit indirect calorimetry can be measured by using Douglas bags to collect expired gases or by online breath-by breath systems. Hopker et al. (2012) demonstrated that the total within-subject variation in GE using the Douglas bag method is 1.5%. Consequently, a change as small as 0.4% may be reliably detected using this technique. In comparison, previous studies using breath-by-breath gas analysers report a coefficient of variation (CV) of 4.2% during a graded exercise protocol with increments every 3 min (Noordhof *et al.* 2010; Moseley and Jeukendrup 2001) and a CV of 4.4% from 6 min stages at 45%, 55% and 65% of participants' power at $\dot{V}$O2max (Noordhof *et al.* 2010). Noordhof et al. 2010 conclude that an increase or decrease in GE of 0.6% can be reliably detected using online breath-by-breath gas analyser.

Efficiency

2.4.4 Calculation of gross efficiency

During exercise, the amount of aerobic and anaerobic metabolic energy utilised to produce a given amount of mechanical power output or speed is determined by whole body efficiency. The most common definition of whole-body efficiency is GE (equation 2.5) (Ettema and Lorås 2009) where the ratio of mechanical power output (PO) to metabolic energy cost or power input (PI) is expressed as a percentage (Schenau and Cavanagh 1990; Ettema and Lorås 2009).

$$GE = PO / PI \times 100 \qquad \text{Equation 2.1}$$

Metabolic PI can be calculated by multiplying steady state $\dot{V}O_{2max}$ (L.s-1) by the oxygen equivalent and respiratory exchange ratio (RER) (Garby and Astrup 1987) as shown in Equation 2.6:

$$PI = \dot{V}O_2 \times (4940 \times RER + 16040) \qquad \text{Equation 2.2}$$

As efficiency is potentially different for aerobic and anaerobic ATP resynthesis (Krustrup *et al.* 2003) conventionally calculated GE (CGE) using the above equations is determined when $\dot{V}O_2$ is in steady state and the RER is at or below 1.0.

As mentioned above, indirect calorimetry assumes that the amount of O_2 consumed and CO_2 produced is dependent on whether protein, fat or carbohydrate is is the substrate being oxidised. Within the cycling efficiency literature, four main equations have been commonly used to calculate the PI from gas exchange data under steady state conditions (Lusk 1924; Brouwer 1957; Garby and Astrup 1987; Péronnet and Massicotte 1991). The Péronnet and Massicotte (1991) equation has previously been found to yield the highest calculated metabolic power and thus the lowest estimated GE in both running (Kipp, Byrnes and Kram 2018) and cycling (Bossi, Timmerman and Hopker 2020). In trained male runners performing 5-min submaximal trials at 8 – 18km/hr there is a 3.6 – 3.8% difference in calculated metabolic power when compared to the PI equation of (Garby and Astrup 1987). Similarly, when looking at cycling gross efficiency, the (Péronnet and Massicotte 1991) equation produces the lowest GE compared to equations by Lusk (1924), Brouwer (1957), and Garby and Astrup (1987) at 60%, 70% and 80% gas exchange threshold (GET) with a 0.8% difference in absolute GE units between the equations of Péronnet and Massicotte (1991) and Garby and Astrup (1987). This difference in energy expenditure calculation can be attributed to Péronnet and Massicotte (1991) reporting a more meticulous account of the energy contributions of both glucose and fat oxidation whilst also noting that both oxygen and carbon dioxide are not ideal gasses as previous energy expenditure (EE) equations have treated them. One mole of oxygen as an ideal gas occupies 22.41 L whereas under standard temperature (0°C) and pressure (760 mmHg) and dry (no water vapour) (STPD) one mole of oxygen occupies 22.3858.

$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O + 2817\ kJ$ *Equation 2.3*

Using the oxidation of glucose where 2817 kJ of heat is released (see equation 2.7) Kipp, Byrnes and Kram (2018) demonstrate that ratio of heat produced to oxygen consumed is 20.95 kJ/L when oxygen is treated as an ideal gas. However, when the STPD oxygen is used 20.97 kJ/L is released. Accounting for differences in higher energy fat species differences

utilised and using STPD oxygen and carbon dioxide in the Perronet and Massicote (1991) results in significant differences in metabolic power values and GE.

However, in line with previous GE reliability studies where breath-by-breath gas analysis systems typically produce a mean coefficient of variation of 4.2% (Moseley and Jeukendrup 2001) and 4.4% (Moseley and Jeukendrup 2001) GE appears to be a reliable measure with a mean coefficient of variation of 4.2% across a GET intensity of 60%, 70% and 80% regardless of the PI equation used (Bossi, Timmerman and Hopker 2020). For ease of comparison with previous studies investigating the effect of cycling exercise on gross efficiency after time-trials and high-intensity exercise (Uitslag *et al.* 2010; Noordhof *et al.* 2015b; Koning *et al.* 2013; Groot *et al.* 2018) this thesis will be using the Garby and Astrup (1987) equation throughout.

Efficiency can also be defined as net efficiency, work efficiency, and delta efficiency. These definitions whilst distinct are unified in their reliance on a baseline subtraction (Ettema and Lorås 2009). Net and work efficiency are calculated by subtracting a baseline measurement at rest (net efficiency) or during unloaded cycling (work efficiency) from the total energy expenditure during exercise. Delta efficiency is calculated by dividing the increase in PO by the increase in PI between two exercise bouts.

Muscle efficiency is the product of metabolic efficiency (oxidative phosphorylation) and mechanical efficiency (contraction coupling) (Whipp and Wasserman 1969; Gaesser and Brooks 1975) and is predicted to be ~30% (Stainbsy *et al.* 1980; Poole *et al.* 1992). Due to the additional costs of other cellular functions (e.g. ion pumps) and supporting organ functions (e.g. circulation, ventilation, nervous system impulses, stabilising muscles), muscle efficiency will be higher than GE (Poole, Gaesser and Hogan 1992). In comparison, the average GE in competitive cyclists have been reported to be as high ~25% (Lucia *et al.* 2002) with a variation between 18.5% – 23.5% in endurance trained cyclists cycling at 300W (Joyner and Coyle 2008).

Using baseline subtractions to calculate efficiency have been criticized in the literature (Stainbsy *et al.* 1980; Cavanagh and Kram 1985) as they assume that energy expended at baseline is constant, independent and unchanged under all the conditions that it is applied.

However, as exercise intensity increases there is an increase in the energy cost of physiological variables such as ventilation (Hesser, Linnarsson and Bjurstedt 1977; Vella, Marks and Robergs 2006). Stainbsy et al. (1980) concluded that baseline subtractions of exercise efficiencies do not indicate muscle efficiency and thus this thesis will use GE to calculate whole-body efficiency. To correctly determine changes in GE during exercise of different intensity and duration the mechanical power output (PO) needs to be accurately measured. Cycling power meters can precisely measure mechanical PO with an accuracy of ±1% (Paton and Hopkins 2001; Gardner *et al.* 2004; Bouillod *et al.* 2017). Therefore, cycling exercise was used in this thesis to calculate GE.

2.4.5 Intensity and duration of exercise and the measurement of GE

The measurement of GE via indirect calorimetry utilises measurements of $\dot{V}O2$ and $\dot{V}CO2$ to calculate the caloric equivalent. $\dot{V}O2$ measurement is needed because the caloric equivalent for O_2 is dependent on the proportion of fat (19.6 kJ) and carbohydrate (21.1 kJ) metabolised (Brooks 2013). The caloric equivalent is then calculated using the respiratory exchange ratio (RER) (equation 2.3) of the expired gas which is an estimation of the metabolic respiratory quotient.

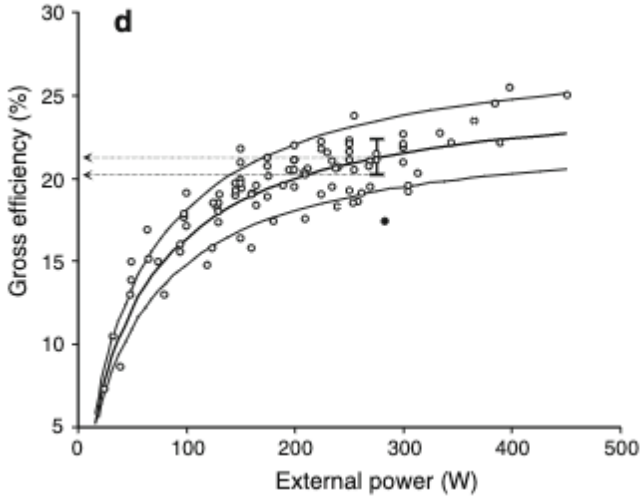

Figure 2.6 *Work rate _GE relationship depicting a possible error of measurement of 5%. Thick curve is the average curve, based on the regression line in b. Thin curves indicate ranges if both metabolic rate and external power have deviation (error) of 5%, but in opposite directions. A thick vertical error bar indicates the same range if only one of the measures has*

a 5% deviation; the thin horizontal arrows indicate the efficiency difference following from this error. Taken from Efficiency in cycling: a review by Ettema and Loras (2009)

Different intensities and durations have been shown to influence changes in GE. A review by Ettema and Lorås (2009) plotted the calculated GE and work rate of a large number of studies investigating GE in cycling which demonstrates a curved work rate-GE relationship (with a decreasing relative contribution of baseline metabolism as GE increases) (see figure 2.6)

Numerous studies have also shown that GE decreases significantly after prolonged (>90min) low or moderate intensity exercise at a constant power output (Hagberg, Mullin and Nagle 1978; Hagan, Weis and Raven 1992; Passfield and Doust 2000; Hopker, O'Grady and Pageaux 2016; Almquist *et al.* 2019) However, an exact measurement of energy expenditure is only possible during steady-state submaximal exercise as there is an increase in the ventilatory cost, anaerobic metabolism, lactate production and bicarbonate buffering. This leads to a rise in $\dot{V}CO2$ and RER above 1.0 that is not attributable to substrate metabolism. Consequently, expired gas measured at the mouth during high-intensity exercise does not reflect total energy expenditure. Therefore, changes in GE during acute high-intensity exercise or prolonged duration exercise interspersed with high-intensity intervals remains unknown.

2.4.6 The back-extrapolation method for estimating gross efficiency during high-intensity cycling exercise

Conventional GE (CGE) is calculated during steady-state exercise where energy expenditure from aerobic processes can be accounted for via expired gases measures at the mouth. However, cyclists frequently train and race at intensities above lactate threshold. As HIE involves both aerobic and a significant anaerobic contribution, conventionally calculated GE CGE during a post-HIE bout may overestimate GE during HIE.

A novel method for estimating GE during HIE uses linear regression of post-HIE GE values and back extrapolates these values to estimate GE at the end of the HIE bout (see figure 2.8)

(Koning *et al.* 2013). An unknown factor in any changes in GE after HIE is an elevated $\dot{V}O2$ caused by the anaerobic work performed during the HIE bout. De Koning et al. (2013) believe that using a 1-minute period at 25W immediately after the HIE bout to allow the $\dot{V}O2$ to decrease below that during the warm-up period allows the $\dot{V}O2$ during the recovery period to be representative of the metabolic cost of riding at 50% maximal aerobic power (MAP) (see figure 2.7).

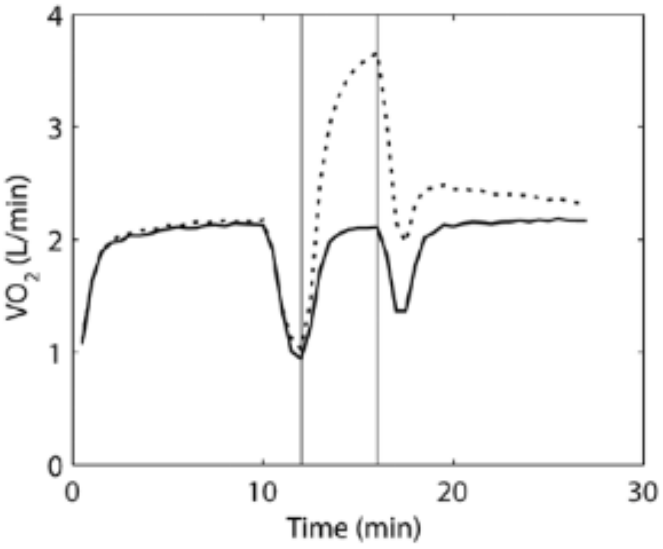

Figure 2.7 *Mean response during 80%MAP (solid line) and high-intensity (dotted line) for oxygen uptake ($\dot{V}O2$). The protocol consisted of 10 min at 50%MAP, 2 min at 25W, 4 min HIE bout (either 80%MAP or 100%MAP), 1 min at 25W and 10 min at 50%MAP. Taken from An approach to estimating gross efficiency during high-intensity exercise (de Koning et al. 2013)*

This decline in BGE during high-intensity exercise has a potential impact on both pacing and performance. Using this BGE method it has been shown that whilst CGE remained constant during 4-min cycling at 100%$_{MAP}$ BGE declined by 2.5% (see figure 2.2) (Koning *et al.* 2013). GE has also been shown to decrease significantly after maximal time-trial exercise with the magnitude of GE reduction inversely proportional to time-trial distance. The authors also found that whilst the decline in GE is linear during short time-trials (1000m and 4000m), GE decline hyperbolically during the 40,000m time-trial (Noordhof *et al.* 2015b) with the final

reduction in GE during the 40,000m time-trial almost attained at 50% of the final time. Noordhof et al. (2015b) speculate that the anaerobic contribution during the start of the trial may be the main cause of the decrement in GE.

Assuming a decline GE using the BGE method, the estimated anaerobic contribution to a 4-min bout at $100\%_{MAP}$ is 32% larger than assuming a constant GE using CGE (see figure 2.9) (23.7kJ vs 17.9kJ) (Koning *et al.* 2013). Similarly, the anaerobic contribution during cycling time trials of 4000m or less has been found to be 30% higher when using BGE compared to CGE (Mulder *et al.* 2015a; Noordhof *et al.* 2015a).

However, the reliability and validity of the BGE method during HIE of different intensity and duration has not been investigated. Additionally, the rate of GE decline and recovery after multiple intervals of HIE or during prolonged mixed intensity exercise is unknown.

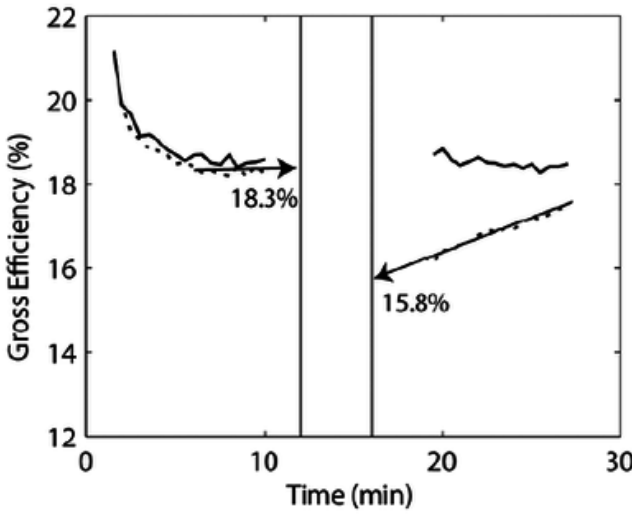

Figure 2.8. *Mean responses during the control (solid line) and high-intensity (dotted line) rides for gross efficiency (GE). The 100%-peak-power-output (PPO) work bout and control ride were from minutes 12 to 16. GE at the end of the warm-up was 18.3%, while the back-extrapolated GE at the end of the 100%-PPO ride was 15.8%. Taken from An approach to estimating gross efficiency during high-intensity exercise (de Koning et al. 2013)*

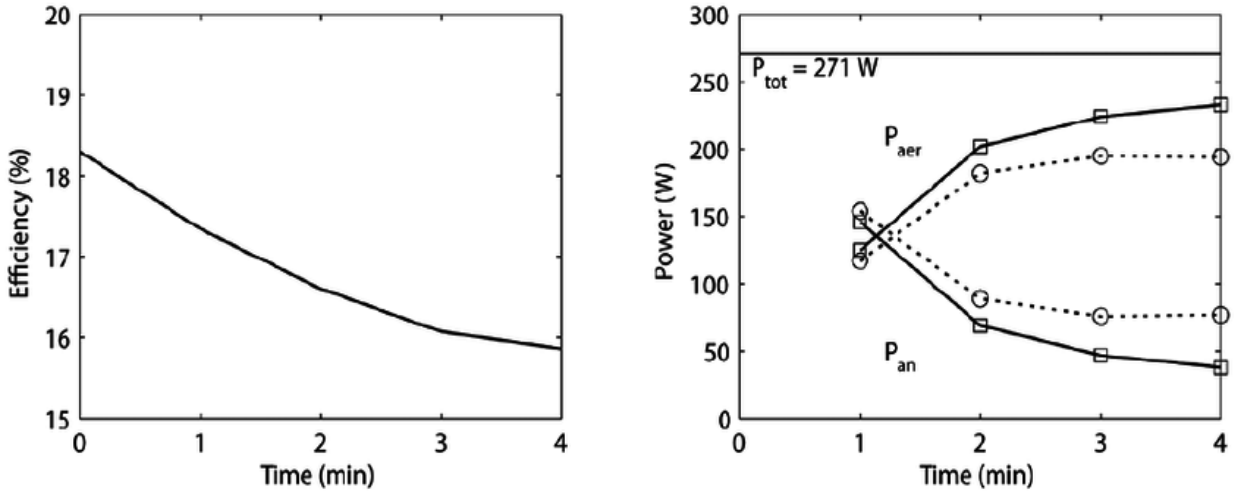

Figure 2.9 *Left: the change in gross efficiency (GE) across the 4-min time trial. Right: aerobic power output (P aer) and anaerobic power output (P an) calculated on the basis of an assumed constant GE of 18.3% (solid line, square) vs a variable GE (from Figure 1; dotted line, circle).*

2.5 Measuring muscle oxygenation using near-infrared spectroscopy

By using near-infrared spectroscopy (NIRS) during exercise we can determine both oxygenation and deoxygenation of skeletal muscle tissue non-invasively whilst also providing insights into whether changes in GE are mirrored by changes in oxygen consumption within exercising skeletal muscle.

There are a number of ways of ascertaining information regarding skeletal muscle cellular function. Muscle biopsies have shown differences in cellular signalling as well as mitochondrial content and function after both acute exercise and training (Perry *et al.* 2010). Local oxygenation of skeletal muscle can also be measured by measuring blood flow and the arterial-venous O2 concentration difference across the muscle (Bangsbo *et al.* 2000) can also be measured However both techniques are invasive, costly and time consuming. Non-invasive in vivo measurement of skeletal muscle oxidative metabolism has previously been undertaken utilizing phosphorus magnetic resonance spectroscopy (P-MRS) and the known kinetics of phosphocreatine (PCr) resynthesis after exercise to assess mitochondrial capacity

(Walter *et al.* 1997). Whilst the recovery time constant of PCr has been shown to be correlated with mitochondrial enzyme activity in skeletal muscle obtained via invasive muscle biopsy techniques (Chance *et al.* 1986) P-MRS is an expensive technique that requires large equipment and technical expertise.

NIRS (is a low cost, non-invasive, portable optical method that can be utilized in ascertaining the balance of microvascular (arterioles, capillaries and venules) oxygen delivery and extraction directly in active skeletal muscle. NIRS uses infrared waves to exploit the differential absorption properties of the chromophores haemoglobin (Hb) and myoglobin (Mb). At a wavelength of 850nm these chromophores occur in their oxygenated state whereas at a lower wavelength of 760nm they occur in their deoxygenated state. Thus, NIRS provides a real time assessment of relative changes in oxygenated haemoglobin (HbO_2) deoxygenated haemoglobin (HHb) and total haemoglobin (tHb). Absolute changes in chromophore concentration can be accommodated for by the use of spatially resolved spectroscopy (SRS) which is insensitive to light scattering. Therefore, the diffusion equation for light transport can be used to give an absolute measure of tissue oxygen saturation (TSI%).

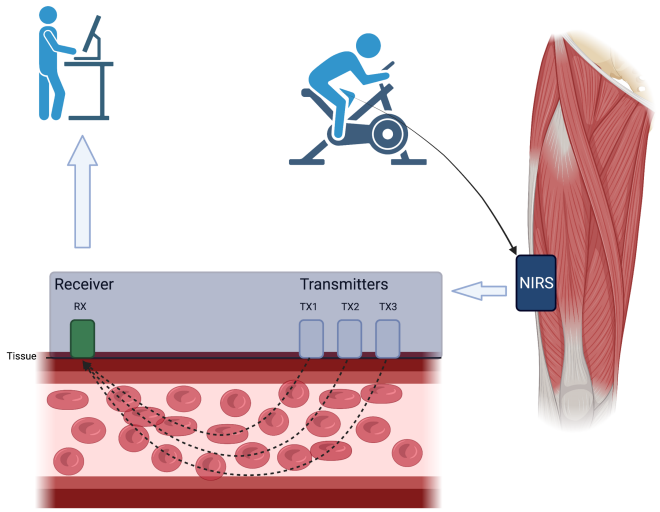

Figure 2.10 *The NIRS optode is placed on the vastus lateralis muscle during exercise. Real time changes in HbO_2, HHb and tHb are wirelessly transmitted to an external computer for analysis*

NIRS measurements have been found to have good agreement with phosphorus magnetic resonance spectroscopy indexes of skeletal muscle oxidative capacity (Ryan *et al.* 2013) and

are well correlated with in-situ high resolution respirometry in permeabilized muscle fibres (Ryan *et al.* 2014) which suggest that NIRS is a valid method of assessing mitochondrial function.

NIRS has been used to investigate the kinetics of muscle deoxygenation during both incremental (Boone *et al.* 2010; Salvadego *et al.* 2013) and constant load exercise (Belardinelli *et al.* 1997; Grassi *et al.* 2003).

Ryan, Brizendine and McCully (2013) NIRS devices have been shown to accurately and reliably measure mitochondrial function (Ryan, Brizendine and McCully 2013), oxygen consumption and blood flow (Lucero *et al.* 2017) after voluntary exercise of varying intensity. NIRS has previously been used to investigate changes in muscle oxygen consumption during a bout of prolonged constant load cycling. It has been shown that 90 mins of cycling at 60% maximal minute power resulted in an increased muscle oxygen consumption, and reduction in whole body GE (Hopker, O'Grady and Pageaux 2016). Whether the same relationships are seen during acute cycling bouts of varying intensity or duration has yet to be investigated.

Previous research has concluded that ventilation (Hopker *et al.* 2013), fat metabolism, body temperature and lactate metabolism do not account for this increase in oxygen cost after sustained moderate-intensity exercise (Hagberg, Mullin and Nagle 1978; Hagan, Weis and Raven 1992).

2.6 Epigenetic regulation of the physiological adaptation after exercise

2.6.1 microRNAs and exercise

Many of the studies that have previously investigated changes in performance with changes in cellular and metabolic adaptation of skeletal muscle in response to exercise of different intensity and duration have utilized invasive muscle biopsies to assess for changes in mRNAs and downstream proteins that influence beneficial physiological adaptation (Burgomaster *et al.* 2007; Gibala *et al.* 2009; Perry *et al.* 2010; Little *et al.* 2011). Recent advancements in molecular physiology have identified short, non-coding RNAs (microRNAs) that regulate post-transcription gene expression which have been used as disease biomarkers due to their ease of acquisition, stability and amplification (Garzon, Marcucci and Croce 2010; Zhou *et al.* 2018;

Qadir *et al.* 2019). These microRNAs can be released in the circulation (ci-miRNAs) and can act as intercellular messengers affecting physiological adaptations such as redox hormesis (Zhang *et al.* 2012b), angiogenesis (Liu *et al.* 2011) and muscle contractility (Urbich, Kuehbacher and Dimmeler 2008) that may affect the metabolic and mechanical efficiency of skeletal muscle. As ci-miRNAs are secreted into biofluids such as serum, plasma, saliva and urine (Chen *et al.* 2008; Mitchell *et al.* 2008; Chen *et al.* 2012) it has potential as a less invasive biomarker of cellular adaptations that may influence changes in GE.

MicroRNAs play a major role in cellular proliferation, differentiation and apoptosis and are thus a key component in the regulation of skeletal muscle adaptation. It has been shown that a single miRNA has the potential to regulate hundreds of downstream mRNA and proteins (Lim *et al.* 2005; Bartel 2004) with specific miRNAs affecting mitochondrial biogenesis, skeletal muscle differentiation and angiogenesis (Nielsen *et al.* 2010). The plasticity of skeletal muscle is reflected by the dynamic and time-specific expression of miRNAs in many tissues and their release into the circulation from the cells in response to a given stimulus (Nielsen *et al.* 2010; Baggish *et al.* 2011; Aoi 2014; Russell and Lamon 2015).

We selected two candidate c-miRNAs, ci-miR-21 and ci-miR-222 that have previously been shown to be upregulated or downregulated after exercise (table 2.1) (Baggish *et al.* 2011; Nielsen *et al.* 2014) and affect cellular processes and validated targets involved in exercise adaptation (see table 2.2) (Horak *et al.* 2018).

Ci-miRNA-21 and ci-miRNA-222 have been linked to the regulation of cellular processes that influence both cardiovascular and skeletal muscle adaptation. Possible mechanisms for ci-miRNA-21 involve the regulation of muscle contractility, redox metabolism and hypoxic adaptation (Urbich, Kuehbacher and Dimmeler 2008; Liu *et al.* 2011; Zhang *et al.* 2012a). Ci-miRNA-222 has been shown to also influence redox metabolism (Dubois-Deruy *et al.* 2017), as well as protecting against adverse cardiac remodelling and dysfunction after ischaemic injury (Liu *et al.* 2015)

ci-miRNA-21 and ci-miRNA-222 have been found to be significantly higher in endurance athletes compared to strength athletes (Wardle *et al.* 2015) and significantly upregulated in response to both acute incremental cycling exercise to exhaustion and after chronic rowing training (Baggish *et al.* 2011). This suggests that these ci-miRNAs may play an important role

in aerobic endurance adaptation. It has been shown that acute constant cycling for 1 hour at 65% maximal aerobic power does not significantly change the expression of miR-21 (Nielsen *et al.* 2014). Thus, the expression of ci-miRNA in response to different acute exercise prescriptions remains unclear. The expression of both ci-miRNA-21 and ci-miRNA-222 appear to be related to markers of endurance performance. In a cohort including both male and female participants it was found that levels of ci-miRNA-222 was found to be higher in the low $\dot{V}O_{2max}$ group ($p = \leq 0.05$) whilst ci-miRNA-21 was increased in male participants with a low $\dot{V}O_{2max}$ ($p = \leq 0.05$) after performing acute incremental cycling exercise to exhaustion (Bye *et al.* 2013). However, the expression of ci-miRNAs potentially affecting gross efficiency in response to different acute high-intensity exercise prescriptions or mixed intensity prolonged exercise has not been characterized.

Table 2.1 *c-miRNAs responses to acute and chronic endurance exercise in plasma or serum.*

c-miRNA	Participants	Exercise type	Responses	Reference
miR-21	Male student athletes (n = 10)	1) Exhaustive incremental cycling exercise test; 2) 90 days rowing training 1–3 h/day	↑(immediately after exercise) ↑ (after training vs pre-training baseline)	(Baggish *et al.* 2011)
	Healthy men (n = 38) and women (n = 38)	None; participants categorized in high (145.2 ±20.7 ml/kg0.75/min) or low (101.1 ±18.0 ml/kg0.75/min) V O2max categories	↑ (Low V O2max vs. high V O2max (men only))	(Bye *et al.* 2013)
	Healthy, trained men (n = 32)	1) 60 min cycle at 65% P_{max} 2) 5 sessions over 12 weeks of endurance exercise	↓(post-training)	(Nielsen *et al.* 2014)
	Male strength (n = 10) and endurance (n = 10) competitive athletes, and non-exercising controls (n = 10)	None; participants compared based on training type	↑Endurance compared with strength athletes	(Wardle *et al.* 2015)
miR-222	Male student athletes (n = 10)	1) Exhaustive incremental cycling exercise test; 2) 90 days rowing training 1–3 h/day	↑ (immediately after exercise) ↑ (after training vs pre-training baseline) ↑ (after training, immediately after exercise)	(Baggish *et al.* 2011)
	Healthy men (n = 38) and women (n = 38)	None; participants categorized in high or low V O2max categories	↑ (Low V O2max vs. high V O2max)	(Bye *et al.* 2013)
	Male strength (n = 10) and endurance (n = 10) competitive athletes, and non-exercising controls (n = 10)	None; participants compared based on training type	↑ Endurance compared with strength athletes	(Wardle *et al.* 2015)

Table 2.2. *Candidate miRNAs and target mRNAs implicated in cellular exercise adaptation. Adapted from* (Horak *et al.* 2018)

miRNA	Targeted gene function	Target genes
ci-miR-21	Proliferation, differentiation, cell cycle regulation	BTG2; CDC25A; CDK2AP1; DUSP10; E2F1;E2F2; EGFR; IL1B; JMY; MEF2c; MyC;PDCD4; PPARA; PTEN; PTX3; RASA1; SASH1; SP1; STAT3; TGFBR2; TP53BP2; YOD1
	Apoptosis	APAF1; BCL2; DAXX; FASLG; IGF1R; IL1B; MYC; SASH1; STAT3; TNFAIP3; TNFRSF10B; TP53BP2
	Tissue remodelling, angiogenesis	HIF1A; MMP2; MMP9; PLAT; RECK; TIMP3; VEGFA
	Inflammation	CCL20; CCR1; HPGD; IL1B; PPARA; PTX3
	Muscle contraction	TPM1
	Antioxidant	SOD3
	Glucose transport	MAP2K3
ci-miR-222	Proliferation, differentiation, cell cycle regulation	CDKN1B; CDKN1C, CERS2; CORO1A; DIRAS3; ETS1; FOS; HIPK1; KIT; PPP2R2A; PTEN; TP53; TP53BP2
	Apoptosis	BBC3; CORO1A; ETS1; FOS; FOXO3; STAT5A; TNFSF10; TP53; TP53BP2
	Tissue and remodelling, angiogenesis	ETS1; KIT; MMP1; RECK; TIMP3
	Mitochondrial function	SOD2
	Heart contraction	GJA1
	RNA interference	DICER1

Summary

GE is an important determinant of endurance performance. Until recently, measurement of GE was based on the assumption that $\dot{V}O2$ during submaximal exercise is representative of GE during HIE. A novel method of measuring GE during HIE using a back-extrapolation method (BGE) of $\dot{V}O2$ data points after HIE suggests that GE is overestimated compared to assuming a constant GE. However, the validity and reliability of this method has not been elucidated.

Whilst the BGE method has been utilised to assess changes after short acute maximal 4-min HIE and time-trials of varying length the rate of GE decline during and after acute mixed intensity exercise and exercise of different duration has yet to be investigated.

Finally, the mechanisms underlying changes in GE during exercise of different intensity and duration remain inconclusive. This thesis will examine the potential role of minimally invasive techniques in assessing factors that may contribute to changes in GE. Using NIRS may offer insights into whether changes in GE during cycling exercise of varying intensity or duration are mirrored by localised changes in oxygen delivery or extraction in skeletal muscle. Additionally, changes in ci-miRNAs and GE will be compared after acute exercise with intervals of different intensity and duration. Ci-miRNAs in human biofluids may have a potential role in regulating the production and function of proteins essential for beneficial physiological adaptations that change GE.

Thesis aims and hypotheses

The overall aim of this thesis is to investigate the effects of intensity and duration on gross efficiency in cycling. Research studies examining how high-intensity or prolonged duration affect gross efficiency are scarce and warrant further investigation. In addition, the physiological mechanisms and epigenetic regulation of factors that may affect gross efficiency have not been fully explored. This thesis presents a series of studies that contribute to the overall research aim.

The specific aims and hypotheses of each of the experimental chapters are as follows:

1) **Is back-extrapolated gross efficiency a valid and reliable method of estimating gross efficiency during high-intensity exercise?**
    - Aims:
        - Investigate the validity and reliability of BGE
        - To compare conventionally calculated GE (CGE) and BGE before and after 4 min HIE at 80% maximal aerobic power (MAP) and 100%$_{MAP}$.
    - Hypotheses:
        - BGE is a valid and reliable method of estimating gross efficiency
        - BGE will be significantly lower than CGE post HIE at 100%$_{MAP}$.

2) **Does prolonged duration cycling interspersed with repeated sprints result in greater decreases in gross efficiency during and after exercise compared to prologed low-intensity cycling**
    - Aims:
        - Compare the effects of prolonged low-intensity continuous cycling exercise (LCIE) and prolonged low-intensity continuous cycling interspersed with sprint intervals (LCIE_SI) on CGE and BGE during and after exercise
    - Hypotheses:
        - Compared to LCIE, CGE will be significantly lower after LCIE_SI
        - BGE will be significantly lower than CGE during LCIE_SI

3) **Does increasing intensity of acute exercise results in greater decreases in gross efficiency?**

- Aims:
    - Compare the effect of three protocols: a constant interval of 40min (CONT); 4 x 5 min intervals (LI); two sets of 10 x 30s intervals (SI), on CGE and BGE
    - Assess the reliability of measuring CGE and BGE after HIE
- Hypotheses:
    - Compared to CONT, BGE and CGE will be significantly lower after LI and SI
    - CGE and BGE will not recover to pre-exercise levels
    - CGE and BGE are reliable methods of assessing GE after HIE

4) **Does acute high-intensity and sprint-intensity exercise result in an increase in circulating miRNAs?**

- Aims:
    - Compare the effects of three protocols: a constant interval of 40min (CONT); 4 x 5 min intervals (LI); two sets of 10 x 30s intervals (SI), on CGE and fold change expression in ci-miRNAs -21 and -222
- Hypotheses
    - Compared to CONT, BGE and CGE will be significantly lower after LI and SI
    - Compared to CONT, LI and SI will have a higher expression of ci-miRNA-21 and -222
    - Changes in ci-miRNA-21 and -222 will be associated with changes in CGE

3 The back-extrapolation method is a reliable and valid method of calculating gross efficiency after an acute bout of high-intensity exercise.

3.1 Abstract

Purpose: To evaluate the reliability of calculating gross efficiency (GE) conventionally and using a back extrapolation (BE) method during high intensity exercise (HIE).

Methods: 12 trained participants completed two HIE bouts (P1 = 4-min 80% Maximal Aerobic Power (MAP); P2 = 4-min at 100%$_{MAP}$). GE was calculated conventionally in the last 3 min of submaximal (50%$_{MAP}$) cycling bouts performed before and after HIE (Pre50%$_{MAP}$ and Post 50%$_{MAP}$). To calculate GE using BE (BGE), a linear regression of GE submaximal values post-HIE were back extrapolated to the end of the HIE bout.

Results: BGE was significantly correlated with Post50%$_{MAP}$ GE in P1 (r= 0.64; P = 0.01), and in P2 (r = 0.85; P = 0.002). Reliability data for P1 and P2 BGE demonstrate a mean CV of 7.8% and 9.8% with limits of agreement of 4.3% and 4.5% in relative GE units respectively. P2 BGE was significantly lower than P2 Post50%$_{MAP}$ GE (18.1 $\pm$ 1.6% vs 20.3 $\pm$ 1.7%; P= 0.01). Using a declining GE from the BGE method, there was a 44% greater anaerobic contribution compared to assuming a constant GE during 4 min HIE at 100%$_{MAP}$.

Conclusion: HIE acutely reduced BGE at 100%$_{MAP}$. A greater anaerobic contribution to exercise as well as excess post oxygen consumption at 100%$_{MAP}$ may contribute to this decline in efficiency. The BGE method may be a reliable and valid tool in both estimating GE during HIE and calculating aerobic and anaerobic contributions.

Keywords: anaerobic capacity, excess post-exercise oxygen consumption, pacing strategy, performance, maximal exercise

3.2 Introduction

Gross efficiency (GE) is defined as the ratio of work generated to the metabolic energy cost and has been shown to be a key component of cycling performance (Joyner and Coyle 2008; Jobson, Hopker and Korff 2012). The calculation of GE is conventionally determined from steady state measures where energy expenditure from purely aerobic processes can be accounted for via expired gases measured at the mouth.

However, during high-intensity exercise (HIE) with a significant anaerobic energy contribution, $\dot{V}O_2$ measured at the mouth cannot be used to estimate the total energy expenditure. Therefore, conventional measurement of GE during submaximal exercise may not be a valid estimate of GE during HIE. A novel approach to estimate GE during high intensity exercise has recently been proposed (Koning *et al.* 2013). This method uses linear regression of post-HIE GE values and back extrapolates these values to estimate GE at the end of the HIE-bout. Using this back-extrapolation (BGE) method, de Koning et al. (2013) found GE declined by 2.5% during 4 minutes of cycling at $100\%_{MAP}$. As a result, the calculated anaerobic contribution to their HIE bout was 32% larger when assuming a declining vs constant GE (23.7kJ vs 17.9kJ) (Koning *et al.* 2013).

The BGE method has previously been used to investigate the impact of GE on high intensity cycling performance (Mulder *et al.* 2015a). It has been demonstrated that the estimated anaerobic contribution to cycling time trial performance is 30% larger during time trials of less than 4000m when a declining rather than constant GE is assumed (Mulder *et al.* 2015a) A declining GE and a higher anaerobic contribution could therefore have important implications for both pacing strategy and performance. However, the reliability and validity of the BGE method in estimating GE during HIE has not previously been assessed.

There is also debate in the literature regarding possible physiological factors that might be influential in the reduction of GE seen following both prolonged submaximal (Hagberg, Mullin and Nagle 1978; Hagan, Weis and Raven 1992; Coyle, Sidossis and Horowitz 1992; Hopker, O'Grady and Pageaux 2016) and high intensity bouts of exercise (Noordhof *et al.* 2015b; Vanhatalo *et al.* 2011). Near-infrared spectroscopy (NIRS) has previously been used to investigate changes in muscle oxygen consumption during a bout of prolonged constant load cycling. It has been shown that 90 mins of cycling at 60% maximal minute power resulted in

an increased muscle oxygen consumption, and reduction in whole body GE (Hopker, O'Grady and Pageaux 2016). Whether the same relationships are seen during short duration high intensity cycling is yet to be elucidated.

The purpose of this study is: 1) to assess the reliability and validity of measuring whole body GE in HIE calculated using the BGE method, compared to traditional submaximal methods 2) to investigate the relationship between changes in whole body GE and NIRS parameters from high intensity exercise.

3.3 Methods

3.3.1 Participants

Thirteen trained male (mean ± SD: age 35 ± 5 yr, mass 75 ± 7 kg, $\dot{V}O_{2max}$ 63± 7 ml·kg^{-1}·min^{-1}, Maximal Aerobic Power (MAP) 389 ± 46 W) and 2 female (age 25 ± 5 yr, mass 60 ± 1 kg, $\dot{V}O_{2max}$ 50 ± 2 ml·kg^{-1}·min^{-1}, MAP 272 ± 39 W) participants who trained for a minimum of 6 hours per week volunteered to participate in the study. The study was conducted with the approval of the School of Sport and Exercise Science Research Ethics Advisory Group (ethical approval number: 78_2014_2015) and after obtaining informed written consent from all participants

3.3.2 Experimental design

Participants attended the exercise testing laboratory on four separate occasions. Visit 1 consisted of a maximal incremental exercise test to determine $\dot{V}O_{2max}$ and MAP. On 2 subsequent visits participants completed 2 high intensity bouts in the same order. Each HIE bout was preceded and followed with a submaximal exercise bout at 50%$_{MAP}$. One HIE was performed at 80%$_{MAP}$, and the second HIE bout at 100%$_{MAP}$. All tests were performed on an electromagnetically braked ergometer (Schoberer Rad Messtechnik, Germany). Handlebar and saddle height were adjusted for each individual during visit 1, and then replicated for each subsequent visit. Participants used their own clipless pedals. Respiratory exchange data was collected on a breath-by-breath basis during each visit using an online gas analyser (Metalyser 3B; CORTEX Biophysik GmbH, Germany).

3.3.3 Maximal Incremental Exercise Test

Upon reporting to the laboratory, body mass was measured to the nearest 0.1 kg using beam balance scales (Seca, Germany), and stature was measured to the nearest 0.5 cm using a stadiometer (Seca, Germany). Participants undertook a maximal incremental cycling test to ascertain their $\dot{V}O_{2max}$ and MAP. The test started with a 10-min warm up at 100W. After a 1-min rest the cycling output increased by 5 W every 15 s until the participant reached volitional exhaustion (defined as a cadence of <60 revolutions/minute despite strong verbal encouragement). $\dot{V}O_{2max}$ was determined as the highest measured 60 s $\dot{V}O_2$ achieved during the incremental test. MAP was calculated as the average power output over the final minute of the ramp test.

3.3.4 High Intensity Exercise Tests

Participants completed two HIE 4-min bouts of exercise in the same order per visit, the first HIE bout at 80%$_{MAP}$ (P1) and the second HIE bout at 100%$_{MAP}$ (P2) (see Figure 3.1). Each HIE bout was preceded by 6-min cycling at 50%$_{MAP}$ and 2-min at 25 W and followed by 1-min at 25 W and 10-min at 50%$_{MAP}$. P1 and P2 were separated by 30-minute rest. Participants were instructed to maintain a cadence of 80 rev·min^{-1} throughout the testing protocols.

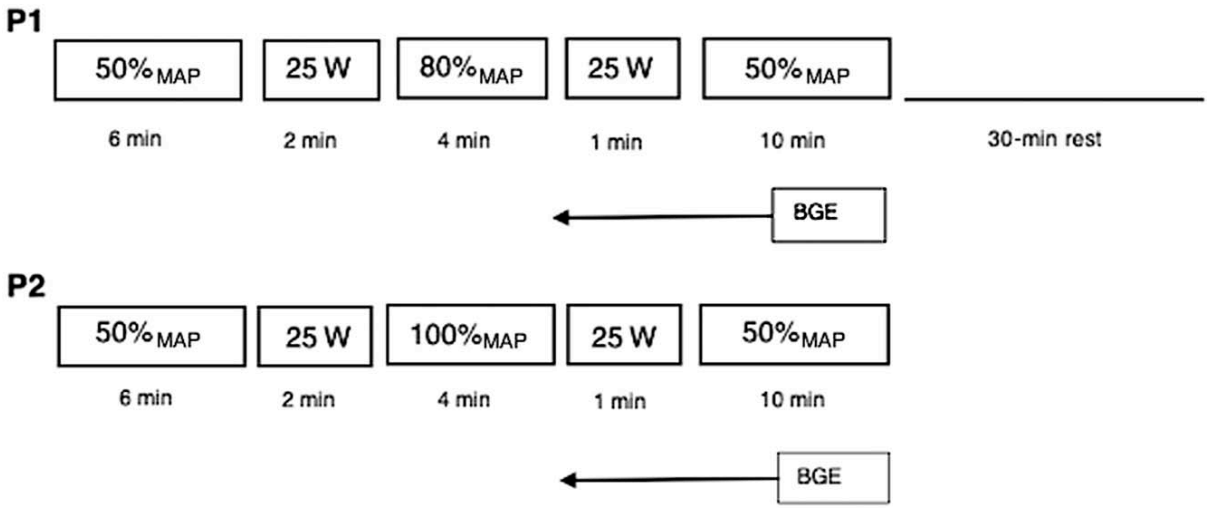

Figure 3.1. *HIE protocols with 4-minute HIE at 80%$_{MAP}$ during P1 and 100%$_{MAP}$ during P2. BGE indicates gross efficiency using back extrapolation*

Muscle oxygenation was measured continuously throughout the trials via near-infrared spatially resolved dual-wavelength spectrometry (Portamon, Artinins Medical Systems, Netherlands), emitting light at 760 nm and 850 nm wavelengths. The device was placed on the right thigh over the belly of the vastus lateralis muscle, 10cm proximal to the knee joint. Relative concentration changes in deoxygenated haemoglobin (HHb) were calculated from an arbitrary baseline value taken for 2-min prior to the start of each exercise protocol. An absolute measure of tissue oxygen saturation (TSI%) was also recorded throughout the exercise trial. Skinfold thickness was measured at the site of the NIRS device over the vastus lateralis muscle in the seated position using Harpenden skinfold callipers (British Indicators Ltd, Burgess Hill, UK). Adipose tissue thickness (ATT) was calculated by taking the median of three skinfold measurements and dividing the skinfold thickness by two with a mean value of 6.8 $\pm$ 2.5 mm (Weits, Beek and Wedel 1986).

Blood lactate was taken via a finger-prick blood sample immediately prior to the start and immediately at the end of the trial (Biosen C-Line analyser, EKF diagnostics, Wales).

3.3.5 Data analysis

Expired gas data measured at steady state during the last 3-min of the 50%$_{MAP}$ bouts (Noordhof *et al.* 2010) prior to, and after, HIE (Pre50%$_{MAP}$ and Post50%$_{MAP}$ respectively) was used to calculate GE conventionally with equations 3.1 and 3.2:

GE (%) = (Power input (W) / Energy expenditure (W)) x 100 *(Equation 3.1)*

Energy expenditure = ($\dot{V}O_2$) x (RER $\cdot$ 4904) +16040)/60) *(Equation 3.2)*

$\dot{V}O_2$ in equation 3.2 is expressed in L·min^{-1}. $\dot{V}O_2$ data points in the last 3 minutes that had an RER >1.0 were excluded.

BGE was calculated by fitting a linear regression to the GE data points in the last 8-min of the Post50%$_{MAP}$ bout; only data points with an RER < 1.0 were included in this 8-min period. These values were then back-extrapolated to the end of the HIE bout to give an estimate of change of GE (Koning *et al.* 2013). The decline in GE during the HIE bout was subsequently calculated

by plotting a linear relationship between GE during Pre50$_{\%MAP}$ and calculated BGE values. Whilst, Mulder et al. (2015) found that a linear relationship can be used for short bouts of HIE, an exponential relationship should be used for HIE of longer duration (Mulder *et al.* 2015b) . Total work, aerobic work and anaerobic work were estimated by calculating the total power output, aerobic power and anaerobic power over time during HIE in P1 and P2 as previously described elsewhere.(Koning *et al.* 2013; Mulder *et al.* 2015a; Serresse *et al.* 2008)

3.3.6 Statistical analysis

Shapiro-Wilk tests were conducted to assess for normality of distribution. To assess validity of BGE, the relationship between submaximal GE and BGE was assessed using a partial correlation controlling for Pre50% efficiency. The magnitude thresholds to assess the strength of the validity correlation are based on Cohen's effect sizes (Cohen 1988). To assess the reliability of GE, BGE, HHb and TSI, data from all three visits were used. Within-participant variation across the three repeated visits was calculated using Coefficient of Variation (CV) and 95% limits of agreement (Hopkins 2000). A repeated measures ANOVA was conducted to assess differences between the repeated visits in terms of GE, BGE, TSI and HHb. Statistical significance was set at P = ≤0.05. All values are presented as mean ± SD unless otherwise stated. Statistical analysis was conducted using the SPSS statistical software package (IBM SPSS Statistics, Rel. 25.0, SPSS, Inc, Chicago, USA).

3.4 Results

All participants completed the P1 HIE bouts at all visits. Three participants failed to complete the P2 HIE bout on two or all three visits; therefore, their P2 GE data were excluded from the analyses.

3.4.1 Reliability of conventional and back-extrapolation GE methods

A mean CV of 7.8% (95% CL: 5.9 – 11.7%) for P1 and 9.8% (95% CL: 7.3 – 15.1%) in P2 BGE was found. For GE Pre50%$_{MAP}$ there was a mean CV of 7.6% (95% CL: 5.8 – 11.6%) for P1 and 8.8% (95% CL: 6.8%– 12.9%) in P2. For GE Post50%$_{MAP}$ a mean CV of 6.2% (95% CL: 4.4 – 10.8%) for P1 and 6.8% (95% CL: 5.3 – 10.6%) in P2 was found. The mean limits of agreement in relative GE percentage point units were ± 3.6% for Pre50%MAP and ± 3.7% for BGE in P1, and ± 4.2% for Pre50%MAP and ± 4.1% for BGE in P2.

Figure 3.2 illustrates the limits of agreement between the three repeated visits for P1 and P2 using Pre50%$_{MAP}$ and BGE.

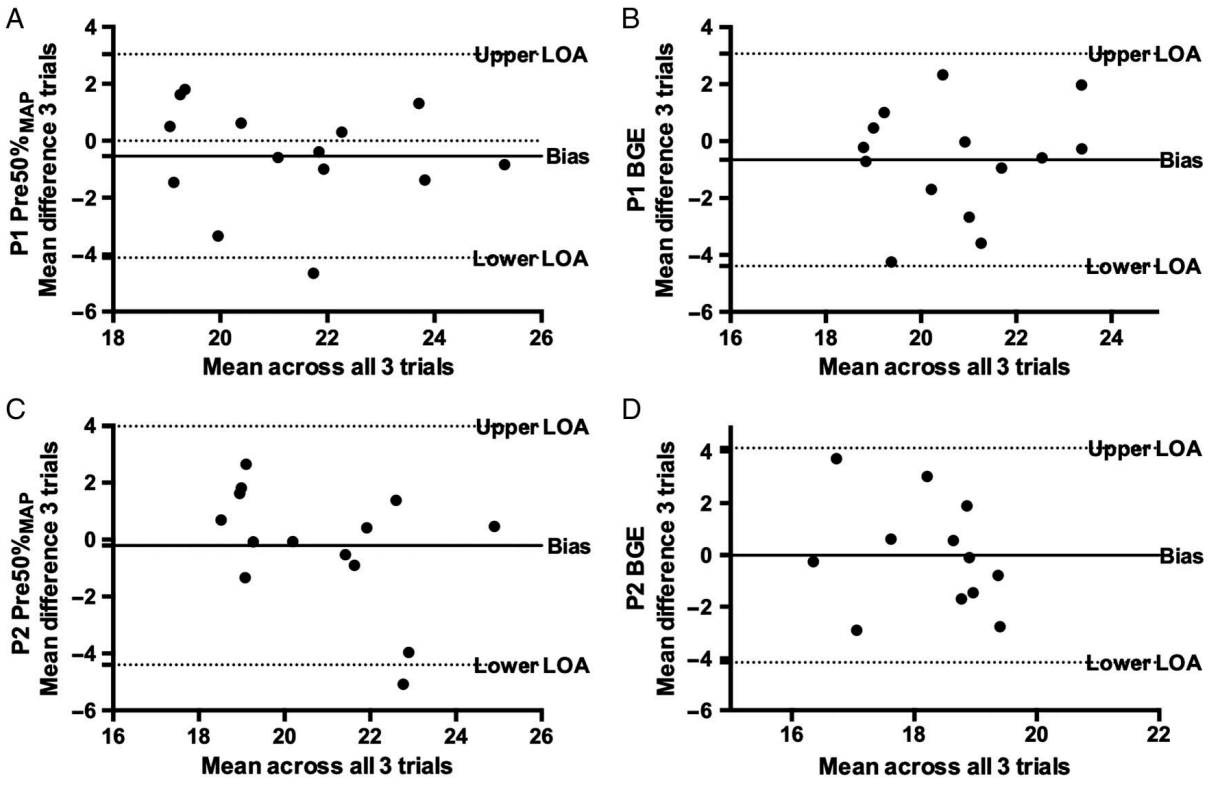

Figure 3.2 *— Bland–Altman plots with 95% LOA for GE across all 3 trials for each HIE protocol. (A) P1 Pre50%MAP GE, (B) P1 BGE, (C) P2 Pre50%MAP GE, (D) P2 BGE.*

3.4.2 Reliability of GE change

There was a mean CV of 0.86% (95% CL: 0.66 -1.23%) for the change in GE observed between Pre50%MAP and BGE in P1 and 0.99% (95% CL: 0.74 – 1.59%) for the change observed between Pre50%MAP and BGE in P2.

3.4.3 Reliability of anaerobic contribution to high-intensity exercise

The mean CV for the anaerobic contribution using a constant GE in P1 were 3.5% (95% CL: 2.6-5.5 %) vs 2.9% (95% CL: 2.2-4.4%) using BGE. The mean CV for the anaerobic contribution using a constant GE in P2 were 6.8% (95% CL: 5.2-10.8%) vs 5.0% (95% CL: 3.9-7.1%) using BGE.

3.4.4 NIRS reliability analysis

Mean CVs were calculated for the 4-min HIE bout at both intensities. A mean CV of 6.9% (95% CL: 5.5 – 9.8%) for P1 TSI and 9.7% (95% CL: 7.8 – 13.9%) in P2 TSI was found. For HHb there was a mean CV of 19.4% (95% CL: 15.5 – 27.11%) for P1 and 17.3% (95% CL: 13.8% – 23.8%) in P2.

3.4.5 Physiological responses to high-intensity exercise

Mean blood lactate concentration was significantly different pre- vs post-HIE (1.49 ± 1.05 vs. 3.06 ± 0.57 mmol·L^{-1} ; $P \leq 0.05$) in P1, and also in P2 (2.06 ± 0.73 vs. 5.52 ± 1.73 mmol·L^{-1}; $P \leq 0.05$). There was a significant interaction effect between intensity and timepoint (P=0.006).

Figure 3.3 shows the calculated GE Pre50%$_{MAP}$, BGE and GE Post50%$_{MAP}$ in both P1 and P2. BGE was significantly correlated with GE Post50%$_{MAP}$ in P1 ($r = 0.98$; $P = 0.01$) and in P2 ($r = 0.80$; $P = 0.01$) (figure 3.4a and 3.4b). Repeated measures ANOVA demonstrated that there was no significant difference between P1 GE Pre50$_{\%MAP}$ and P1 BGE (21.1% vs 20.9%; $P = 0.29$). P1 GE Pre50%$_{MAP}$ and GE Post50%$_{MAP}$ were not significantly different (21.1% vs. 21.0%; $P = 0.65$). A greater reduction in BGE is seen following HIE from P2 compared to P1 (-3.0% vs

0.27%, absolute GE units, figure 5a). HIE in P2 resulted in a significantly lower BGE compared to P2 GE Pre50$_{\%MAP}$ (18.1 ± 1.6% vs 21.1 ± 2.2%; P =0.01), and P2 GE Post50$_{\%MAP}$ (20.3 ± 1.7% P= 0.01). P2 Post50%$_{MAP}$ GE was also significantly different than P2 Pre50%$_{MAP}$ GE (20.3 ± 1.7 vs 21.1% ± 2.2%)

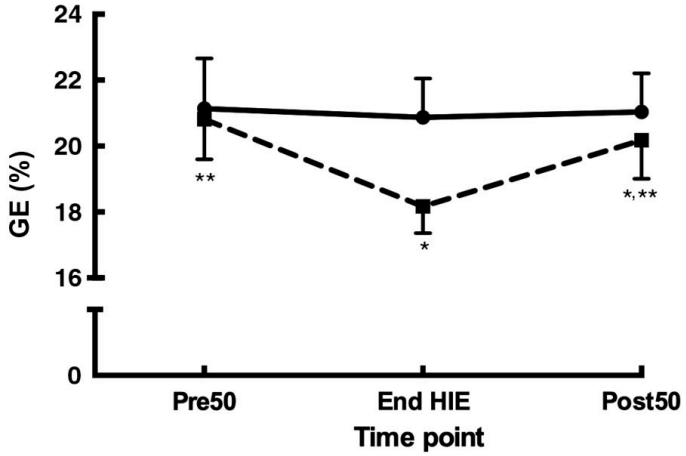

Figure 3.3 — *Mean GE changes (with 95% CIs) at 80%MAP (solid line, circle) and 100%MAP (dotted line, square) calculated Pre and Post 4-minute HIE and using the BGE method to the end of the 4-minute HIE bout. *Significant difference from Pre50%MAP (P = ≤0.05). **Significant difference from BGE (P = ≤0.05).*

Mean total energy expenditure during the 4-min HIE bouts were 72.8kJ in P1 and 92.5 kJ in P2. Calculated anaerobic contribution at the end of the 4min HIE bout in both P1 and P2 were higher at the end of the HIE bout using the BGE method compared to assuming a constant GE from the Pre50%$_{MAP}$ bout (P1 = 6.8 kJ vs 6.1kJ, P = 0.89; P2 = 20.9kJ vs 11.8kJ, P = 0.034). This resulted in a 9% and 44% difference in anaerobic work contributions at the end of the 80%$_{MAP}$ (P1) and 100%$_{MAP}$ (P2) bouts respectively (figures 3.5 c and d).

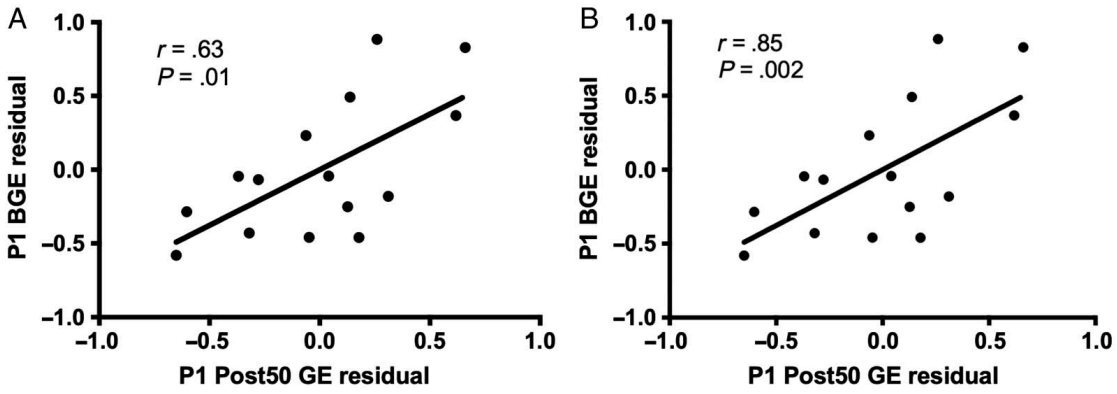

Figure 3.4 *— Linear partial correlation using GE residuals between Post50 and BGE in (A) P1 and (B) P2.*

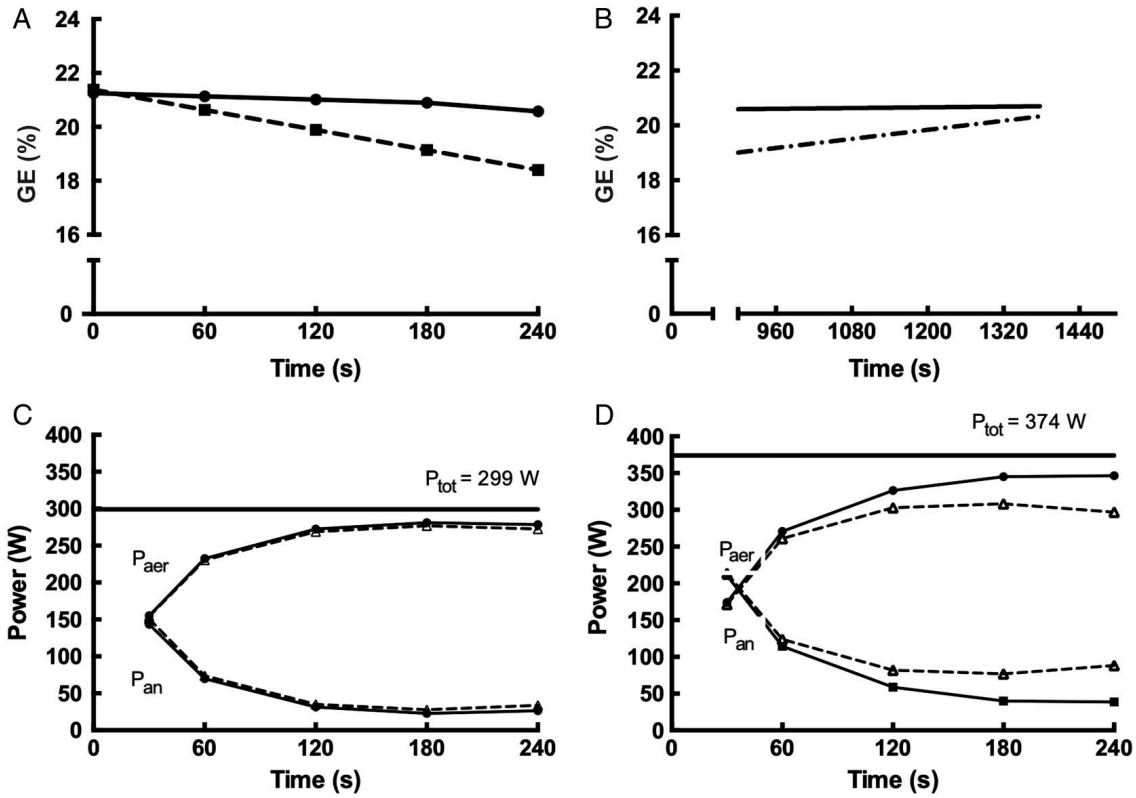

Figure 3.5 — *(A) Mean change in GE calculated using the BGE method during the 4-minute HIE bout at 80% MAP (solid line, squares) and 100% MAP (dotted line, circles). (B) Mean BGE values from the end of P1 (solid line) and P2 (dotted line) from the end of the 10-minute post-HIE recovery to the end of the 4-minute HIE bout. Mean aerobic (Paer) and anaerobic (Pan) power contributions during 4-minute HIE bout at (C) 80%MAP and (D) 100%MAP using a constant* Pre50%MAP GE (solid line, circle) and a variable BE50 GE (dotted line, triangle).

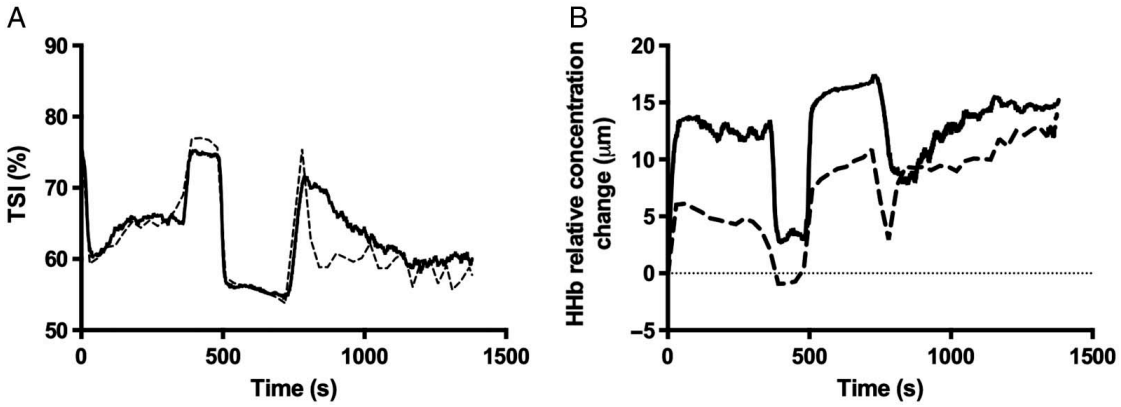

Figure 3.6 *— (A) A typical TSI (%) and (B) HHb concentration changes for P1 (dotted line) and P2 (solid line).*

A typical NIRS trace for TSI and HHb is shown in figure 3.6a and 3.6b, respectively. Table 3.1 presents mean data and changes from baseline for TSI and HHb for both P1 and P2 before the HIE, during HIE, at 5-min after HIE (post5), and at 10-min after HIE (post10). There were no significant differences across all TSI timepoints in P1. However, there was a significant difference in TSI between 4-min HIE vs Post5 (P = 0.02), and Post5 vs Post10 (P = 0.02) in P2. There were no significant differences across all HHb timepoints in P1. However, there was a significant difference between Post5 vs Post10 (P = 0.009) in P2.

Table 3.1. *Mean and changes in baseline of NIRS parameters pre, during 4-min HIE, post5 and post10.* [*] *significantly different from 4-min HIE (P = 0.02).* [§] *significantly different from Post 5 (P = 0.009)*

	Pre50	Δbaseline	4min HIE	Δbaseline	Post5	Δbaseline	Post10	Δbaseline
P1								
TSI(%)	68.6 (±1.0)	-3.8 (±0.4)	68.5 (±3.8)	-8.7 (±0.8)	69.2 (±1.2)	-5.9 (±0.3)	68.6 (±1.0)	-5.1 (±0.3)
HHb (μm)	10.2 (±1.1)	9.1 (±0.6)	9.9 (±4.9)	11.8 (±1.2)	9.9 (±1.0)	4.9 (±0.3)	9.7 (±1.4)	6.7 (±0.4)
P2								
TSI(%)	67.8 (±1.0)	-3.5 (±0.2)	66.6 (±4.4)	-10.3 (±1.3)	70.0 (±2.0) [*]	-7.9 (±0.6)	68.6 (±2.0) [§]	-8.1 (±0.8)
HHb (μm)	9.7 (±1.2)	10.3 (±0.3)	10.8 (±4.6)	10.4 (±0.7)	11.0 (±1.7)	8.9 (±0.3)	9.8 (±1.7) [§]	7.2 (±0.2)

3.5 Discussion

The main findings of this study are 1) the BGE method is valid and reliable to estimate GE during HIE; 2) using the BGE method, GE declines during HIE accompanied by a significant reduction in TSI; 3) assuming a declining GE during HIE, resulted in a larger calculated anaerobic contribution compared to using an assumed constant GE.

BGE was significantly correlated with GE Post50%$_{MAP}$ in both P1 (r = 0.63; P = 0.01), and in P2 (r = 0.85; P = 0.002). The "medium-large" correlation (Cohen 1988) between conventional GE and BGE measured in P1 and P2 suggests that BGE is a valid measure of GE during high intensity exercise such as that used in the current study.

The total within-participant variation in GE using the Douglas bag method has previously been reported to be as low as 1.5% during submaximal exercise (Hopker *et al.* 2012). However, in line with the current study, previous research using breath-by-breath online gas analysers have reported higher mean CVs of 3.2- 6.4%; closer to that of the current study (Noordhof *et al.* 2010; Moseley and Jeukendrup 2001). Moreover, the mean bias of all trials using Pre50%$_{MAP}$ GE and BGE were almost zero, indicating a similar level of repeatability for both methods. By assessing reliability, the smallest important difference (SID) in GE can also be ascertained. This is calculated by square rooting the difference between the observed standard deviation of the difference scores, by the typical error (Hopkins 2000). This measure is useful in monitoring any beneficial or detrimental changes in an individual's GE. SID values for GE Post50%$_{MAP}$ compared to BGE were 0.29 and 0.27 absolute GE units in P1, and 0.30 and 0.10 absolute GE units and P2, respectively. This suggests that BGE may be a more sensitive method in detecting important differences from high intensity work rates.

The decline in GE of 2.5% seen during 4-min at 100%$_{MAP}$ in de Koning et al. (2013) is comparable to the 3% seen at the same intensity (P2) in the present study. As illustrated in Figure 3, the differential effects of the two different HIE protocols on GE (using both CGE and the BGE methods) suggests that its determining mechanisms are intensity dependent. Indeed, GE has been shown to be decreased as a result of both HIE (Koning *et al.* 2013; Mulder *et al.* 2015a; Noordhof *et al.* 2015b) and prolonged exercise (Hopker, O'Grady and Pageaux 2016; Passfield and Doust 2000). However, this study is the first to demonstrate that the relative intensity of HIE may play a major role in the observed decrease in GE. Specifically, the present study demonstrated that BGE was significantly lower in P2, but not P1 where GE was not different across the measurement time points (Figure 3).

In agreement with previous work using BGE (Koning *et al.* 2013; Mulder *et al.* 2015a), the current study demonstrates a larger anaerobic work contribution during the 4-min HIE bout at 100%$_{MAP}$ results in a reduction in GE. However, as indicated by similar calculated energetic contributions using conventional and BGE methods, cycling for 4-min at an intensity of 80%$_{MAP}$ does not appear to have a large reliance on anaerobic metabolism (figure 5c and 5d), and hence does not result in a reduction in GE. Moreover, the current study demonstrates a greater decrease in the TSI in P2 compared to P1 (Table 1), accompanied by a corresponding significant reduction in BGE. It is likely that these changes in TSI when cycling at an intensity

of 100%$_{MAP}$ arise from the greater metabolic demand than oxidative supply, and therefore greater anaerobic energy contribution to power production, compared to 80%$_{MAP}$.

It is important to note that part of the estimated GE using BGE may be affected by recovery processes that increase $\dot{V}O_2$ post-HIE. Whilst there were no significant differences in P1 GE or NIRS parameters, the reduction of BGE in P2 was accompanied by significant differences in P2 GE and TSI and HHb at both Post5 and Post10 timepoints (see table 1). This suggests that exercise at 100%$_{MAP}$ may create a larger oxygen deficit as well as a greater excess post-exercise oxygen consumption where there is an increase in adenosine triphosphate and creatine phosphate re-synthesis, as well as increased lactate removal. Thus, at supramaximal intensities, or longer durations of HIE (Passfield and Doust 2000), there may be greater reductions in calculated BGE leading to greater calculated anaerobic contribution and a prolonged period of recovery.

3.6 Practical applications

From a practical perspective, our data suggests that performing short duration HIE involving a large anaerobic energy expenditure, will subsequently reduce GE. However, should exercise intensity reduce sufficiently for the oxygen deficit to be repaid, GE will likely recover. Therefore, in bicycle races combining high-intensity exercise and prolonged duration (e.g. criteriums, stage races) there may be reductions in GE through a combination of both recovery related processes and reductions in muscle contraction-coupling efficiency (Hopker, O'Grady and Pageaux 2016). Consequently, competition or pacing strategy may have to be modified to minimize the negative impact of reductions in GE on performance. To date, much of the research utilising the BGE method has investigated on constant load or maximal intensity exercise. Further investigation is needed to explore how the BGE changes during mixed intensity exercise.

3.7 Conclusion

This study demonstrates that the BGE method to estimate GE during HIE is a valid and reliable measure that is potentially more sensitive in detecting important changes in GE after HIE. Assuming a declining GE during short duration HIE at an intensity of 100%$_{MAP}$ there is a significantly greater anaerobic contribution compared to cycling at 80%$_{MAP,}$ leading to a larger O_2 deficit, and thus contributing to a reduction in GE. Following short-duration HIE, GE may recover if the intensity is sufficiently low to allow the oxygen debt to be repaid. Further work is needed to characterise changes in GE during prolonged exercise interspersed with HIE.

4 The effect of prolonged low-intensity cycling with repeated sprint intervals on gross efficiency

4.1 Abstract

Purpose: To investigate the effect of prolonged low-intensity continuous cycling (LCIE) and low-intensity continuous cycling interspersed with 15 x10 second sprint intervals (LCIE_SI) on conventionally calculated gross efficiency (CGE) and back-extrapolated gross efficiency (BGE).

Methods: A total of 10 male trained participants completed 2 protocols: LCIE (90 min at 50% maximal aerobic power ($50\%_{MAP}$) and LCIE_SI (90 min at $50\%_{MAP}$ interspersed with 15x10s sprint intervals). CGE was calculated in the last 3 min of 100W cycling bouts before (PreCGE) and after (PostCGE) each protocol. BGE was calculated after each sprint interval (SI) by linear regression of GE submaximal values to the end of the sprint interval. A two-way repeated measures ANOVA (protocol x timepoint) was used to assess for differences in CGE, BGE, TSI and HHb.

Results: PostCGE was significantly lower than PreCGE by 1.35 ± 0.3% in LCIE (P= 0.04) and by 2.06 ± 0.1% in LCIE_SI (P = 0.0001). There was no difference in PostCGE change between the protocols (P = 0.12). LCIE CGE and LCIE_SI BGE showed differences in change between protocols (mean CGE 19.96 ±0.5% vs mean BGE 17.9 ±0.6%, P = 0.01). In LCIE_SI there was a progressive decrease in mean average power between SI (9 ± 4W, P = 0.05), SI average power (70 ±14W, P = 0.04) and SI maximal power (93 ± 31W, P = 0.02). Tissue saturation index (TSI) was significantly lower post-protocol compared to pre-protocol for LCIE (73.58 ± 7.78 vs 65.40 ±9.25%, P = 0.01) and LCIE_SI (74.86 ±6.74 vs 68.66 ±9.33%, P = 0.02). Deoxygenated haemoglobin (HHb) was significantly higher post-protocol compared to pre-protocol for LCIE (3.85 ± 3.59 vs 11.95 ±6.10, P = 0.01) and LCIE_SI (4.30 ± 4.18 vs 12.10 ±8.12, P = 0.007).

Conclusion

CGE and BGE decrease as a function of time. A decrease in both BGE and TSI with an increase in HHb over repeated sprint intervals is reflected in a decline in average and maximal sprint interval power.

Keywords: excess post-exercise oxygen consumption, repeated sprint intervals, Near-infrared spectroscopy

4.2 Introduction

Chapter 3 demonstrates that BGE is a valid and reliable method for estimating GE during HIE. This allows the investigation of how GE may be affected in mixed intensity exercise such as road race cycling. Criteriums are a common road racing format that are typically 60-90 min in duration with performance mainly determined by $\dot{V}O2_{max}$, lactate threshold and GE (Joyner and Coyle 2008). For tactical reasons, long duration races are interspersed with repeated high-intensity or sprint-intensity efforts (Ebert *et al.* 2006). Whilst previous research has investigated changes in CGE during prolonged low-intensity exercise with repeated sprint-intervals (Almquist *et al.* 2019) it is currently unknown how BGE changes during prolonged mixed intensity exercise.

It has previously been shown that GE decreases after prolonged moderate exercise (Hopker, O'Grady and Pageaux 2016) and time-trials of varying duration and intensity (Noordhof *et al.* 2015b) and that this can affect performance (Passfield and Doust 2000). During constant work rate efforts above the lactate threshold in 0.5-40km time-trials and during prolonged (>2hrs) moderate intensity endurance exercise (Hopker, O'Grady and Pageaux 2016; Passfield and Doust 2000) there is an increase in $\dot{V}O_2$ and a reduction in GE which correlates (r= 0.91) with a reduced 5-min all-out performance (r = 0.91) and 30s peak power in trained cyclists (Passfield and Doust 2000). More recently, Almquist et al. (2019) has shown reductions in GE during 4 hours of low intensity exercise with 3x30s maximal sprints at the end of each of the first 3 hours. Results suggested that efficiency decreased as a function of time, rather than

due to the effects of the maximal repeated sprints. However, in this study there was only one bout of sprints each hour, with no sprints in the last hour. Moreover, changes in GE after the 30s sprint bouts were calculated from data obtained 4 min following the sprint (1 min passive rest, 3 min cycling at 100W prior to data collection) due to the need to limit the potential influence of anaerobic energy metabolism. Therefore, GE is unlikely to reflect the true metabolic consequences of the sprint exercise due to recovery processes.

Conventionally, the metabolic energy cost is calculated at submaximal intensities with a RER <1.00 where aerobic processes can be accounted for via expired gases measured at the mouth (Garby and Astrup 1987). However, decisive attacking efforts during competitive cycling occur at power outputs above the lactate threshold (Ebert *et al.* 2006; Sanders and Erp 2020), accompanied by a significant anaerobic contribution (Koning *et al.* 2013; Mulder *et al.* 2015b; Ebreo, Passfield and Hopker 2020). An increase in both lactate production and bicarbonate buffering leads to an increase in $\dot{V}$CO2 that is not attributable to substrate metabolism (McArdle, Katch and Katch 2009), and as such is problematic for the determination of GE (Koning *et al.* 2013; Ebreo, Passfield and Hopker 2020). Using BGE, a significant 2.5% reduction in GE (P = <0.01) has been shown after a 4-min bout of HIE at 100%$_{MAP}$ (Koning *et al.* 2013). This reduction is associated with a 44% greater anaerobic contribution compared to assuming a constant GE using the conventional submaximal GE method (Ebreo, Passfield and Hopker 2020).

Previous research has concluded that substrate utilisation, ventilation, body temperature and lactate metabolism do not account for the increase in $\dot{V}$O2 associated with the reduction in GE (Hagan, Weis and Raven 1992; Hagberg, Mullin and Nagle 1978; Passfield and Doust 2000; Almquist *et al.* 2019). Other mechanisms that have been proposed are a progressive decline in the efficiency of type I fibres due to peripheral fatigue (Vanhatalo *et al.* 2011; Almquist *et al.* 2019) and the additional recruitment of less efficient type II muscle fibres (Borrani *et al.* 2001; Krustrup *et al.* 2004). However, the proportion of muscle fibre types does not appear to influence cycling efficiency at low-intensity relative or absolute work rates in trained cyclists (Hopker *et al.* 2013). A more recent study using near-infrared spectroscopy (NIRS) to non-invasively measure muscle oxygenation during prolonged moderate intensity cycling showed a significant reduction in GE associated with an increase in the oxygen cost of exercise

(Hopker, O'Grady and Pageaux 2016). The authors suggest that the reduction in GE despite no changes in power output may be secondary to an increase in mitochondrial or contractile inefficiency (Hopker, O'Grady and Pageaux 2016). However, it has been suggested that the mechanisms underpinning changes in GE during short acute bouts of HIE may be related to oxygen debt and excess post-exercise oxygen consumption (EPOC) (Ebreo, Passfield and Hopker 2020). Therefore, the physiological mechanisms underpinning changes in GE may be different depending on the intensity and duration of exercise. Furthermore, the mechanisms that affect GE after multiple sprint intervals during prolonged low intensity exercise remain unclear.

The aim of this study is to investigate the relative effects of varied intensity and duration on whole body gross efficiency and local muscle oxygenation.

4.3 Methods

4.3.1 Participants

Ten trained male participants (mean ± SD: age 38 ± 9 years, weight 82 ±11 kg, $\dot{V}O_{2max}$ 49 ±5 ml.kg^{-1}.min^{-1}, Maximal aerobic power [MAP] 377 ±52 W) volunteered to participate in the study. All participants had previously completed a minimum of three years of cycle training consisting of three training sessions a week on average including at least one high-intensity exercise session. The study was conducted ethical approval obtained from the School of Sport and Exercise Science Research Ethics Advisory Group (31_2018_19) and after obtaining informed written consent from all participants.

4.3.2 Experimental design

Visit 1 consisted of a maximal incremental exercise test to determine $\dot{V}O_{2max}$ and MAP. Visits 2 and 3 were randomised and participants were asked to complete a 90-min low-intensity endurance exercise (LCIE) or 90-min low-intensity endurance exercise interspersed with 15 x 10 s maximal sprint intervals (LCIE_SI) which were counterbalanced. Participants were

permitted to use a self-selected cadence (see Experimental Protocols). Visits were completed within the same 3 h period of the day within participants to reduce the effects of diurnal variability. Participants were asked to refrain from drinking alcohol and completing any strenuous exercise 24 h before visits. Participants were also instructed to eat a meal no later than 3 h prior to each visit and to replicate food and drink consumption during visits 2 and 3. Participants consumed food, energy drinks and gels without caffeine to prevent dehydration and glycogen depletion. Food and liquid intake was replicated for each protocol.

All tests were performed on an electromagenetically braked ergometer (Cyclus2; RBM elektronik-automation GmbH). Participants used their own bicycle or were provided with a laboratory bicycle with saddle height adjusted and replicated for subsequent visits. Gaseous exchange data was collected continuously throughout all trials on a breath-by-breath basis using an online gas analyser (Metalyser 3B; CORTEX Biophysik GmbH, Germany).

Prior to exercise testing, body mass was measured to the nearest 0.1kg using beam balance scales (Seca, Germany). Stature was measured to the nearest 0.5cm using a stadiometer (Seca, Germany). Participants completed an incremental test to determine $\dot{V}O_{2max}$ and MAP. Participants undertook an incremental exercise test to determine their $\dot{V}O_{2max}$ on the cycle ergometer. The test consisted of a 10 min warm up at 100W before conducting a step protocol where cycling power output increased by 20W every minute until volitional exhaustion (defined as a cadence below 60 revolutions per minute despite strong verbal encouragement). $\dot{V}O_{2max}$ was determined as the highest 60 s $\dot{V}O_2$ achieved during the incremental test. MAP was calculated as the average power output during the last 60 s of the test.

Muscle oxygenation was non-invasively measured during visits 2 and 3 using near-infrared spatially resolved dual-wavelength spectrometry (Portamon, Artenis Medical Systems, Netherlands). The NIRS optode was positioned over the distal vastus lateralis muscle approximately 10cm superior to the lateral epicondyle of the femur. Skinfold thickness was measured at the site of the NIRS device over the vastus lateralis muscle in the seated position using Harpenden skinfold calipers (British Indicators Ltd, Burgess Hill, UK). Adipose tissue

thickness (ATT) was calculated by taking the median of three skinfold measurements and dividing the skinfold measurements by 2 with a mean value of 7.1 ± 2.5 mm (Weits, Beek and Wedel 1986).

The NIRS optode was covered to minimise the unwanted effects of ambient light. Utilising 760nm and 850nm wavelengths, relative concentration changes in HHb were calculated from an arbitrary baseline taken 2-min prior to the start of each exercise protocol. An absolute measure of tissue oxygen saturation (TSI%) was also recorded.

4.3.3 Experimental protocols

The LCIE protocol consisted of 90-min continuous cycling at 50%$_{MAP}$. The LCIE_SI protocol consisted of 90-min cycling at 50%$_{MAP}$ interspersed with 15x10 s maximal sprints (see figure 4.1). Each sprint was performed at intervals of 350s. After each sprint participants were asked to resume cycling at 50%$_{MAP}$. Each protocol was preceded (Pre100) and followed (Post100) with a submaximal bout at 100W to measure GE.

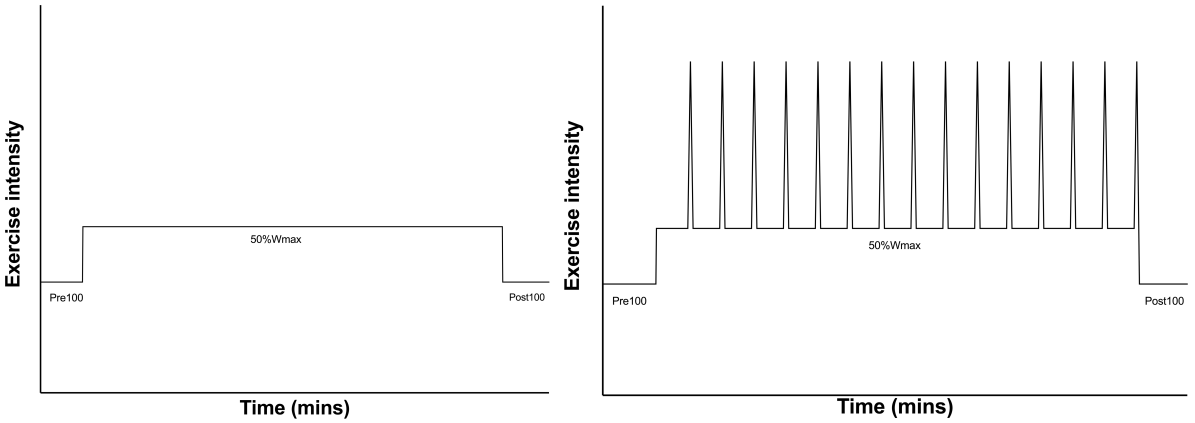

Figure 4.1 Exercise protocols for a) LCIE and b) LCIE-SI

4.3.4 Data analysis

Expired gas data was measured at steady-state during the last 3 mins of 1) the 100W bouts preceding and following the LCIE and LCIE_SI protocols and 2) the 3mins preceding each sprint

interval during the LCIE_SI protocol. GE was calculated using the equations 3.1 and 3.2 in chapter 3.

BGE was calculated by fitting a linear regression to the $\dot{V}O_2$ data points of the last 3 min of the of each stage. $\dot{V}O_2$ points with an RER >1.0 were omitted during this time period. These values were then back-extrapolated to the end of the sprint interval to estimate the GE change. As sprint interval 15 is followed by Post100 this interval was not analysed when comparing changes in CGE and BGE for LCIE and LCIE_SI.

4.3.5 Statistical analysis

Shapiro-Wilk tests were used to assess for normality of distribution. Participant characteristics and exercise testing data are presented as means ± standard deviation. A two-way repeated measures ANOVA (2 protocol x 14 timepoints) was conducted to assess for differences in CGE, BGE, TSI and HHb for LCIE and LCIE-SI. Statistical significance was set at $P \leq 0.05$. All values are presented as mean $\pm$SD unless otherwise stated. Statistical analysis was conducted using the SPSS statistical software package (IBM SPSS Statistics, Rel. 25.0, SPSS, Inc, Chicago, USA).

4.4 Results

4.4.1 Mean conventional and back-extrapolation GE changes

As shown in Figure 4.2a, there was no significant difference in pre-protocol CGE (PreCGE) ($P = 0.52$) or post-protocol CGE (PostCGE) ($P = 0.73$) for LCIE and LCIE_SI. PostCGE was significantly lower than PreCGE by $1.35 \pm 0.3\%$ in LCIE ($P= 0.04$) and by $2.06 \pm 0.1\%$ in LCIE_SI ($P = 0.0001$).

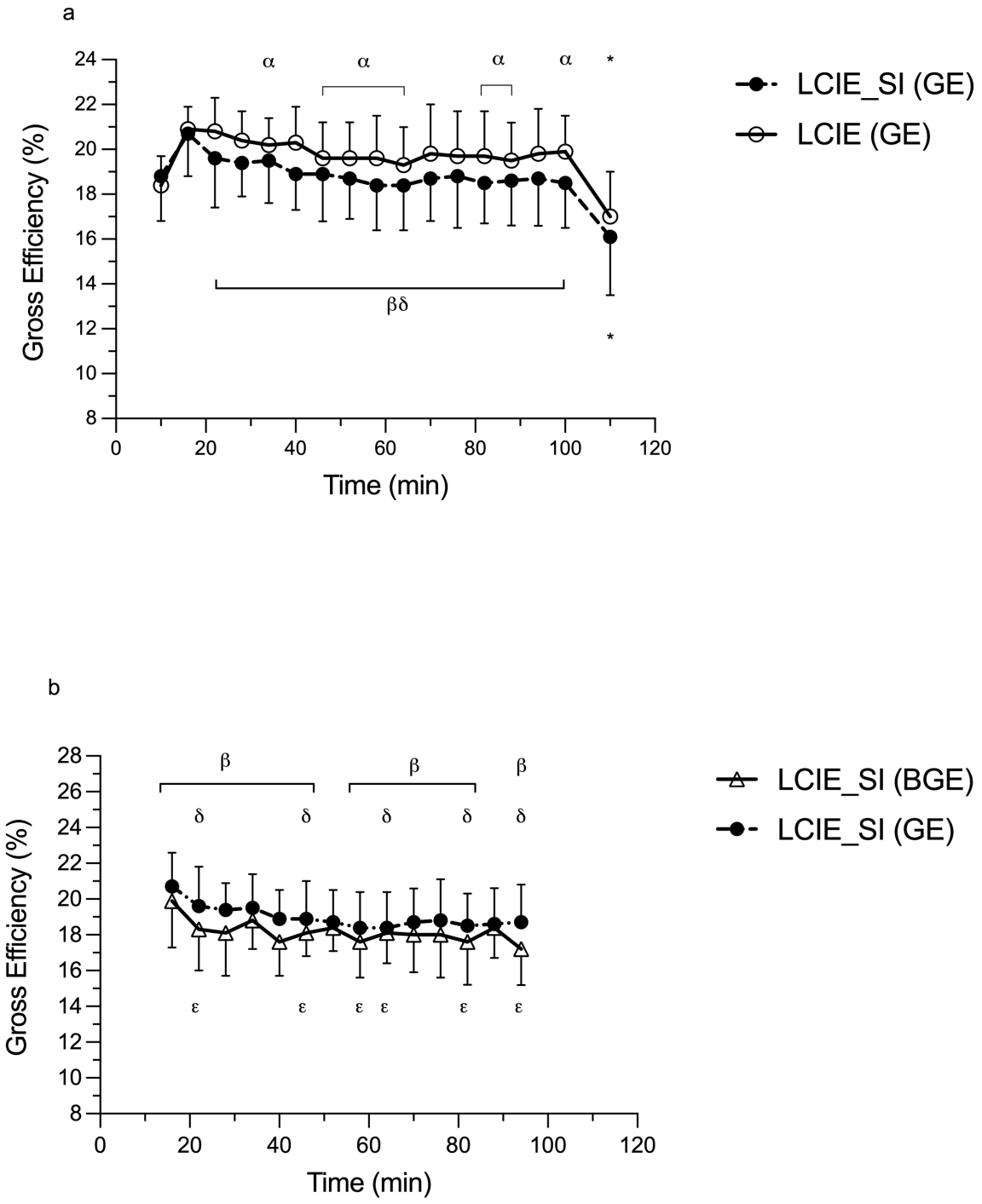

Figure 4.2 *(a) Mean changes in CGE during LCIE (clear dots, solid line) and LCIE_SI (solid dots, dashed line). (b) Mean changes in CGE (solid dots, dashed line) and mean changes in BGE (clear triangle and solid line) during LCIE_SI intervals 1 – 14 (min 16 – 94). * = significant difference in PostCGE compared to PreCGE. α = significant difference in LCIE. δ = significant difference in LCIE_SI. β = significant difference between protocols. Significant differences (P = ≤0.05) compared to baseline (min 16)*

Comparing LCIE and LCIE_SI CGE showed there was no significant overall difference in protocol (F = 3.17, P = 0.12) but there was a significant difference overall between timepoints (F = 6.37, P = 0.01; see Figure 4.2a). Timepoints 2-15 were significantly different between the two protocols (P = ≤0.05). There was no significant interaction effect between condition and timepoint (F = 1.91, P = 0.14). In LCIE, there was a significant difference between timepoints (F = 3.98, P = 0.01) with timepoints 4, 6-9, 12,13 and 15 significantly different to baseline (P = ≤0.05). In LCIE_SI, there was a significant difference in timepoint (F = 5.28, P = 0.005) with 2-15 being lower than baseline (P = ≤0.05).

There was a significant interaction effect between protocol and timepoint (F = 3.36, P = 0.01) when comparing LCIE_SI CGE and LCIE_SI BGE (see Figure 4.2b). There was also a main effect for protocol (19.96 ± 0.50% vs 17.9 ± 0.64% for CGE and BGE respectively, F = 9.03, P = 0.02) and timepoint (F = 4.15, P = 0.008). Timepoints 1-5, 7-12 and 14 were significantly different between the two protocols (P = <0.001). In LCIE_SI BGE, there was a significant difference in timepoint (F = 3.61, P = 0.009) with timepoints 2, 6, 8, 9, 12, 14 significantly different to baseline.

4.4.2 Power

As shown in Figure 4.3a, there was no significant difference between mean power in LCIE and LCIE_SI protocols (not including SI efforts) (F = 2.01, P = 0.19). There was no significant overall difference between timepoints for the two protocols (F = 2.14, P = 0.10) and no significant interaction effects between condition and timepoint (F = 2.56, P = 0.08).

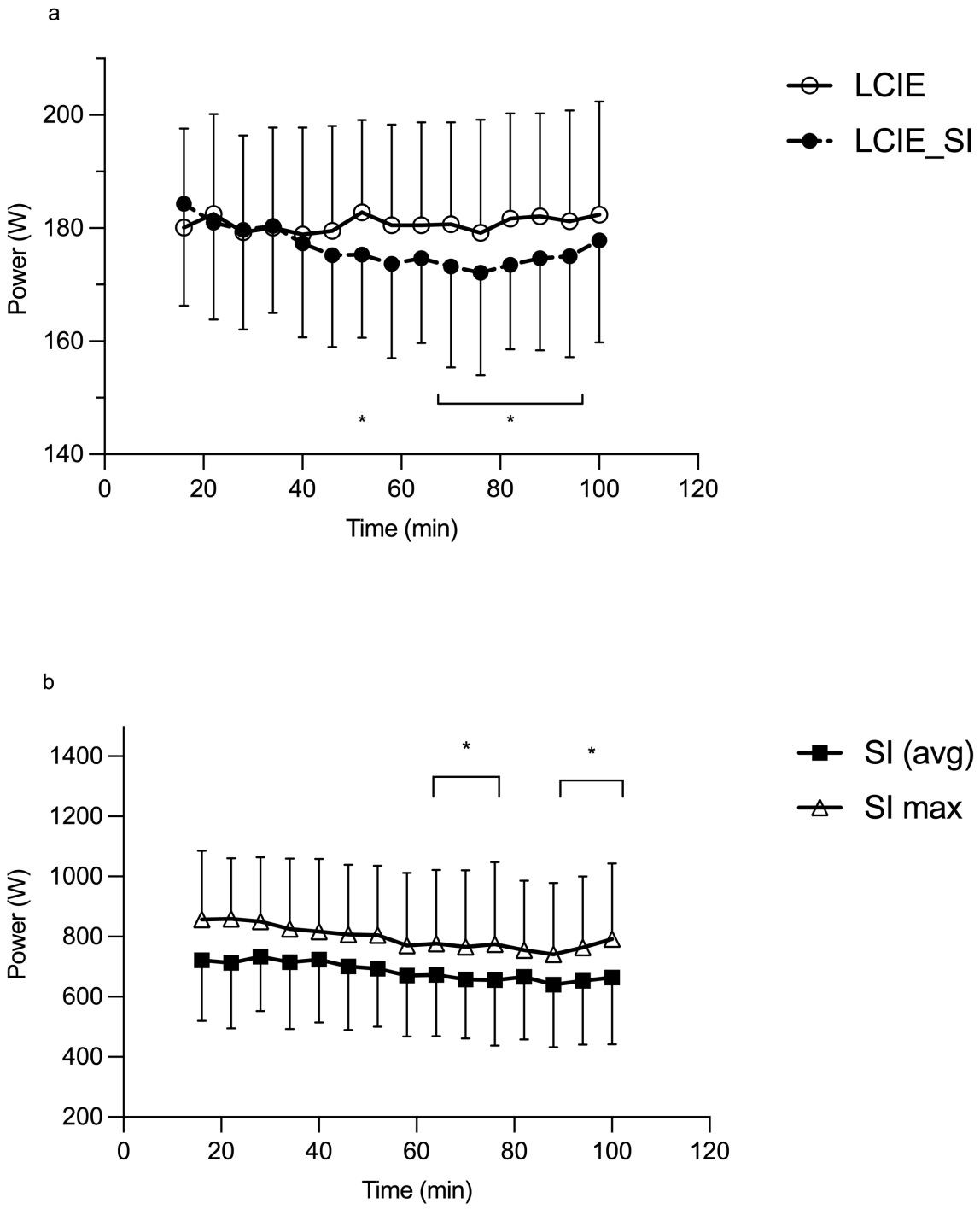

Figure 4.3 *(a) Mean average power during 15 x 6min intervals at 50%$_{MAP}$ for LCIE (clear dots, solid line) and 15 x 5min 50s intervals at 50%$_{MAP}$ for LCIE_SI (solid dot, dashed line). (b) Mean average power during 15 x 10s sprint intervals (solid squares, solid line) and mean maximum power during 15 x 10s sprint intervals (clear triangles, solid line). Mean ± SD, n=10. * = a significant difference (P = ≤0.05) from baseline.*

No significant differences were found for timepoint (see figure 4.3a) in LCIE (F= 0.86, P = 0.47) but there was a significant difference in LCIE_SI (F = 3.06, P = 0.04). During LCIE_SI there was a significant difference in mean average power between sprint intervals for timepoints 7, 9, 10, 11, 12, 13 and 14 (P = ≤0.05) compared to baseline with a mean change of 9 ± 4W.

As shown in Figure 4.3b, in the LCIE_SI protocol there was an overall effect of timepoint observed in the sprint interval average (SI_{av}) (F = 2.90, P = 0.04) for timepoints 7-15 compared to timepoint 3 (P = <0.001) with a mean change of 70 ± 14W. There was a significant overall effect of timepoint in sprint interval maximal power (SI_{Max}) (F = 4.96, P = 0.002) with significant differences in SI_{max} for intervals 8-10, and 12-14 (P = <0.001) compared to baseline with a mean change of 93 ± 31W.

4.4.3 $\dot{V}O_2$, $\dot{V}E$ and RER

Between protocols there was no significant difference in $\dot{V}O_2$ (see figure 4.4) pre-protocol (P = 0.79) or post-protocol (P = 0.48). $\dot{V}O_2$ was significantly higher post-protocol compared to pre-protocol for LCIE (1.61 ± 0.26 vs 1.79 ±0.26 $L{\cdot}min^{-1}$, P = 0.03) and LCIE_SI (1.62 ±0.22 vs 1.87 ±0.16 $L{\cdot}min^{-1}$, P = 0.01).

Repeated measures ANOVA for $\dot{V}O_2$ showed there was no overall effect of protocol (F = 3.05, P = 0.13) but there was a significant effect of timepoint (F = 6.18, P = <0.001; Figure 4.4). Timepoints 2-15 were significantly different between the two protocols (P = <0.001). There was no interaction effect between protocol and time (F = 1.51, P = 0.25). In LCIE, there was no significant difference in timepoint (F = 2.73, P = 0.07). There was a significant difference in timepoint for LCIE_SI (F= 7.90, P = <0.001) with timepoints 2-15 significantly different to baseline (P = ≤0.05).

Between protocols there was no significant difference in $\dot{V}E$ pre-protocol (P = 0.38; Figure 4.5a) or post-protocol (P=0.48). $\dot{V}E$ was significantly higher post-protocol compared to pre-protocol in both LCIE (42.00 ± 5.76 vs 47.97 ±7.78 $L{\cdot}min^{-1}$, P = 0.02) and LCIE_SI (41.03 ± 4.52 vs 53.49 ± 5.07 $L{\cdot}min^{-1}$, P = 0.01). Repeated measures ANOVA for $\dot{V}E$ showed that there was a significant overall effect of protocol (69.06 ± 9.42 $L{\cdot}min^{-1}$ vs 81.81 ± 10.84 $L{\cdot}min^{-1}$, F = 26.46,

P = 0.001) and timepoint (F = 10.82, P = <0.001 and a significant interaction effect between protocol and time (F = 3.49, P = 0.02). Timepoints 2-15 were significantly different between the two protocols (P = <0.001). There was a significant difference for timepoint in LCIE (F = 4.34, P= 0.01) and LCIE_SI (F = 13.08, P = <0.001), with all timepoints higher than baseline.

Between protocols there was no significant difference in RER pre-protocol (P = 0.67; Figure 4.5b) or post-protocol (P=0.71). RER was significantly lower post-exercise compared to pre-exercise for LCIE (0.94 ±0.02 vs 0.89 ± 0.04, P = 0.0001) and LCIE_SI (0.94 ±0.02 vs 0.89 ±0.02, P = 0.007). Comparison of RER showed that there was no significant overall effect of protocol (P=1.0) but there was a significant overall effect of timepoint (F = 12.87, P = <0.001) with timepoints 3-15 significantly different compared to baseline. There was no significant interaction effect between condition and time (F = 1.31, P = 0.29). There was a significant difference for timepoint in LCIE (F = 8.75, P = <0.001) with stages 3, 5, 7-15 significantly different to baseline (P = ≤0.05). There was a significant difference for timepoint in LCIE_SI (F = 9.83, P = <0.001) with all stages significantly lower to baseline.

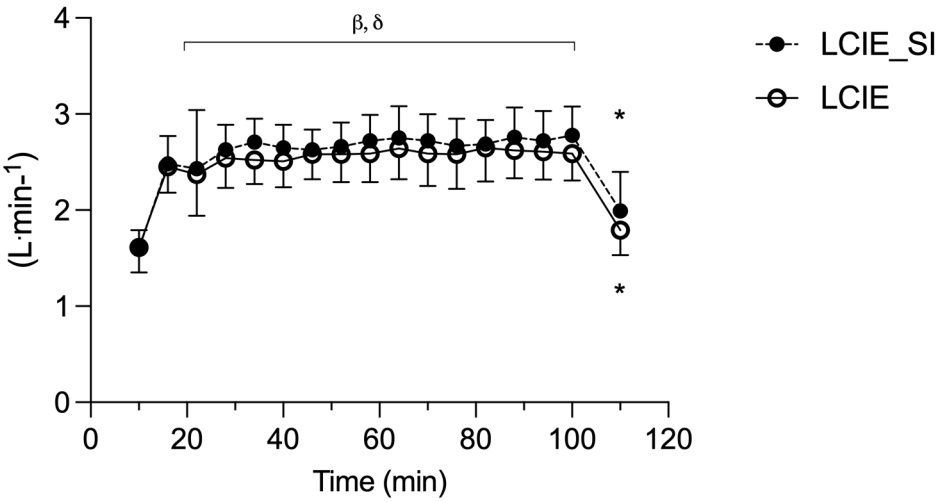

Figure 4.4 *Mean changes in $\dot{V}O2$ for LCIE (clear dots, solid line) and LCIE_SI (solid dots, dashed line). * = significant difference compared to pre-protocol (P = ≤0.05). α = significant difference in LCIE. δ = significant difference in LCIE_SI. β = significant difference between protocols. Significant differences (P = ≤0.05) compared to baseline (min 16)*

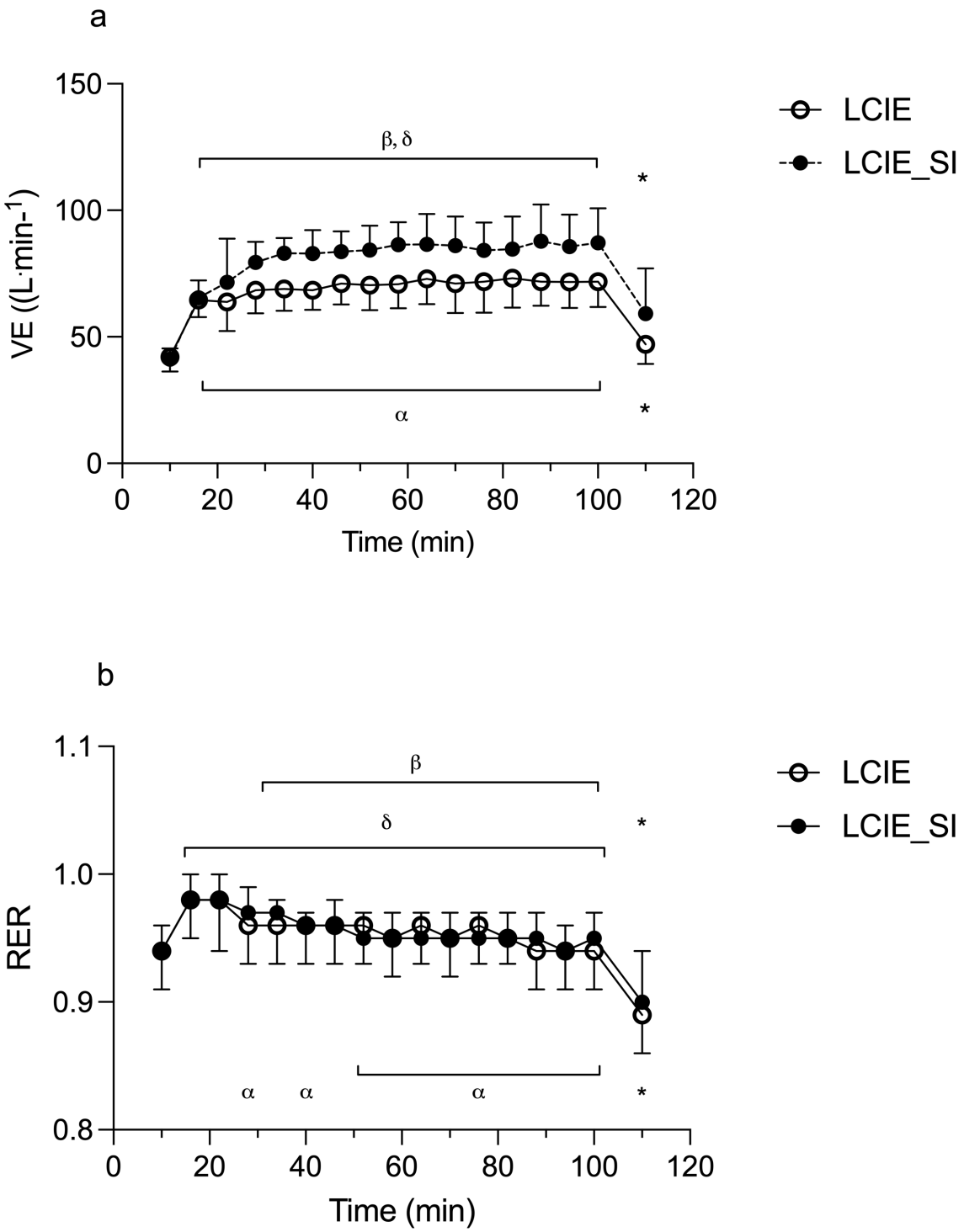

Figure 4.5 *Mean changes in (a) $\dot{V}E$ ($L \cdot min^{-1}$) and (b) RER for LCIE (clear dots, solid line) and LCIE_SI (solid dots, dashed line). * = significant difference compared to pre-protocol (P = ≤0.05). α = significant difference in LCIE. δ = significant difference in LCIE_SI. β = significant difference between protocols. Significant differences (P = ≤0.05) compared to baseline (min 16)*

4.4.4 Energy expenditure

Repeated measures ANOVA of kJ showed that there was no overall effect of protocol (F = 3.80, P=0.08) or timepoint (F = 2.06, P = 0.11) between LCIE and LCIE_SI (Figure 11a). There was no significant interaction effect between condition and time (F = 2.60, P=0.06). A significant overall effect of timepoint was observed in LCIE_SI (F= 3.09, P = 0.04) but not in LCIE (F = 0.53, P = 0.68) or SI (F = 2.17, P = 0.14). In LCIE_SI, timepoints 11-13 and 15 were significantly different to baseline (P = ≤0.05).

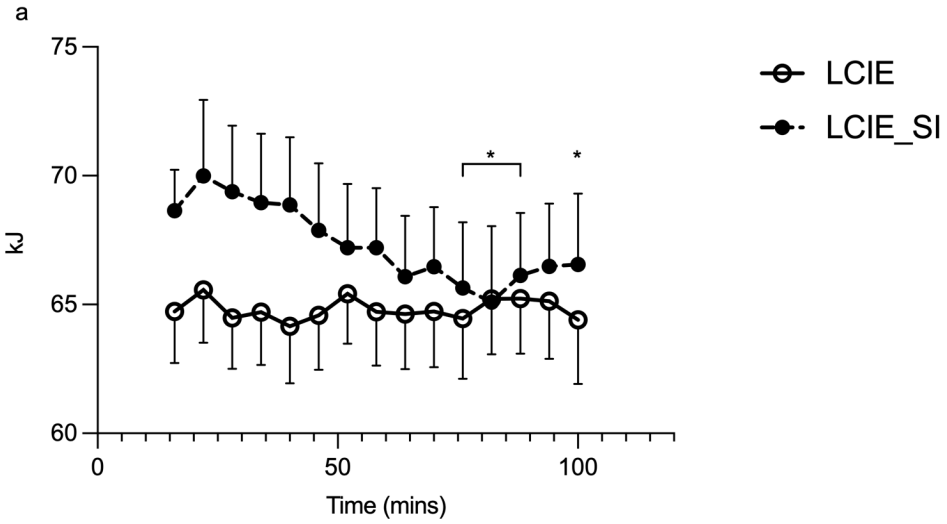

***Figure 4.6** (a) Mean average energy expenditure during 15 x 6min intervals at 50%$_{MAP}$ for LCIE (clear dots, solid line) and for LCIE_SI (solid dot, dashed line).*

4.4.5 NIRS responses

Between protocols there was no significant difference in TSI pre-protocol (P = 0.70) or post-protocol (P = 0.54). TSI was significantly lower post-protocol compared to pre-protocol for LCIE (73.58 ± 7.78% vs 65.40 ±9.25%, P = 0.01) and LCIE_SI (74.86 ±6.74 vs 68.66 ±9.33%, P = 0.02; see Figure 12 a and b).

Comparing TSI during both protocols, there was a significant difference between protocols (F = 8.76, P = 0.03, mean difference +1.06% in LCIE_SI) but no difference in timepoint (F = 0.97, P = 0.43). There was no interaction effect (protocol x timepoint) (F = 1.19, P = 0.35). Within each protocol, there was no effect of timepoint in LCIE (F = 1.43, P = 0.28) or LCIE_SI (F = 0.71, P = 0.55). There was no effect of timepoint for the sprint intervals (F = 1.54, P = 0.26).

Between protocols there was no significant difference in HHb pre-protocol (P = 0.54) or post-protocol (P = 0.97). HHb was significantly higher post-protocol compared to pre-protocol for LCIE (3.85 $\pm$ 3.59 vs 11.95 $\pm$6.10, P = 0.01) and LCIE_SI (4.30 $\pm$ 4.18 vs 12.10 $\pm$8.12, P = <0.001; see figure 4.8c and 4.8d).

Comparing HHb during both protocols, there was a significant difference between protocols (F = 6.92, P = 0.04, mean difference +1.26 in LCIE_SI) but no difference in timepoint (F = 1.67, P = 0.22). There was no interaction effect (protocol x timepoint) (F = 1.20, P = 0.34). Within each protocol, there was no effect of timepoint in LCIE (F = 3.61, P = 0.06) or LCIE_SI (F = 5.92, P = 0.55). There was no effect of timepoint for the SI (F = 0.96, P = 0.42).

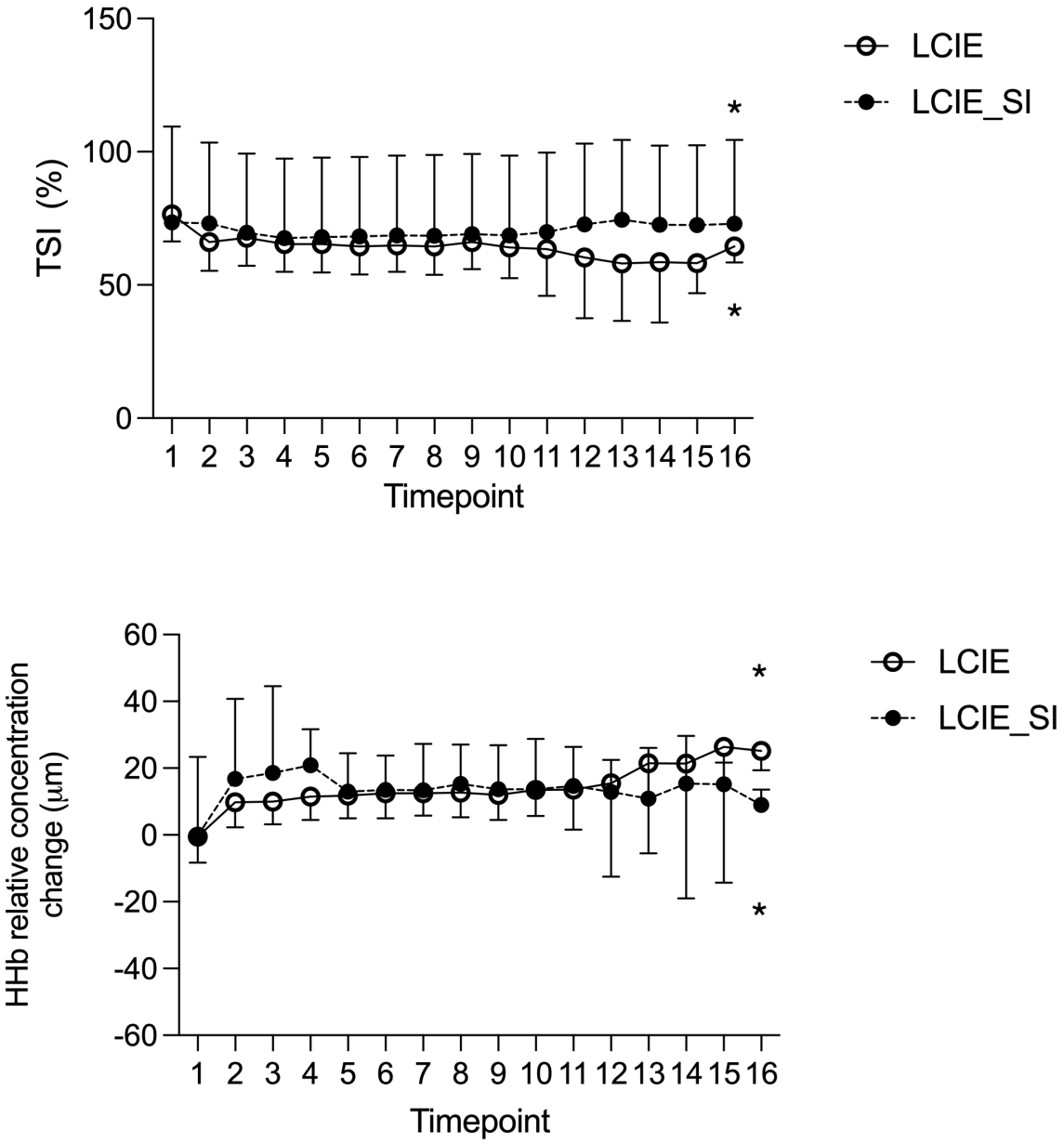

Figure 4.7 *Mean TSI (%) for (a) LCIE and (b) LCIE_SI. Mean HHb concentration change for (c) LCIE and (d) LCIE_SI. TSI indicates tissue saturation index; HHb, deoxygenated haemoglobin. * = significant difference compared to pre-protocol*

4.5 Discussion

The main findings of this study are 1) CGE was reduced after both LCIE and LCIE_SI protocols, 2) BGE was lower than CGE in LCIE_SI, 3) $\dot{V}$E was higher during and after LCIE_SI, 4) TSI and HHb were higher during LCIE_SI but not post-protocol compared to LCIE.

In the present study, the decline in GE (~1%) in the present study is the same after 90 mins cycling at 50%$\dot{V}O_{2max}$ (LCIE) as 240 mins cycling at 50%$\dot{V}O_{2max}$ (Almquist *et al.* 2019). One reason for this difference could be that participants in the Almquist et al. (2019) study were permitted 5-min lavatory breaks every hour. This 5 min break could allow GE to recover towards pre-exercise levels. Therefore, whilst duration of exercise is a factor in GE decline, it is possible that GE could be substantially lower after 240 mins continuous cycling. It is also possible that during constant load intensity cycling there is a maximal level of GE decline. Noordhof et al. (2014) demonstrate that shorter time-trials (1000m and 4000m) exhibit a linear decline in GE whereas a more hyperbolic decline in GE is seen in a time-trial of longer duration (40,000m). Interestingly, the final decrement in GE was attained at 50% of the final time of the 40,000m time-trial with the authors suggesting that the anaerobic contribution at the start of the trial causes this decrement in GE (Noordhof et al. 2014).

CGE reduced by ~2% after LCIE_SI compared to ~1% in the study by Almquist et al. (2008). Whilst both studies had participants cycle at 50% $\dot{V}O_{2max}$, the protocols differed markedly in overall duration (90 mins in the present study vs 240mins), and the number and duration of sprint intervals (15 x 10s sprints in the present study vs 3 x 30s sprints at the end of each hour). The higher number and frequency of sprint intervals in the present study could potentially yield a greater anaerobic contribution and oxygen deficit creating a greater EPOC between sprint intervals that cannot recover fully at submaximal intensities above 50%$_{MAP}$. The fact that there was a similar decline in GE after both 240 mins cycling at 50%$\dot{V}O_{2max}$ and 240 mins cycling at 50%$\dot{V}O_{2max}$ interspersed with sprints in the study by Almquist et al. (2019) would suggest that any oxygen debt incurred from the sprint intervals has been resolved. This contrasts with previous research which has shown that GE does not fully recover after a 20,000m time-trial during 30-min of submaximal cycling at a 55%$_{MAP}$ (Groot *et al.* 2018).

Further investigation is needed to assess how an active recovery bout at a lower intensity (below 50%$\dot{V}O_{2max}$) or longer duration would affect GE.

In agreement with Almquist et al. (2019), $\dot{V}O_2$ and $\dot{V}E$ increased over time with a reduced CGE post-protocol after both low intensity cycling and low intensity cycling interspersed with sprint intervals. The rise in $\dot{V}O_2$ after prolonged low to moderate intensity exercise and prolonged low intensity exercise with interspersed sprints is in line with previous research (Passfield and Doust 2000; Hopker, O'Grady and Pageaux 2016; Almquist *et al.* 2019). It is possible that the reduction in GE may be secondary to changes in localised oxygen supply and/or utilization within exercising muscle (Hamaoka *et al.* 1996). In agreement with (Hopker, O'Grady and Pageaux 2016), there is a progressive localised vastus lateralis desaturation seen in the present study (as indicated by an increasing HHb and decreasing TSI). It has previously been shown that BGE and HHb increases and TSI decreases after 4 min HIE at 100%$_{MAP}$ suggesting a larger oxygen deficit and a greater excess post-exercise oxygen consumption compared to cycling at 80%$_{MAP}$ (see chapter 3). However, in agreement with previous studies examining prolonged cycling exercise (Almquist *et al.* 2019) or time-trials of 20,000m (Groot *et al.* 2018), CGE does not return to pre-exercise levels after a period of submaximal cycling. This suggests that an intensity lower than 50%$_{MAP}$ or a longer duration of recovery is required for GE to fully recover.

In agreement with previous studies $\dot{V}E$ was increased and $\dot{V}O_2$ was decreased after prolonged low to moderate intensity cycling (Passfield and Doust 2000; Hopker, O'Grady and Pageaux 2016; Almquist *et al.* 2019), and $\dot{V}E$ was higher and $\dot{V}O_2$ lower at all timepoints after prolonged low intensity cycling interspersed with sprints compared to low intensity cycling (Almquist *et al.* 2019). Using the work of (Vella, Marks and Robergs 2006) an increase in VE has been calculated to account for a variance of 0.2-0.3% GE units (Hopker *et al.* 2013). However, this was calculated with the assumption that the oxygen cost of ventilation ($\dot{V}O2_{VENT}$) was between 2.14 and 2.74 ml·L^{-1} (35 – 50% maximal ventilation) with participants cycling at 150W (Hopker *et al.* 2013). Both high-intensity exercise and sprint-interval exercise involve VE rates higher than 50% maximal ventilation and would therefore induce a higher $\dot{V}O2_{VENT}$. (Vella, Marks and Robergs 2006) estimate that the $\dot{V}O2_{VENT}$ to be between 2.18 and

2.53 ml·L at 75 – 100% maximal ventilation, therefore, the variance in GE may be greater at maximal and supramaximal intensities. In endurance trained males it has been estimated that the oxygen uptake of respiratory muscles can be as high as 15% of $\dot{V}O_{2max}$ (Aaron *et al.* 1992) with ~15% of cardiac output taken up by the respiratory muscles during maximal exercise (Coast *et al.* 1993; Harms *et al.* 1997). Respiratory fatigue initiates a metaboreflex mediated increase in muscle sympathetic nerve activity causing vasoconstriction in the exercising limb muscle (Harms et al. 1997; Hill et al 2000; St. Croix et al. 2000) with a subsequent reduction of both blood flow and oxygen delivery to the working skeletal limb muscle which exacerbates limb fatigue and compromises exercise performance (Harms et al. 2000). In the present study, whilst there was a significant difference in $\dot{V}E$ between the two protocols there were no significant differences in TSI or HHb suggesting that 10s sprint intervals separated by 5 min 50s is not sufficient to compromise oxygen delivery to skeletal muscle.

It has been speculated that this localised skeletal muscle desaturation during prolonged cycling may be secondary to mitochondrial uncoupling during exercise with an alteration in the ratio between mitochondrial ADP phosphorylation and oxygen consumption (P/O ratio) which is a reflection of the efficiency of oxidative phosphorylation (Hopker, O'Grady and Pageaux 2016). It has been shown that mitochondrial coupling is higher during state 4 (resting) respiration compared to state 3 (exercise) (Sakellariou, Jackson and Vasilaki 2013; Ji, Kang and Zhang 2016) with acute exercise stimulating the increased expression of uncoupling proteins in skeletal muscle mitochondria (Mogensen *et al.* 2006). These uncoupling proteins have been shown to move protons from the intermembrane space to the matrix and thus potentially reduce the efficiency of oxidative phosphorylation (Mogensen *et al.* 2006; Jiang *et al.* 2009) . These uncoupling proteins also protect mitochondria against oxidative stress by decreasing ROS formation (Brand *et al.* 2005; Jiang *et al.* 2009; Sahlin *et al.* 2010).

High levels of ROS have been shown to cause muscle damage and contractile dysfunction (Powers and Jackson 2008) with ROS levels exerting a biphasic influence on muscle force production (Reid *et al.* 1992; Reid, Khawli and Moody 1993; Reid *et al.* 2005) which may have contributed to the decrease in LCIE_SI average power, SI_{av} and SI_{max} towards the end of the LCIE_SI protocol with a subsequent decrease in BGE. (St-Pierre *et al.* 2002; Sakellariou *et al.* 2013). An increase in oxygen consumption in skeletal muscle could also be secondary to additional mitochondrial ATP generation to decrease ROS from other potential sources

including NADPH oxidase which is associated with intense muscle contractions (Sakellariou *et al.* 2013) and xanthine oxidase activation secondary to AMP degradation during an aerobic exercise and an increase in xanthine and hypoxanthine following anaerobic sprint intervals (Mastaloudis, Leonard and Traber 2001; Kang *et al.* 2009; Radak *et al.* 2013).

Increased muscle fibre recruitment may be a cause of the reduction in the $\dot{V}O2$ slow component and the reduction of GE in the present study. Noordhof et al. (2015) found during time-trials of varying distance that there was a small but not significant mean increase in iEMG of the gluteus maximus, vastus medialis, vastus lateralis and biceps femoris but there was a mean effect of time. More recently, Almquist *et al.* (2019) showed a gradual recruitment of muscle motor units over time during prolonged (240 minutes) low-intensity cycling at 50%$\dot{V}O2_{max}$ using iEMG in the vastus lateralis and vastus medialis with the authors speculating that changes in motor-unit recruitment may reflect a decreasing efficiency of already recruited fibres. Vanhatalo et al. (2011) suggest that the decrease in efficiency during an all-out test may be secondary to a combination of factors which include the slow kinetics of the initially recruited type 2 muscle fibres, reduced contractile efficiency (due to the accumulation of metabolites such as H+, Pi, ADP and ROS or greater depletion of substrates such as muscle phosphocreatine) and EPOC. In the present study, there is a greater decrease in CGE and BGE seen both during and after LCIE_SI which may be due to a combination of progressive muscle recruitment and increasing metabolic and mechanical inefficiency. A limitation of the current study is that muscle motor unit recruitment was not assessed and so the effect of additional muscle motor unit recruitment on GE during prolonged exercise interspersed with repeated SI remains unknown.

In agreement with previous research (Passfield and Doust 2000; Hopker, O'Grady and Pageaux 2016) there was a reduced RER after LCIE indicating a change in substrate utilisation. RER has been found to decrease during prolonged moderate intensity cycling (60%$\dot{V}O_{2max}$) after 120min (Hopker, O'Grady and Pageaux 2016; Passfield and Doust 2000) but not after 240min at 50%$\dot{V}O_{2max}$ (Almquist *et al.* 2019).

Food, energy gels, water and energy drinks were consumed ad libitum and were matched between experimental protocols in the present study. A limitation of the present study is that there was no familiarisation trial on which participants could formulate a nutrition strategy for the experimental protocols. Furthermore, the food and drink consumed between participants was not standardised leading to differences in macronutrients absorbed. Therefore, as the calculation of GE takes into account changes in substrate utilisation, together with a rise in $\dot{V}O_{2max}$ this could play a part in the reduction in GE after both LCIE and LCIE_SI. Another potential limitation of the current study is that participants were allowed to cycle at their preferred cadence during each protocol as well as pre- and post-protocol. However, previous research has shown that the preferred self-selected cadence adopted by cyclists (80 – 100 revolution per minute) are higher than what is deemed to be a metabolically optimal cadence (50 – 70 revolutions per minute) (Brisswalter *et al.* 2000; Sarre *et al.* 2003; Vercruyssen *et al.* 2005; Vercruyssen and Brisswalter 2010).

4.6 Conclusions

This study demonstrates that performing repeated sprint intervals during prolonged low-intensity exercise reduces BGE and TSI with an increase in HHb which is reflected in a decline of both SI average and SI maximal power. Furthermore, during prolonged low-intensity exercise with repeated short supramaximal intensity intervals GE does not recover to pre-exercise levels.

5 The effect of different acute iso-effort protocols on gross efficiency

5.1 Abstract

Purpose: To investigate the reliability of measuring changes in conventionally calculated gross efficiency (GGE) and using a back-extrapolation method (BGE) during exercise of different intensities and durations.

Methods: 17 trained participants randomly completed three different iso-effort (self-paced maximum sustainable intensity exercise) protocols three times; a constant interval of 40min (CONT); 4 x 5 min intervals (LI); four sets of 10 x 30s intervals (SI). Each protocol ended with a 5 min bout of active recovery (AR5). To calculate BGE, a linear regression of post-exercise $\dot{V}O_2$ values were back extrapolated from AR5 to the end of the interval bout. Dynamic tissue oxygenation of the vastus lateralis was measured using Near-Infrared Spectroscopy (NIRS). A three-way repeated measures ANOVA (protocol x timepoint x visit) was used to compare differences between CONT, LI and SI for CGE, BGE, TSI and HHb.

Results: Reliability data for CONT, LI and SI demonstrated a mean CV of 12.1%, 12.3% and 9.0% respectively. Mean power output for CONT (262 ±40W), LI (310 ±45W) and SI (382 ±54W) were significantly different ($P \leq 0.05$). Mean CGE, TSI (Tissue Saturation Index) or HHb (deoxygenated haemoglobin) were not significantly different between the protocols. BGE was significantly different compared to Pre150 ($P = 0.02$) and Post150 ($P = 0.03$) between protocols.

Conclusion: All protocols GE, BGE, TSI and HHb recovered to pre-protocol levels after AR5. Acute exercise reductions in GE following 40 min of exercise of different intensity appear to be transient and short-term caused by an incurred oxygen deficit and an excess post-exercise oxygen consumption that has resolved by the end of an AR5. This suggests that the duration of exercise has a greater impact on GE recovery than intensity.

Keywords: iso-effort, excess post-exercise oxygen consumption, recovery

The following experimental chapter was undertaken in collaboration with another PhD researcher at the University of Kent, Ciaran O'Grady. Participants were shared and undertook identical exercise protocols. Whilst physiological markers (gas exchange, NIRS, blood lactate and RPE) were shared the analyses performed were different consistent with the separate aims of our respective theses.

5.2 Introduction

Chapter 3 found that BGE is a valid and reliable method of estimating GE during high-intensity exercise. Both Chapter 3 and 4 demonstrate that GE decreases after an acute bout of HIE and during acute prolonged low-intensity exercise interspersed with repeated sprint intervals. Metabolic energy cost is usually measured during steady state exercise where aerobic processes can be accounted for via expired gases measured at the mouth. This presents a challenge when investigating HIE involving both aerobic and anaerobic energy contributions. During HIE, together with an increase in ventilatory cost of exercise there is an increase in anaerobic metabolism, lactate production and bicarbonate buffering. This leads to an increase in $\dot{V}O2$ that is not attributable to substrate metabolism. Consequently, expired gas measured at the mouth during HIE does not reflect total energy expenditure. However, the effect of different acute bouts of exercise of different interval intensities and duration on both CGE and BGE remains unknown. Furthermore, chapter 3 demonstrates that the BGE method has been shown to be a valid and reliable method of estimating GE after a short (4 minute) bout of HIE at $100\%_{MAP}$. However, the reliability of measuring BGE after different interval intensities and duration has yet to be elucidated.

The efficiency of energy transfer within skeletal muscle mitochondria may be a contributory factor in whole body GE changes (Passfield and Doust 2000; Hopker, O'Grady and Pageaux 2016). Indeed, using spatially resolved near-infrared spectroscopy it has been shown that both deoxygenated haemoglobin (HHb) and Tissue Saturation Index (TSI) decrease after prolonged moderate cycling which could be indicative of a decrease in muscle contraction-coupling efficiency and mitochondrial inefficiency (Hopker, O'Grady and Pageaux 2016), also

reflected by a reduction in GE. Conversely, it has been suggested that the mechanisms underpinning reductions in GE after a 4 min bout at 100%$_{MAP}$ may be related to oxygen debt and EPOC (Ebreo, Passfield and Hopker 2020). Chapter 4 demonstrated that GE decreases after LCIE_SI, however the relationship between changes in GE and NIRS parameters after acute cycling exercise bouts of different intensities has yet to be investigated.

GE has been shown to increase during a competitive season when trained cyclists performed two HIE sessions a week for six weeks (Hopker *et al.* 2010). Numerous studies have demonstrated that HIE training (Little *et al.* 2011; Bartlett *et al.* 2012; Gibala *et al.* 2012; MacInnis and Gibala 2016)and SIT (Burgomaster 2005; Gibala *et al.* 2006; Burgomaster *et al.* 2008) induce beneficial cellular adaptations (Perry *et al.* 2010; Serpiello *et al.* 2012; McGinley and Bishop 2016; MacInnis and Gibala 2016) with a rise in performance determinants such as $\dot{V}O2_{max}$ (Helgerud *et al.* 2007). However, the acute effect of different interval intensity and duration on GE has yet to be investigated.

Chapter 3 demonstrated that CGE recovers fully to pre-HIE levels when cycling at 50%$_{MAP}$ for 10 minutes after a 4 min HIE bout at 100%$_{MAP}$ (Ebreo, Passfield and Hopker 2020) which suggests that the decrease in GE may be due to a transient oxygen deficit and EPOC. Conversely, CGE does not recover to pre-exercise levels when cycling at 50%$_{MAP}$ for 10 minutes after LCIE and LCIE_SI which may be due to a combination of progressive muscle recruitment and increasing metabolic and mechanical inefficiency (see chapter 4). Similarly, Groot et al. (2018) demonstrate that GE does not fully recover after time-trials of 2,000m or 20,000m during 30 minutes of cycling exercise at 55%$\dot{V}O2_{max}$. However, it is unknown whether GE can fully recover after acute maximal effort exercise of varying interval intensity and duration after a submaximal bout below 50%$_{MAP}$ and whether this recovery is secondary to a transient oxygen deficit and EPOC.

The purpose of this study is: 1) to assess the reliability and validity of the BE method in estimating GE during intervals of different intensity and duration 2) to investigate and compare changes in GE in response to a constant interval of 40 min (CONT); 4 x 5 min intervals (LI); two sets of 10 x 30s intervals (SI), 3) to assess the recovery of GE after exercise of different

intensities, 4) to investigate the relationship between changes in whole body GE and NIRS parameters from CONT, LI and SI exercise.

5.3 Methods

5.3.1 Participants

Seventeen healthy, trained male cyclists aged 18-55 years old participated in the study (Mean ±SD) age 34 ± 12 years, mass 71 ± 11kg, $\dot{V}O_{2max}$ 59 ± 8ml·kg^{-1}·min^{-1}, MAP 376 ± 46W. All participants had completed a minimum of three years of cycle training consisting of three training sessions a week on average, inclusive of at least one high-intensity exercise session. The purpose of the study, possible risks and potential discomfort were explained to the participants prior to obtaining written consent. The study was conducted with full ethical approval from the School of Sport and Exercise Science Research Ethics Advisory Group (ethics approval number: 106_2015_2016) and was conducted in accordance with the Declaration of Helsinki.

5.3.2 Experimental design

Participants attended the exercise testing laboratory on 10 separate occasions. Visit 1 consisted of a maximal incremental test to ascertain $\dot{V}O_{2max}$ and associated maximal aerobic power (MAP). Visits 2-10 consisted of three different exercise protocols (see section 5.3.4) each performed three times in a randomized order in order to test for the reliability of measuring GE after the three different acute HIE protocols. An iso-effort model was used as this has been shown to be more consistent with how endurance athletes train (Seiler and Sjursen 2004).

All tests were performed on an electromagnetically braked ergometer (Cyclus2; RBM elektronik-automation GmbH). Participants either used their own bicycle or were provided with a laboratory bicycle. If a laboratory bicycle was used, saddle height was adjusted for each individual during visit 1 and replicated for each subsequent visit. Gaseous exchange data was collected on a breath-by-breath basis during each visit using an online gas analyser (Metalyser 3B; CORTEX Biophysik GmBh, Germany). Each protocol was preceded and followed with a 5 min bout at 150 W to measure gross efficiency. Expired gas data was

measured at a steady state during the last 3-min of the 150W bouts preceding and following the iso-effort protocols.

Muscle oxygenation was continuously measured during visits 2 to 10 using near-infrared spatially resolved dual-wavelength spectrometry (Portamon, Artinis Medical Systems, Netherlands). The NIRS optode was covered to minimise the unwanted effects of ambient light. The device was placed on the belly of the vastus lateralis muscle 20 cm from the joint line of the right knee. Utilising 760nm and 850nm wavelengths, relative concentration changes in HHb were calculated from an arbitrary baseline taken 2-min prior to the start of each exercise protocol. An absolute measure of tissue oxygen saturation (TSI%) was also recorded. Skinfold thickness was measured at the site of the NIRS device over the vastus lateralis muscle in the seated position using Harpenden skinfold callipers (British Indicators Ltd, Burgess Hill, UK). Adipose tissue thickness (ATT) was calculated by taking the median of three skinfold measurements and dividing the skinfold measurements by 2 with a mean value of 6.5 $\pm$ 1.4 mm (Geraskin, Boeth and Kohl-Bareis 2009).

Rating of perceived exertion (RPE) using the Borg 6-20 scale (Borg 1982) was used before and immediately after every work bout (and at the end of a set in the case of the CONT and SI protocols). Finger prick blood lactate samples (Biosen C-Line analyser, EKF diagnositics, Wales) were collected prior to and at the end of each protocol as well as at the beginning and end of each work bout/set within a protocol (see figure 13).

5.3.3 Maximal Incremental Exercise Test

On arrival at the laboratory, body mass was measured to the nearest 0.1kg using beam balance scales (Seca, Germany). Stature was measured to the nearest 0.5cm using a stadiometer (Seca, Germany). Participants undertook an incremental exercise test to determine their $\dot{V}O_{2max}$ on the cycle ergometer. The test consisted of a 10-min warm up at 100W before conducting a step protocol where cycling power output increased by 20W every minute until volitional exhaustion (defined as a cadence below 60 revolutions per minute despite strong verbal encouragement). $\dot{V}O_{2max}$ was determined as the highest 60s $\dot{V}O_2$

achieved during the incremental test. MAP was calculated as the average power output over the final 60s of the test.

5.3.4 Protocols

As shown in Figure 5.1, the three protocols consisted of: 40 min of CONT cycling followed by 5 min active recovery interval; LI consisting of 4 x 5 min intervals interspersed with 4 x 5 min active recovery intervals; SI consisting of 4 sets of 10 x 30 s intervals interspersed with 30 s active recovery (sets 2 and 3 are separated with 5 min active recovery). There was a 5 min active recovery (AR5) interval immediately after each protocol which preceded the submaximal 5 min bout at 150W.

Participants were instructed to cycle at their maximal sustainable intensity for each of the protocol intervals (iso-effort) so that they 1) completed the prescribed interval and 2) with an even or slightly progressive power from the 1st to 4th interval (Seiler and Sjursen 2004).

Participants were allowed to cycle at their preferred cadence pre, post and during each prescription. Each bout was preceded (Pre150) and followed (Post 150) with a 5 min bout at 150 W to measure GE.

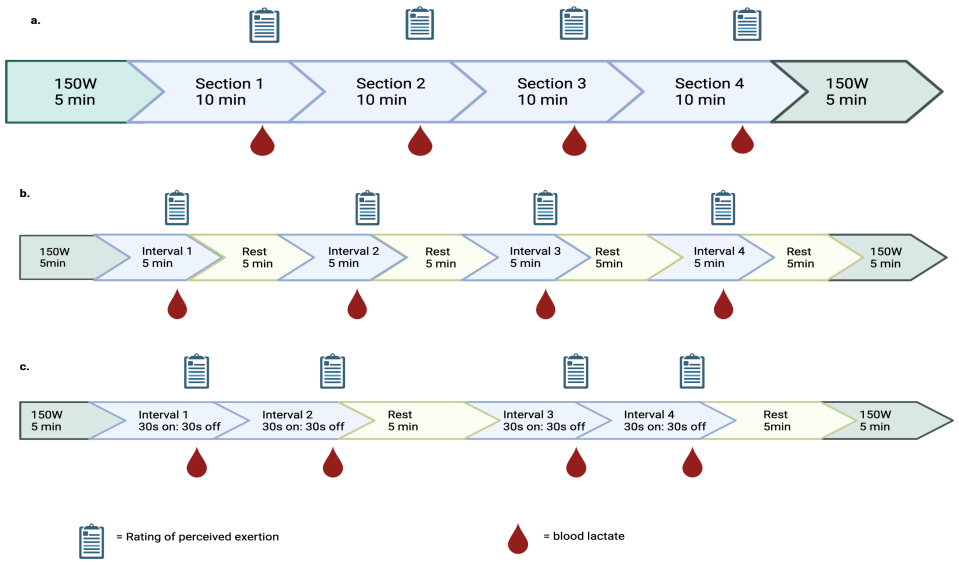

Figure 5.1 *Acute iso-effort protocols (a) Continuous Protocol Format. (b) = Long Interval Protocol Format. (c) = Short Interval Protocol Format*

5.3.5 Data analysis

GE was calculated using the equations 3.1 and 3.2 in chapter 3. $\dot{V}O_2$ data points in the last 3 min of each submaximal bout that had an RER >1.0 were excluded to avoid the contribution of unmeasured anaerobic work (Schenau and Cavanagh 1990). Previous research has shown that 3 minutes are needed for $\dot{V}O_2$ to reach a steady-state (Whipp and Wasserman 1972; Barstow and Mole 1991). Steady state conditions for the calculation of GE was accepted if the mean difference in $\dot{V}O_2$ between minute 3 and 4 and 5 and 6 expressed relative to the mean $\dot{V}O_2$ over the corresponding 3 minutes was <5.00%

BGE was calculated by fitting a linear regression to the GE data points of the last 3 min of the Post 150W period. GE data points with an RER >1.0 were omitted during this time period. These values were then back-extrapolated to the end of the interval protocol to estimate the GE change from Pre150 to Post150 (Koning *et al.* 2013; Ebreo, Passfield and Hopker 2020)

Each protocol was divided into 4 discrete intervals (as CONT is one long interval this has been divided into sections to demarcate timepoints where blood lactate and rating of perceived exertion have been obtained; see figure 5.1) to assess for between and within protocol differences in terms of CGE, BGE, $\dot{V}O_2$, VE, RER, TSI, HHb, lactate and rating of perceived exertion (RPE).

5.3.6 Statistical analysis

Shapiro-Wilk tests were used to assess for normality of distribution. All three visits of each protocol were used to assess reliability of CGE and BGE. Within-subject variation across the three repeated visits of each protocol was calculated using coefficient of variation (Hopkins WG., 2000). Relative changes in GE pre to post prescription were assessed using change scores both within and between each prescription (Hopkins WG.,2000). Three-way repeated measures ANOVA (protocol x timepoint x visit) was conducted to assess for differences between in dependent variables of CGE, BGE, $\dot{V}O_2$, $\dot{V}E$, RER, TSI, HHb, lactate and RPE. Data are presented as mean values for the whole protocol (work and rest intervals), work intervals

only, and rest intervals only for each of the protocols. Statistical significance was set at P ≤0.05 and confidence intervals (CI) calculated. All values are presented as mean ± SD unless otherwise stated. Statistical analysis was conducted using the SPSS statistical software package (IBM SPSS Statistics, Rel. 25.0, SPSS, Inc, Chicago, USA).

5.4 Results

Reliability of CGE and BGE

For Pre150 CGE there was mean CV of 6.7% (95% CL, 5.2% – 10.0%), 4.4% (95% CL, 3.4% - 6.3%) and 4.7% (95% CL, 3.8 – 6.5%) for LI, SI and CONT respectively. There was a mean CV of 5.4% (95% CL, 4.4% - 7.5%) in LI, 4.3% (95% CL, 3.3% - 6.1%) in SI and 5.8% (95% CL, 4.4% - 8.5%) in CONT for Post150 CGE.

For BGE there was mean CV of 12.3% (95% CL, 9.3% – 18.2%), 9.0% (95% CL, 6.9% - 13.0%) and 12.1% (95% CL, 9.3 – 18.2%) for LI, SI and CONT respectively (see table 5.1).

Table 5.1 *Mean typical error (CV) with upper and lower confidence levels (CL) for LI, SI and CONT for Pre150 CGE, Post150 CGE and BGE*

LI	**Pre150**	**Post150**	**BGE**
Typical error (CV)	6.7	5.4	12.3
Upper CL	5.2	4.4	9.3
Lower CL	10.0	7.5	18.2
SI	**Pre150**	**Post150**	**BGE**
Typical error (CV)	4.4	4.3	9.0
Upper CL	3.4	3.3	6.9
Lower CL	6.3	6.1	13.0
CONT	**Pre150**	**Post150**	**BGE**
Typical error (CV)	4.7	5.8	12.1
Upper CL	3.8	4.4	9.3
Lower CL	6.5	8.5	18.2

5.4.1 Changes in GE

There was a significant difference between timepoints (F = 6.05, P = 0.01) but not between protocols (F = 1.66, P = 0.23) or visit (F = 24.40, P = 0.06) when comparing CONT, LI and SI. Timepoints Pre150 (P = 0.02) and Post150 (P = 0.03) were significantly different to BGE between CONT, LI and SI (see figure 5.2).

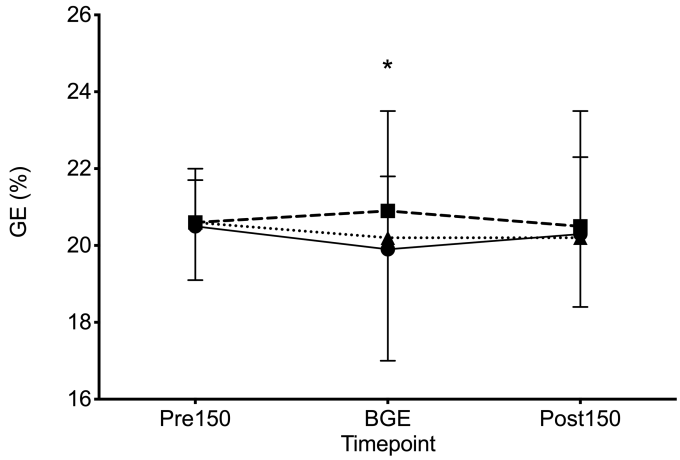

Figure 5.2 *Mean CGE across participants (Pre150, Post150) and using back-extrapolation (BGE) for CONT (circle, solid line), LI (square, dashed line) and SI (triangle, dotted line). * = significant difference in Pre150 and Post150 compared to BGE (P = ≤0.05)*

There was a significant interaction effect between protocol x visit between CONT, LI and SI (F = 0.94, P = 0.03) but there were no significant interaction effects between protocol, timepoint and visit (F = 0.52, P = 0.07), between protocol x timepoint (F= 0.03, P = 0.39) or between timepoint x visit (F= 2.45, P = 0.07).

There was an interaction effect between timepoint and visit for CONT (F = 0.34, P = 0.79) but there were no significant differences between Pre150 CGE (20.7 ± 1.7%) BGE (20.6 ±2.3%) or Post150 CGE (20.0 ± 2.9%) for timepoint (F = 0.18, P = 0.93) or visit (F = 0.95, P = 0.07).

In LI, there were no significant differences between Pre150 CGE (20.6 ± 1.1%) BGE (20.5 ± 1.8%) or Post150 GE (20.9 ± 2.6%) for timepoint (F = 2.8, P = 0.11) or visit (F =1.00, P = 0.39). There was no interaction effect between timepoint or visit for LI (F= 0.37, P = 0.69)

There were no significant differences in SI between Pre150 CGE (20.6 (±1.3%) BGE (20.7 (±2.7%) or Post150 GE (20.2 ±3.3%) for timepoint (F = 0.06, P = 0.86) or visit (F =0.23, P = 0.98) and no interaction effect between timepoint or visit for SI (F = 0.45, P = 0.78).

Across visits for CONT, 12 participants were outside the typical error (CV) confidence limits within these participants there was mean GE change of +1.3% and -0.78% (see figure 5.3). For LI, 10 participants were outside the typical error (CV) confidence limits with a mean GE change within these participants of +1.0% and -1.5%. For SI, 10 participants were outside the typical error (CV) confidence limits with a mean GE change of +1.7% and -1.1%.

There were no significant differences in mean GE (%) change scores in protocol (F = 0.87, P = 0.44; figure 5.4), visits (F = 0.50, P = 0.62) for CONT (-0.12 ±1.6%), LI (-0.27 ±0.9%) or SI (-0.11 ±1.2%). There was no interaction effect between protocol and visit (F = 1.27, P = 0.31).

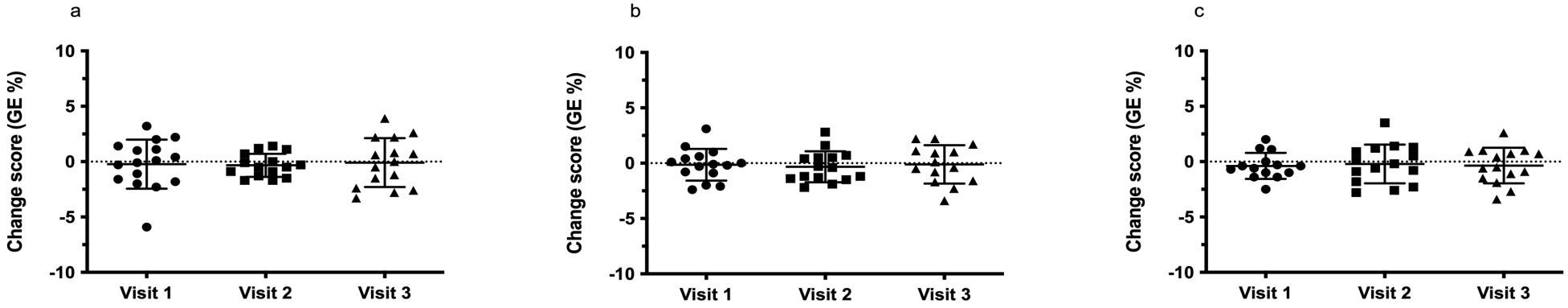

Figure 5.3 CGE (%) change scores (error bars are 95% CI) across all three visits a) CONT Pre-Post150, b) LI Pre-Post150, c) SI Pre-Post150

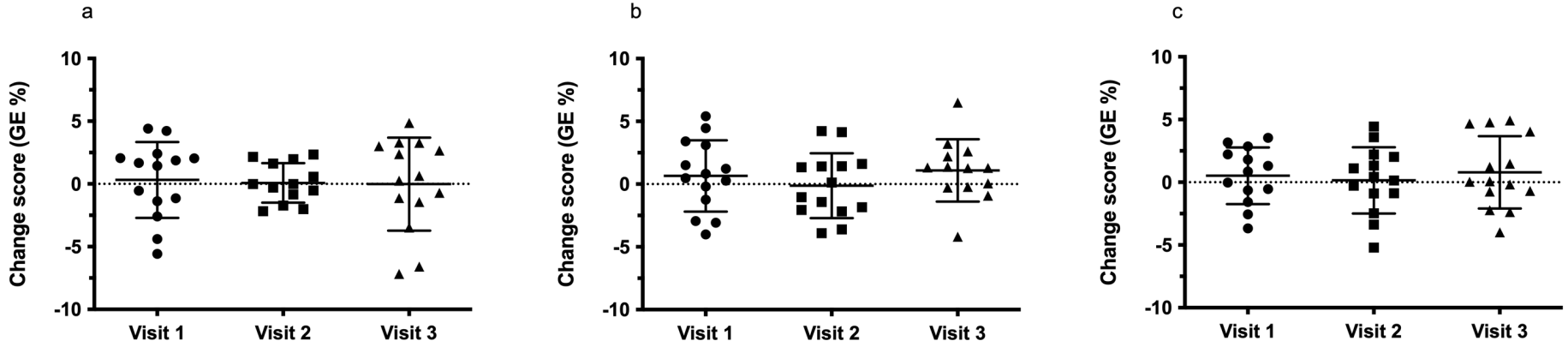

Figure 5.4 BGE (%) change scores (error bars are 95% CI) across all three visits a) CONT Pre-Post150, b) LI Pre-Post150, c) SI Pre-Post15

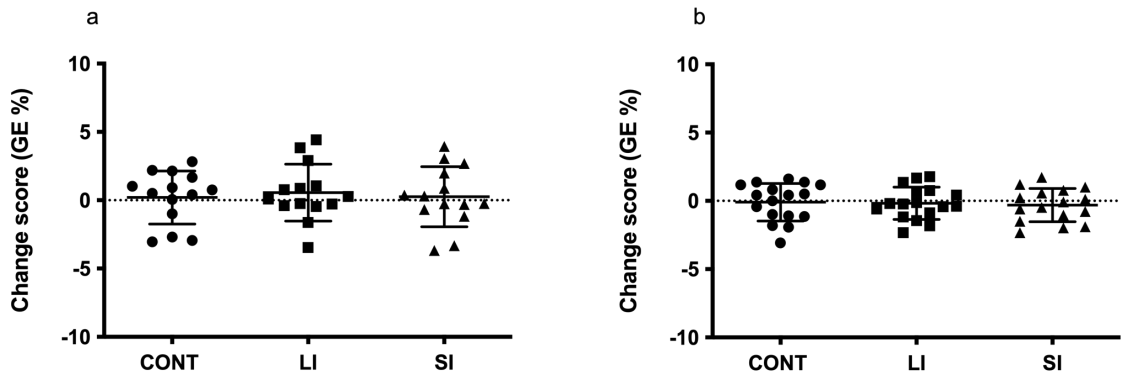

Figure 5.5 *Mean change scores (error bars are 95% CI) in CONT, LI and SI for (a) Pre-Post150 CGE (b) BGE*

5.4.2 Physiological responses

As shown in Figure 5.5, there were no significant differences between pre- and post-protocol for CONT, LI and SI $\dot{V}O_2$ (F = 0.38, P = 0.69), $\dot{V}E$ (F = 0.55, P = 0.58) or RER (F = 1.72, P = 0.21).

For $\dot{V}O_2$, there was significant overall differences in protocol (F = 14.44, P = 0.001) with CONT producing a lower $\dot{V}O_2$ compared to LI (mean difference -0.45 ±0.11 L·min^{-1}, P = < 0.001) but not SI (mean difference 0.81 ±0.72 L·min^{-1}, P = 0.54). There was no significant difference for timepoint (F = 0.75, P = 0.46) or visit (F = 0.64, P = 0.46). There were no significant interaction effects between protocol x timepoint, protocol x visit or timepoint x visit.

For $\dot{V}E$ during each protocol (figure 14b), there were significant differences in protocol (F = 21.39, P = 0.00008) with CONT producing a lower $\dot{V}E$ compared to LI (mean difference -32.60 ±5.21 L·min^{-1}, P = 0.0001) but not SI (mean difference -8.55 ±5.38 L·min^{-1}, P = 0.15). There were significant differences in timepoint (F = 17.16, P = <0.0001) with all timepoints significantly higher than INT1 (P = <0.001). There was a significant interaction effect between protocol x timepoint (F = 0.686, P = <0.001).

For RER during each protocol (figure 14c), there were significant differences in protocol (F = 28.95, P = 0.00002) with CONT producing a lower RER compared to both LI (mean difference

-0.07 ±0.01, P = 0.00003) and SI (mean difference -0.03 ±0.01, P = 0.001). There were significant differences in timepoint (F = 5.94, P = 0.003) with INT2 and INT3 significantly different to INT1 (P = ≤0.05). There were no significant differences between visits (F = 0.42, P = 0.62) but there were significant interaction effects between protocol x timepoint (F = 20.70, P = 0.000009) and protocol x timepoint x visit (F = 2.48, P = 0.05)

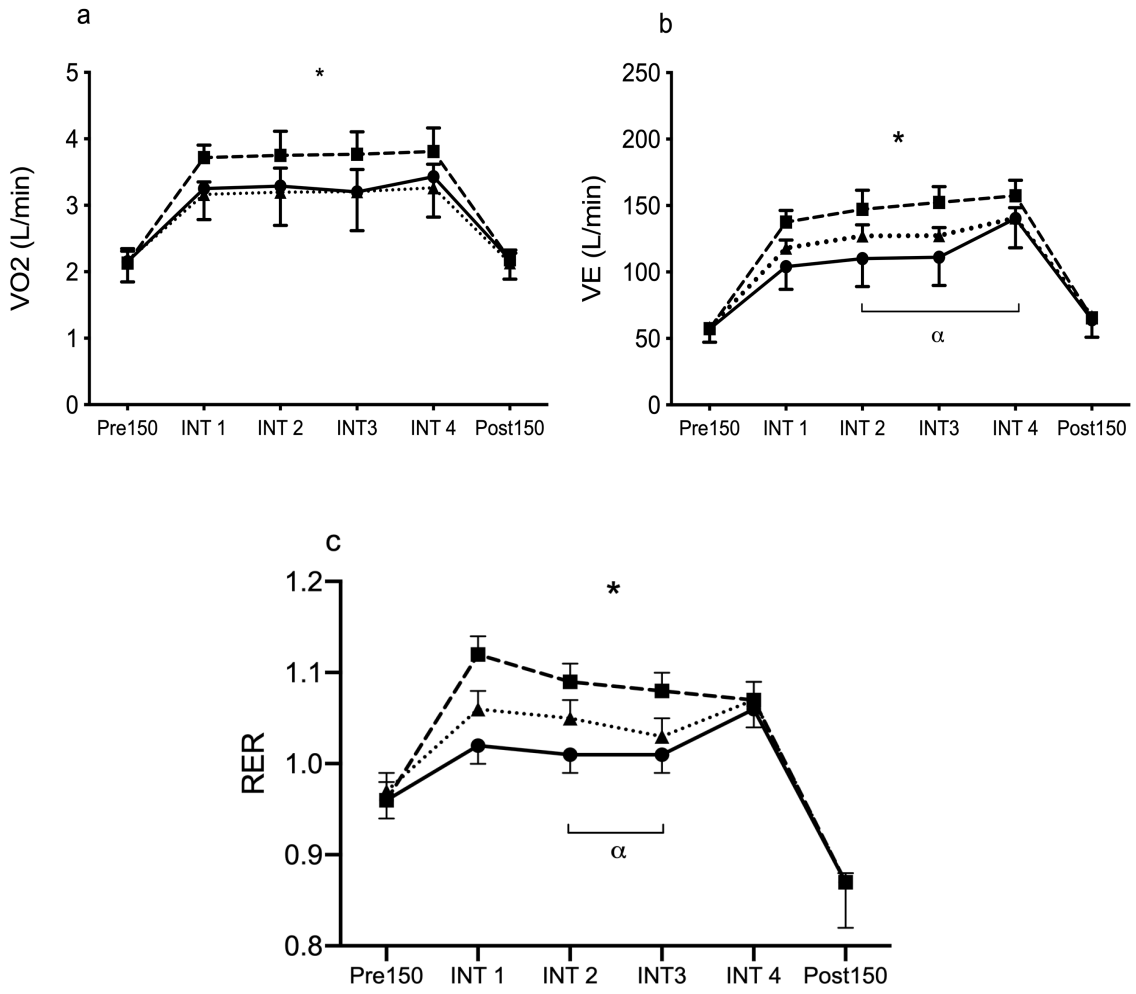

Figure 5.6 *Mean responses for (a) $\dot{V}O_2$, (b) VE and (c) for CONT (solid line, circle), LI (dashed line, square) and SI (dotted line, triangle). * = significant difference between CONT and LI. α = significant difference between timepoints compared to INT1*

5.4.3 Power

There was a significant difference (F = 129.51, P ≤ 0.05) in mean power output between CONT (262 ±40W), LI (310 ±45W) and SI (382 ±54W). Work interval power output was significantly different between LI (291 ±43W) and SI (382 ±12W) (F = 159.44, P ≤0.05) and between LI (80 ±5W) and SI (100 ±7W) for recovery power output between LI (74W ±9W) and SI (100W ±11W) (F= 9.65, P ≤0.05).

There were no differences between CONT, LI or SI work interval output between protocol, timepoint or visits for LI or SI. Recovery power output was significantly different between LI and SI (F = 3.69, P = 0.05). Mean session heart rate was not significantly different between conditions (CONT: 164 ± 10beats.min, LI (163±9bpm) and SI 164 ±9bpm) for timepoint or visit.

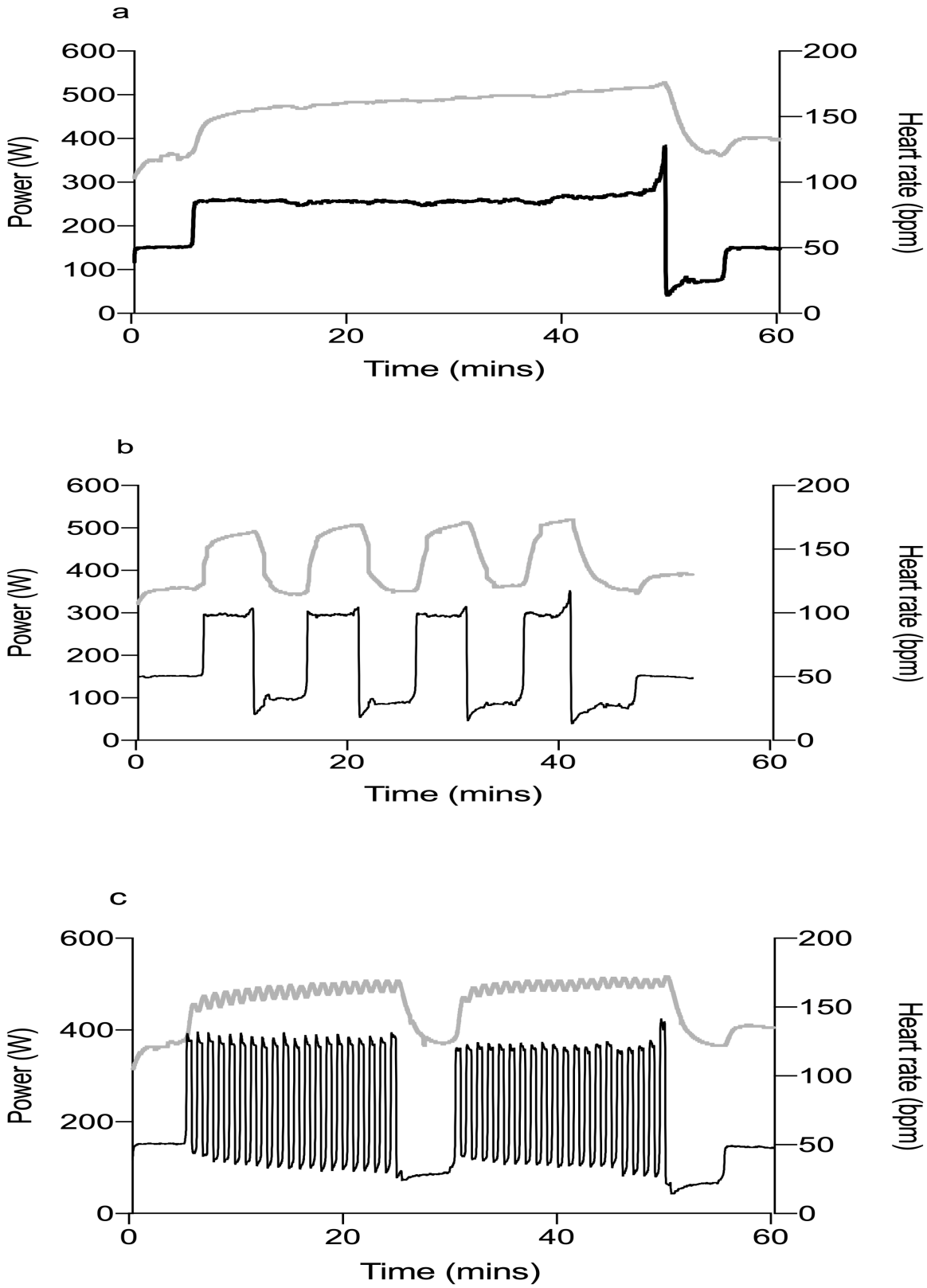

Figure 5.7 *Mean cycling power output (W) (solid line) and mean heart rate (beats·min^{-1}) (dotted line) for (a)CONT, (b) LI and (c) SI*

Whole session mean lactate responses were not different between LI and SI (9.1 ±0.3 mmol.L^{-1} vs. 9.5 ± 0.2 mmol.L^{-1} for LI and SI respectively; P = 0.33), but both were significantly higher compared to CONT (6.3 ±0.3 mmol.L^{-1}; F = 43.24, P ≤0.05). There were no significant differences in whole session mean lactate responses across all timepoints or visits.

All session protocols were matched for final session effort (RPE 19.2 ±0.8, 19.4 ±0.8 and 19.4 ±0.7 for CONT, LI and SI respectively; P = 0.30). There were significant differences in post work bout/set RPE responses between CONT and LI and SI (F = 23.57, P ≤0.05) but not between LI and SI (P= 0.32) for intervals 1-3.

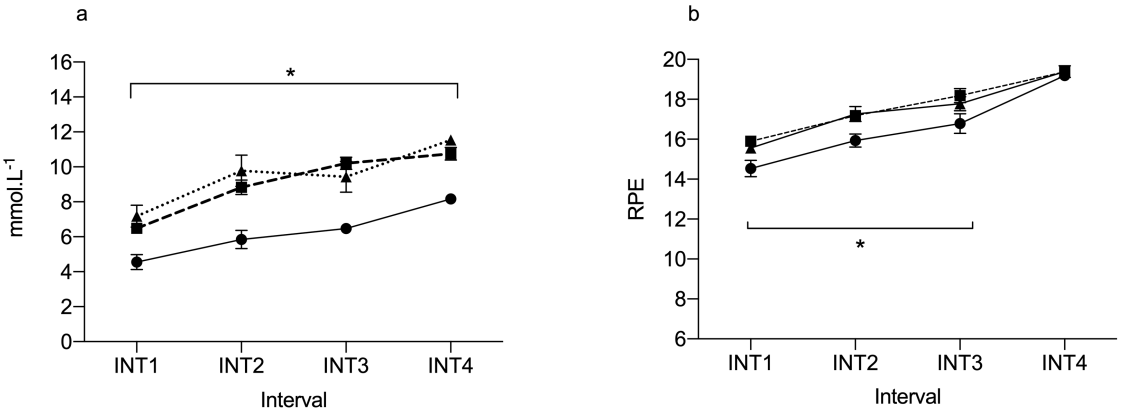

Figure 5.8 *(a) mean lactate responses and (b) mean RPE responses for CONT (circles, solid line), LI (squares, dashed line) and SI (triangles, dotted line). * = significantly different to CONT*

5.4.4 NIRS responses

Comparing Pre150 and Post150 for TSI across all protocols (see figure 5.8a) there were no significant differences in protocl (F= 0.95, P = 0.81), timepoint (F = 0.95, P = 0.81) or visit (F = 4.71, P = 0.62). There were no interaction effects between protocol and timepoint (F = 0.33, P = 0.75), protocol and visit (F = 0.98, P = 0.51) or timepoint and visit (F = 0.51, P = 0.61). There was no interaction effect between protocol, timepoint and visit (F = 198.32, P = 0.45).

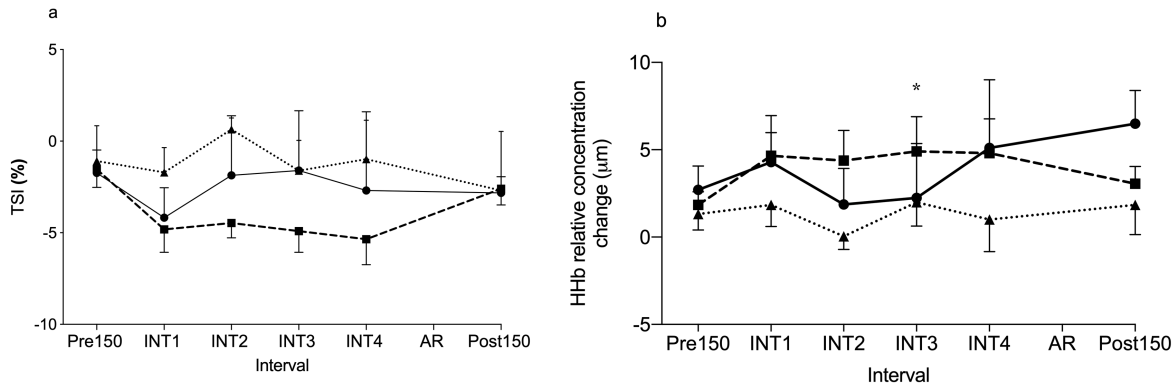

Figure 5.9 *Mean differences between first and last minute of each interval for (a) TSI (%) and (b) mean HHb concentration for CONT (solid line, circle), LI (dashed line, square) and SI (dotted line, triangle) protocols. AR = active recovery interval. * = INT 3 significantly different from INT2 for SI*

Across all protocols there were no significant TSI interval differences (see figure 5.8a) in protocol (F = 1.42, P = 0.34), timepoint (F = 3.29, P = 0.20) or visit (F = 2.12, P = 0.24). There were no interaction effects between protocol and timepoint (F = 1.94, P = 0.29), protocol and visit (F = 1.24, P = 0.38) or protocol, timepoint and visit (F = 0.41, P = 0.62).

Comparing Pre150 and Post150 for HHb across all protocols there were no significant differences in protocol (F= 1.52, P = 0.32), timepoint (F = 1.57, P = 0.73) or visit (F = 2.21, P = 0.23). There were no interaction effects between protocol and timepoint (F = 4.97, P = 0.64) or protocol and visit (F = 2.12, P = 0.28), timepoint and visit (F = 2.64, P = 0.19) or between i protocol , timepoint and visit (F = 0.32 P = 0.45).

Across all protocols there were no significant HHb interval differences in protocol (F = 1.97, P = 0.29), timepoint (F = 2.12, P = 0.27) or visit (F = 0.58, P = 0.60). There were no interaction effects between protocol and timepoint (F = 1.12, P = 0.40), protocol and visit (F = 1.97, P = 0.50) or protocol, timepoint and visit (F = 1.56, P = 0.33).

5.5 Discussion

The main findings of this study are 1) the BE method is a valid and reliable measure of estimating GE during HIE of varying intensity and duration 2) there are no significant

differences in mean conventionally calculated GE, BGE, TSI or HHb across different protocols when AR5 precedes submaximal cycling post-prescription.

The mean CV of BGE across all three protocols and repeated visits was 9.0 – 12.3% which is comparable to the findings of chapter 3 and previous research measuring the reliability of CGE using breath-by-breath online gas analysers (Moseley and Jeukendrup 2001; Noordhof *et al.* 2010). The SID for BGE (0.42 – 0.68 absoulte GE units) across the three protocols was higher than CGE (0.33 – 0.36 absolute GE units). This is in contrast to chapter 3 where after one 4-min HIE bout at 100%$_{MAP}$ the SID was lower than CGE 0.10 vs 0.30 absolute GE units) perhaps indicating that BGE is slightly less sensitive in detecting important differences during repeated high-intensity intervals

It would be expected that the greater aerobic and anaerobic energy demands for both LI and SI would create a greater EPOC compared to CONT and would subsequently lower GE.The present study shows that an AR5 potentially allows both CGE, BGE, TSI and HHb to recover to pre-exercise levels regardless of whether a CONT, LI or SI iso-effort prescription was performed. This is in contrast to the study by (Groot *et al.* 2018) where CGE did not recover after time-trials of 2000m or 20,000m whilst cycling at 55%$_{MAP}$ for 30 min. However, in the present study the participants cycled for 5 min at ~70W prior to completing an efficiency trial at 50%$_{MAP}$.

Whilst initially both TSI and HHb are similar at INT1 for LI and CONT, TSI remains lower and HHb remains higher for INT2, INT3 and INT4 during LI compared to CONT. In addition, lactate is significantly different for during all intervals for LI and SI compared to CONT. This suggests that the LI protocol requires a higher demand on both oxidative aerobic pathways and anaerobic glycolysis. Conversely, the SI protocol has a higher TSI and a lower HHb indicating that anaerobic glycolysis is the dominant pathway. The results of this study suggest that an AR5 bout is sufficient for the oxygen deficit incurred in both LI and SI to be repaid and for CGE to recover to pre-protocol levels.

It has been shown that during prolonged low intensity cycling interspersed with 30-second sprints that GE only transiently decreases between the sprints and recovers during a 40-

minute period of low intensity cycling at 50%$\dot{V}O2_{max}$ (Almquist *et al.* 2019). Previous studies using the BGE method to assess efficiency during 4 min HIE bouts with minimal recovery (1-min at 25W), found it was significantly lower than conventionally calculated post-exercise GE (Koning *et al.* 2013; Ebreo, Passfield and Hopker 2020). BGE was significantly lower compared to Pre150 and Post 150 CGE. Therefore, if the AR5 bout were removed in the present study, BGE for all protocols could potentially be lower. In line with previous research (Ebreo, Passfield and Hopker 2020), this suggests that the reduction in GE is likely to be due to an incurred oxygen deficit and EPOC that has resolved by the end of the AR5 bout.

In contrast, it has been shown that GE does not fully recover whilst cycling at 55% $\dot{V}O2_{max}$ after completing either a 2,000m or 20,000m time-trial (Noordhof *et al.* 2015b). The authors found that the extent of recovery was quicker after the shorter time-trial with 93% of the total recovery of post time-trial GE attained after 14 min of the allotted 20 min of submaximal cycling at 55% $\dot{V}O2_{max}$. Moreover, the authors propose that the larger the difference between the BGE and the submaximal recovery GE the greater the rate of GE recovery and suggest that either a lower submaximal intensity or absolute rest is needed for GE to fully recover. In the present study a combination of 5-min active recovery at a participant self-selected power (mean power of 70W across protocols) and 5-min cycling at 150W is sufficient for GE, TSI and HHb to fully recover. Future studies are needed to ascertain the rate of GE recovery after exercise bouts of different duration.

Whilst no mean statistically significant GE changes were found in the present study after HIE, there were GE change scores both within and between protocols which lie outside of the 95% confidence limits. This demonstrates that these individuals were affected by a given protocol to a larger extent then the measurement error suggesting that a significant change in GE has occurred. This could indicate that for these individuals, a longer duration of active recovery is needed for GE to fully recover.

5.6 Conclusions

Despite varied work intensities and interval duration it is possible for GE to fully recover within 5 min of active recovery (~70W) after 40 min of cycling. However, within a race orientated

scenario it may not be possible to reduce power output to such an extent as this would inevitably incur a tactical disadvantage. Future studies are needed to ascertain the optimal work rate that would allow GE to fully recover, whilst not negatively impacting on a race scenario. Furthermore, the BGE method may be a useful tool for evaluating recovery in training.

6 The effect of different acute iso-effort exercise protocols on gross efficiency and circulating miRNAs

6.1 Abstract

Purpose: To investigate the effect of acute exercise of varying intensity on gross efficiency and circulating miRNAs (ci-miRNA) involved in skeletal muscle angiogenesis and redox metabolism.

Methods: Eleven healthy trained male participants completed (in random order) three acute iso-effort (self-paced maximum sustainable intensity exercise) protocols on three separate occasions: continuous cycling (CONT) for 40 min; 4 x 5 min intervals (LI); 4 sets of 10 x 30s intervals interspersed with 30s recovery (SI). BGE and CGE were calculated using data collected from 150W cycling before (Pre150) and after (Post150) each protocol. EDTA plasma ci-miRNAs were detected pre, immediately post and 1 hour post-exercise (1Hr) using Taqman-based quantitative PCR and normalised to ci-miRNA-423. Two-way ANOVA (protocol x timepoint) was used to assess differences between CGE, BGE, with cohen's d used for effect size estimations of ci-miRNA fold changes.

Results: For LI, there was a significant difference for timepoint ($F = 4.84$, $P = 0.02$) between Post150 CGE (20.50 $\pm$0.67%) and BGE (21.9 $\pm$0.64%) ($P = 0.01$) but not between Pre150 CGE and Post CGE ($P = 0.96$) or BGE ($P = 0.06$). CONT revealed a large increase in ci-miRNA-21 (cohen's $d = 0.90$) but only small increases in LI (cohen's $d = 0.30$) and a moderate increase in SI (cohen's $d = 0.68$) post-protocol. There was a large increase in ci-miRNA 222 pre-1Hr in LI (cohen's $d = 1.04$) but only small increases in CONT (cohen's $d = 0.27$) and in SI (cohen's $d = 0.49$). There was a negative correlation between SI CGE (%) difference between visit 3 and visit 1 with mean pre-post fold changes in ci-miRNA-21 ($r = -0.77$, $P = 0.03$). The LI CGE (%) difference between visit 3 and visit 1 negatively correlated with mean fold changes in ci-miRNA-222 ($r = -0.65$, $P = 0.04$) pre-post exercise.

Conclusion: Acute iso-effort LI and SI are associated with the upregulation of ci-miRNAs involved in angiogenesis and redox metabolism which are negatively correlated with progressive increases in GE over time.

Keywords: iso-effort, circulating microRNA, angiogenesis, redox metabolism, circulating miRNA-21, circulating miRNA-22

The following experimental chapter was undertaken in collaboration with another PhD researcher at the University of Kent, Ciaran O'Grady. Participants were shared and undertook identical exercise protocols. Whilst physiological markers (gas exchange, NIRS, blood lactate and RPE) were shared the analyses performed were different consistent with the separate aims of our respective theses.

6.2 Introduction

The previous chapter demonstrated that despite varying interval intensity and duration in acute exercise, GE and NIRS parameters can recover to pre-exercise levels after AR5. Additionally, chapter 3 shows that after a short HIE bout GE and NIRS parameters can recover after 10 minutes at 50%$_{MAP}$. This suggests that the oxygen deficit and metabolic cost of recovery processes of fatigued fibres can be ameliorated after acute exercise. However, chapter 4 demonstrates that CGE significantly declines after both LCIE and LCIE_SI with corresponding reductions in both HHb and TSI. This is in agreement with Groot et al. (2017) who show that GE does not recover to pre-exercise levels after 2,000m and 20,000m time-trials.

Whilst an improvement in GE is clearly important for endurance performance success (Joyner and Coyle 2008), it has been shown that mitochondrial dysfunction (metabolic inefficiency), in older adults (Conley, Jubrias and Esselman 2000; Menshikova *et al.* 2006; Carter, Chen and Hood 2015) and younger inactive adults (Buso *et al.* 2019) can lead to decreases in muscle quality (mechanical inefficiency) and an increase in oxidative stress (Echtay *et al.* 2002; Peterson, Johannsen and Ravussin 2012). Endurance training and high-intensity interval training have been shown to improve cycling efficiency in active males (Hopker, Coleman and Wiles 2007; Hopker, Coleman and Passfield 2009; Sassi *et al.* 2008) as well as increase mitochondrial energy coupling efficiency (Conley *et al.* 2013). However, the molecular mechanisms underpinning changes in GE have yet to be elucidated.

Progressive activation and/or repression of specific cell signalling pathways in response to exercise training regulate both the transcription and translation of proteins, enzymes and genes that are key to exercise adaptation (Pilegaard, Saltin and Neufer 2003; Perry *et al.* 2010). The plasticity of skeletal muscle means that over time skeletal muscle undergoes both functional adaptation and remodelling affecting the regulation of the genes that influence contractile muscle function and enhanced substrate metabolism (Hawley, Hargreaves and Zierath 2006; Holloszy 2009), angiogenesis (Russell *et al.* 2003), mitochondrial biogenesis (Egan and Zierath 2013), mitochondrial content and function (Lundby and Jacobs 2015; Jacobs *et al.* 2013). However, analysis of mRNAs in response to exercise requires invasive muscle biopsies that are inaccessible to the majority of athletes.

MicroRNAs (miRNAs) are small endogenous, short non-coding RNAs negatively regulating gene expression by either inhibition of translation or degradation at the post-transcriptional level (Bartel 2004; Bartel 2009). Over 1,000 different human miRNAs have currently been identified and it is estimated that over 60% of human protein-coding genes are targets of miRNAs (Friedman *et al.* 2009) with a single miRNA potentially regulating hundreds of downstream mRNA and proteins (Bartel 2004; Lim *et al.* 2005). miRNAs have been shown to be key mediators of processes associated with beneficial physiological adaptations such as cardiac and skeletal muscle hypertrophy (Chen *et al.* 2005; Carè *et al.* 2007; Safdar *et al.* 2009; Mann and Rosenzweig 2012), angiogenesis (Wang *et al.* 2008; Weber *et al.* 2010; Kulshreshtha *et al.* 2007) and redox metabolism (Qadir *et al.* 2019; Torma *et al.* 2020a).

miRNAs are actively or passively secreted into the bloodstream packaged in extracellular vesicles (exosomes, microvesicles and apoptotic bodies), proteins or high-density lipoproteins, making them relatively stable and resistant to endogenous RNase activity (Valadi *et al.* 2007; Zernecke *et al.* 2009). Thus, miRNAs have the potential to be a molecular biomarker involved in the acute response to exercise and physiological adaptation. As discussed in chapters 4 and 5, potential causes of GE decrement could be secondary to both oxygenation and ROS formation (metabolic inefficiency) or increasing muscle fatigue and recruitment of less efficient type 2 muscle fibres (contractile inefficiency). Ci-miRNAs-21 have been shown to regulate downstream proteins involved in muscle contractility, redox metabolism and hypoxic adaptation (Urbich, Kuehbacher and Dimmeler 2008; Liu *et al.* 2011; Zhang *et al.* 2012a). Ci-miRNA-222 has been shown to also influence redox metabolism

(Dubois-Deruy *et al.* 2017). Thus, ci-miRNAs may be a useful biomarker in assessing the cellular adaptive response to exercise of varying intensity and duration in tandem with changes in GE.

The purpose of this study is 1) investigate the response of ci-miRNAs-21 and -222 to a constant interval of 40min (CONT); 4 x 5 min intervals (LI); two sets of 10 x 30s intervals (SI), 2) to investigate the relationship between ci-miRNA responses and whole-body GE.

6.3 Methods

6.3.1 Participants

Eleven healthy, trained male athletes aged 18-55 years old participated in the study (Mean ±SD: age 34 ± 12 yrs, mass 71 ± 11kg, $\dot{V}O2_{max}$ 59 ± 8 $ml \cdot kg^{-1} \cdot min^{-1}$, MAP 376 ± 46W). All participants had completed a minimum of three years of cycle training consisting of three training sessions a week on average, inclusive of at least one high-intensity exercise session. The purpose of the study, possible risks and potential discomfort were explained to the participants prior to obtaining written consent. The study was conducted was in accordance with the Declaration of Helsinki and with full university ethical approval from the School of Sport and Exercise Science Research Ethics Advisory Group (ethical approval number: 106_2015_2016).

6.3.2 Experimental design

Participants attended the exercise testing laboratory on 10 separate occasions. Visit 1 consisted of an incremental exercise test to determine their $\dot{V}O2_{max}$ and Maximal Aerobic Power (MAP). On three subsequent visits participants completed three different exercise protocols (see figure 5.1 in chapter 5) in a randomised order. The three protocols consisted of: continuous cycling (CONT) for 40 min followed by a 5 min active recovery interval; 4 x 5

min intervals interspersed with 5 min active recovery intervals (LI); 4 sets of 10 x 30s intervals interspersed with 30s recovery (SI) with a 5 min active recovery interval between sets 2 and 3 and after set 4.

6.3.3 Maximal Incremental Exercise Test

The test consisted of a 10-minute warm up at 100W before conducting a step protocol where cycling power output increased by 20W every minute until volitional exhaustion (defined as a cadence below 60 revolutions per minute despite strong verbal encouragement). $\dot{V}O2_{max}$ was determined as the highest 60s $\dot{V}O2$ achieved during the incremental test. MAP was calculated as the average power output over the final minute of the test.

6.3.4 Protocols

Intensity was prescribed according to an iso-effort model as this has been shown to be more consistent with how endurance athletes train (Seiler and Sjursen 2004). Participants were instructed to use their preferred pedalling cadence throughout each training session whilst cycling at their maximal sustainable intensity for each of the protocol intervals (iso-effort) so that they completed the prescribed interval with an even, or slightly progressive power, from the 1st to 4th interval (Seiler and Sjursen 2004). Each protocol was preceded and followed with a 5-min bout at 150W to measure GE (see figure 6.1)

6.3.5 Experimental measurements

On arrival at the laboratory, stature was measured to the nearest 0.5cm using a stadiometer (Seca, Germany) and body mass was measured to the nearest 0.1kg using beam balance scales (Seca, Germany). All tests were performed on a stationary cycle ergometer (Cyclus2; RBM elektronik-automation GmbH). Participants used their own bicycles or were provided with a laboratory bicycle (saddle height was adjusted and replicated for all visits). Respiratory

exchange data was collected on a breath-by-breath basis during each visit using an online gas analyser (Metalyser 3B; CORTEX Biophysik GmbH, Germany).

6.3.6 Venous sampling

A vacutainer needle was inserted aseptically into the antecubital vein and two EDTA vacutainer tubes containing ten millilitres of blood was collected at baseline (PRE), within 5 min of exercise completion (POST) and after 1 h of rest (1Hr) post-exercise. All blood samples were centrifuged at 1500g for 10min to pellet cellular elements. Cellular components were discarded, and the acellular supernatant was then aliquoted and immediately frozen at -80°C.

6.3.7 Plasma RNA isolation

Individual samples for a given protocol were processed and analysed in a single batch and repeated freeze-thaw cycling of plasma samples was avoided. Total RNA extraction from 300µl plasma was performed using a Nucleospin miRNA Plasma Kit (Macherey-Nagel GmbH and Co, Germany) according to the manufacturer's instructions

6.3.8 Quantification and quality control of miRNA by qRT-PCR

Individual miRNA levels were determined in a two-step process. First, reverse transcription was performed according to the manufacturers protocol using a Taqman MicroRNA Reverse Transcription kit (Thermo Fisher Scientific, MA, USA), miRNA specific primers (Thermo Fisher Scientific, MA, USA). cDNA was amplified according to the manufacturer's instructions using Taqman Universal Mastermix (Thermo Fisher Scientific, MA, USA), Taqman miRNA assays (has-miR-21-5p, hsa-miR-222-3p, Thermo Fisher Scientific, MA, USA). The cycle threshold (Ct) data were determined using default threshold settings of the Lightcycler 96 instrument (Roche Molecular Systems, Inc, UK) and the mean Ct was determined from duplicate PCRs.

To adequately assess the quality of miRNA yield we used the stable endogenous ci-miRNA 423-5p for normalisation of our target ci-miRNAs-21 and -222 (Mestdagh et al. 2009; Nielsen et al. 2014). The relative levels of ci-miRNA-21 and ci-miRNA-222 were calculated using the $2^{-\Delta\Delta Ct}$ method. ΔCt was calculated by subtracting the Ct values of ci-miRNA-423-5p from the average values of the target ci-miRNAs. ΔCt values were then compared (ΔΔCt) with each participant's own resting baseline (Pre timepoint) which was normalised to a fold change of 1.

Haemolysis of plasma samples was assessed by using a ratio between the ΔCt of a known stable ci-miRNA (ci-miR-423) and a ci-miRNA known to be increased in haemolysed samples (ci-miRNA-451). A ΔCt ratio >7 was used to determine if samples were haemolysed (Blondal *et al.* 2013; Faraldi *et al.* 2019).

6.3.9 Data analysis

As this is an initial "proof of concept" study the sample size of this pilot study was limited to 11 participants and only one visit of the CONT, LI and SI protocol was analysed.

GE was calculated using the equations 3.1 and 3.2 in chapter 2. BGE was calculated by fitting a linear regression to the $\dot{V}O_2$ data points of the last 3 min of the of each stage. $\dot{V}O_2$ points with an RER >1.0 were omitted during this time period. These values were then back-extrapolated to the end of the sprint interval to estimate the GE change.

6.3.10 Statistical analysis

Shapiro-Wilk tests were used to assess for normality of distribution. A two-way repeated measures ANOVA was conducted (protocol x timepoint) to assess for differences in CGE, BGE and ci-miRNA ΔCt for CONT, LI and SI at Pre, Post and 1Hr. A Wilcoxin Signed Rank test was performed when data was not normally distributed.

Cohen's effect size (d) was calculated using fold changes for the comparison of pre-post, pre-1hr and post-1hr exercise and for the comparison of delta values (post-pre and 1hr-pre)

between the different exercise protocols CONT, LI and SI (Cohen 1988). Magnitudes of differences in ci-miRNA expression levels were interpreted using the following thresholds of cohen's d: 0.2, 0.5, and 0.8 for small, moderate, and large respectively (Cohen 1988)(Hopkins, Hawley and Burke 1999)

As the intervals were prescribed using an iso-effort approach, GE could potentially be affected by learning effects between visits. Additionally, as only one visit of each protocol was analysed the relationship between fold changes in ci-miRNAs and GE were assessed using a partial correlation controlling for visit. Statistical significance was set at $P \leq 0.05$ and confidence intervals (CI) calculated. Exercise test data are presented as mean $\pm$ SD. Statistical analysis was conducted using the SPSS statistical software package (IBM SPSS Statistics, Rel. 25.0, SPSS, Inc, Chicago, USA).

6.4 Results

6.4.1 Between protocol changes in CGE and BGE

For CGE there was no significant difference between protocol (F = 0.04, P = 0.96), timepoint (F = 1.00, P = 0.36). There were no interaction effects between protocol x timepoint (F = 0.65, P = 0.54).

For BGE there was no significant difference between protocol (F = 0.23, P = 0.80) but there was a significant difference in timepoint (F = 9.59, P = 0.03) between BGE and Pre150 CGE (F = 9.59, P = 0.03) but not Post150 CGE (F = 9.59, P = 0.36). There was a significant interaction effect between protocol x timepoint (F= 2.45, P = 0.04).

For CONT, there was no significant differences for timepoint (F = 0.60, P = 0.56). For LI, there was a significant difference for timepoint (F = 4.84, P = 0.02) between Post150 CGE (20.50 $\pm$0.67%) and BGE (21.9 $\pm$0.64%) (P = 0.01) but not between Pre150 CGE and Post CGE (P = 0.96) or BGE (P = 0.06). For SI, there was no significant differences for timepoint (F = 0.40, P = 0.70).

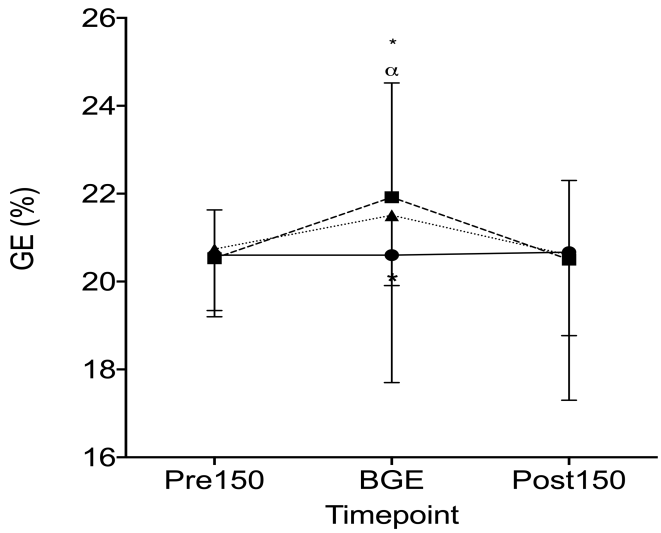

Figure 6.1 *Mean CGE across participants calculated conventionally (Pre150, Post 150) and using back-extrapolation (BGE) for CONT (solid line, circle), LI (dash line, square), SI (dotted line, triangle). * = significant difference in BGE compared to Pre150 CGE across protocols. α = significant difference between LI BGE and Post150 CGE*

6.4.2 Blood plasma quality

The plasma samples of 11 participants were analysed for the degree of haemolysis by measuring the expression of ci-miRNA-451. The expression of ci-miRNA-451 is normally absent in plasma but is highly enriched in erythrocytes (Bruchova *et al.* 2007). Plasma from all 11 participants had a ΔCt ratio <7 and thus passed quality control.

6.4.3 Changes in ci-miRNA-21

Comparing protocols, repeated measures ANOVA showed no effect of timepoint (F =1.13, P = 0.89), no effect of protocol (F = 0.32, P = 0.74) and no interaction effect (protocol x timepoint) (F = 0.40, P = 0.81).

In CONT, there was no effect of timepoint (F = 1.36, P = 0.28) but there was a large increase from pre-post (cohen's d = 0.90) and a small increase pre-1Hr (cohen's d = 0.40). In LI, there was no effect of time (F = 0.15, P = 0.87) but there were small increases from pre-post (cohen's d = 0.30) and from pre-1Hr (cohen's d = 0.81). In SI, there was no effect of time (F = 0.72, P =

0.50) but there were moderate increases from pre-post (cohen's d = 0.68) and large increases from pre-1Hr (cohen's d = 0.88).

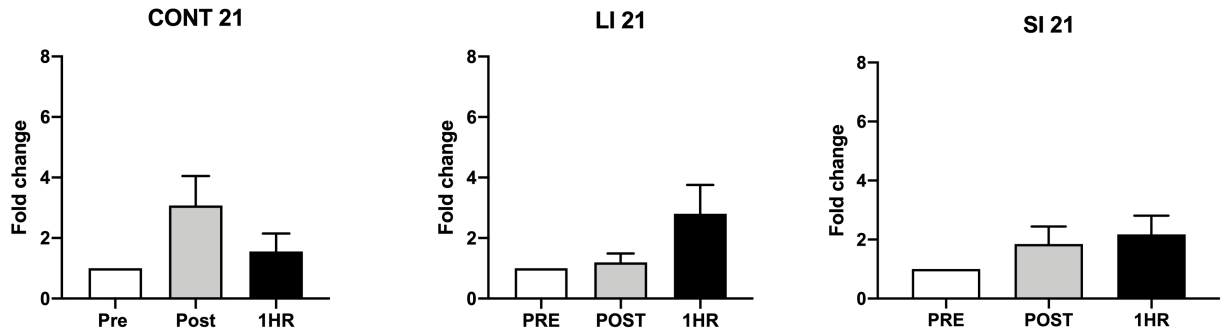

Figure 6.2 *Fold change in ci-miRNA-21 expression pre post- and 1Hr- after CONT, LI and SI.*

6.4.4 Changes in ci-miRNA-222

Comparing protocols, repeated measures ANOVA showed no effect of timepoint (F =1.03, P = 0.40), no effect of protocol (F = 2.71, P = 0.11) and no interaction effect (protocol x time) (F = 0.22, P = 0.70). In CONT, there was no effect of time (F = 1.19, P = 0.31) but there was a moderate increase from pre-post (cohen's d = 0.49) and a small increase pre-1Hr (cohen's d = 0.27). In LI, there was no effect of time (F = 0.76, P = 0.20) but there were small increases from pre-post (cohen's d = 0.38) and a large increase from pre-1Hr (cohen's d = 1.04). In SI, there was no effect of time (F = 0.32, P = 0.97) but there were small decreases from pre-post (cohen's d = -0.03) and a moderate increase from pre-1Hr (cohen's d = 0.49).

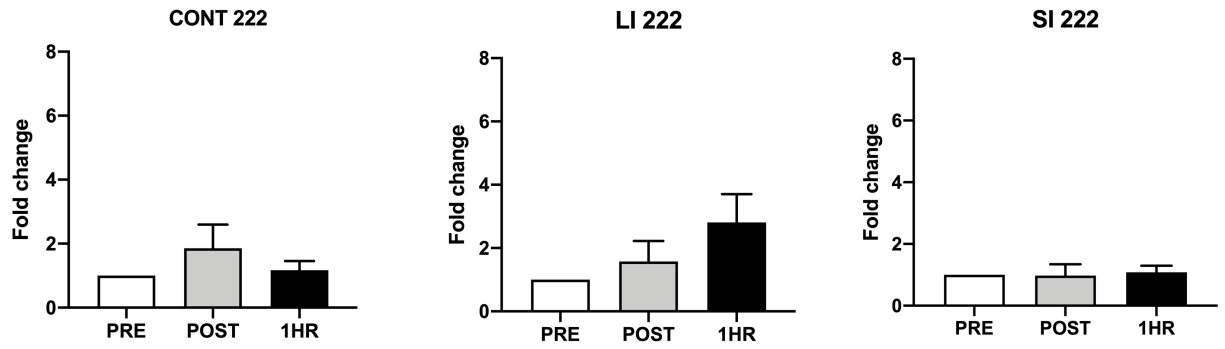

Figure 6.3. *Fold change in ci-miRNA-222 expression post- and 1Hr- after CONT, LI and SI*

6.4.5 Linear correlation between expression of ci-miRNA, gross efficiency

Whilst there were no significant differences in mean CGE or BGE across protocols, the SI CGE (%) difference between visit 3 and visit 1 negatively correlated with mean pre-post fold changes in ci-miRNA-21 ($r = -0.77$, $P = 0.03$; see figure 6.4a). There was a negative correlation for mean ci-miRNA-21 fold changes 1 hour post-exercise and mean SI CGE (%) difference between visit 3 and visit 1. The LI CGE (%) difference between visit 3 and visit 1 correlated with mean fold changes in ci-miRNA-222 ($r = -0.65$, $P = 0.04$); see figure 6.4c. pre-post exercise whilst controlling for visit. There were no significant correlations between the protocols and visits 1 and 2. There were no significant correlations between mean BGE change (pre-post) or mean BGE change between visits 3 and visit 1 across all three protocols.

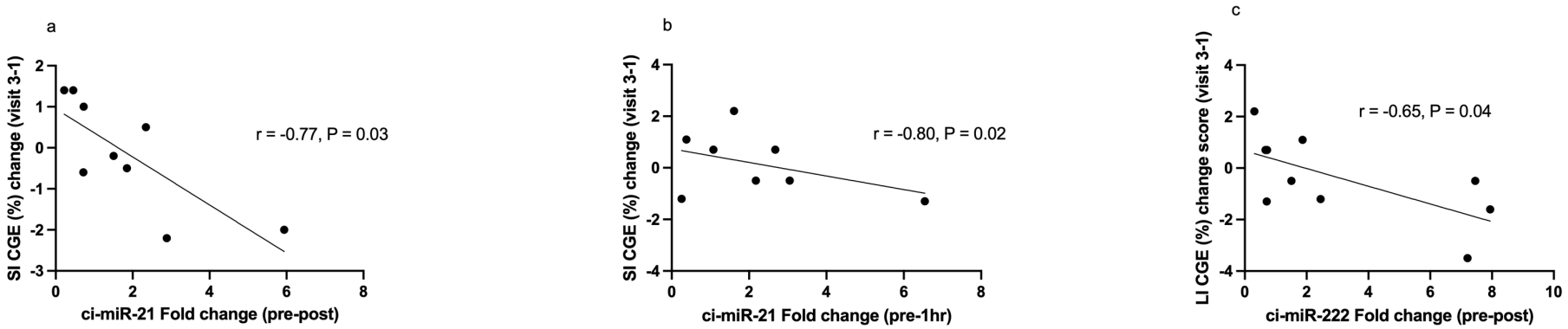

Figure 6.4. *A direct correlation (r= correlation coefficient) is observed between (a) fold change difference (pre-post) of ci-miRNA-212 and the CGE (%) change score between visits 3 and 1 after SI, (b) fold change difference (1hr) of ci-miRNA-21 and the CGE (%) change score between visits 3 and 1 after LI (c) fold change difference (pre-post) of ci-miRNA-222 and the CGE (%) change score between visits 3 and 1 after LI.*

6.5 Discussion

The main findings of this study are 1) mean BGE was significantly higher after LI , 2) the largest increases in ci-miRNA-21 expression were immediately post-CONT and 1Hr after LI, only SI was negatively correlated with GE (%) differences from visit 3 to visit 1, 3) LI revealed the largest increase in ci-miRNA-222 1Hr after exercise and was negatively correlated with GE (%) differences from visit 3 to visit 1,

In contrast to the chapter 5, a significant difference was found in BGE after LI between Post150 CGE (20.50 ±0.67%) and BGE (21.9 ±0.64%), but not between Pre150 CGE and Post CGE (P = 0.96) or BGE (P = 0.06). (Hopker, Coleman and Wiles 2007)

In agreement with previous studies (Nielsen *et al.* 2014; Wahl *et al.* 2016), this study shows that in response to acute exercise of different intensity and work duration that ci-miRNA-21 and ci -miRNA-222 does not significantly increased in trained male cyclists. This is contrary to the findings of Baggish et al. (2011) where both ci-miRNA-21 and ci-miRNA-222 were significantly upregulated. This may be secondary to differences in exercise protocol as in the present study and that of Nielsen et. al (2016) and Wahl et al. (2016) the participants were cycling to exhaustion.

Wahl et al. (2016) also investigated the effect of three different exercise protocols HVT (high volume training: 130 min at 55% peak power output), HIT (high-intensity training: 4 x 4min at 95% peak power output) and SIT (sprint-interval training: 4 x 30s all-out) on ci-miRNA-21 in participants with a higher $\dot{V}$O2max (64 ±9 ml/kg/min). Similarly, no significant changes in ci-miRNA-21 were found but there were small decreases after HIT (cohen's d = -0.28) and large increases after HVT (cohen's d = 0.85) and SIT (cohen's d = 0.85).

In agreement with Wahl *et al.* (2016) there was a larger increase in expression of ci-miRNA-21 immediately post-exercise after both continuous and sprint-intensity exercise compared to high-intensity exercise. Wahl et al. speculate that the increase in ci-miRNA-21 may be caused by localised short-term hypoxic conditions. Wahl et al. found that ci-miRNA-21 decreased after SIT and increased after HIT 1hr after exercise, whilst in the present study ci-miRNA-21 increased in both post and 1Hr after LI and SI. Again, this may be due to differences

in both intensity and duration of exercise between the HIT protocol employed by Wahl et al. (4x4min at 90-95% peak power output) vs LI in the present study (4 x 5 min iso-effort).

This is the first study to display a correlation between GE and ci-miRNAs after different acute exercise protocols. We found that GE changes between visit 3 and visit 1 displays a negative correlation with ci-miRNA-21 expression after SI. This may indicate that repeated sprint-interval training may decrease expression levels of ci-miRNA-21 whilst promoting beneficial exercise adaptations that increase gross efficiency. Wahl et al. (2016) found that ci-miRNA-21 increased immediately post-exercise after sprint-interval training (SIT). The authors speculated that the repeated short-term hypoxic conditions in SIT could explain an increase in ci-miRNA-21 expression. ci-miRNA-21 has been shown to indirectly upregulate the pro-angiogenic protein vascular endothelial growth factor (VEGF) and hypoxia inducible factor-1 (HIF-1α) expression (Liu *et al.* 2011) as well as increasing endothelial nitric oxide synthase activity (Weber *et al.* 2010; Traub and Berk 1998). This may improve muscle oxygenation and recovery of the oxygen deficit incurred after sprint intervals. (Nicoli *et al.* 2010) have also demonstrated that ci-miRNA-21 integrates haemodynamics with VEGF signalling during angiogenesis. Therefore, prolonged shear stress may be a reason for the upregulation of ci-miRNA-21 during CONT.

Strenuous single bouts of acute moderate to intense endurance exercise are known to induce the overproduction of reactive oxygen species (ROS) (Powers and Jackson 2008; Gomes, Silva and Oliveira 2012; Powers, Radak and Ji 2016) with subsequent oxidative damage potentially causing elevated lipid perioxidation, DNA oxidative damage (Mastaloudis et al 2004), immunosuppression (Neilsen et al. 2004), muscle damage and contractile dysfunction (Powers and Jackson 2008). In a series of studies, it has been demonstrated that in an unfatigued muscle, an optimum level of ROS is needed for muscle fibres to generate 100% maximal force production (Reid *et al.* 1992; Reid, Khawli and Moody 1993; Reid 2001; Reid *et al.* 2005). Whilst these studies specifically investigated isometric force production, it is likely that this biphasic influence on skeletal muscle is also applicable to concentric contractions. SIT may therefore induce higher levels of ROS and a subsequent initial increase in ci-miRNA-21 and SOD3 than CONT or LI. As both GE and mean SI power increase over the time-course of subsequent SI trials it can be speculated that skeletal muscle fibres have an increased ability to remove ROS during SI exercise during trials 2 and 3 leading to an increase in GE. This

study also found that changes between visit 3 and visit 1 displays a negative correlation with ci-miRNA-222 expression after LI. Potential targets of ci-miRNA-222 include the proteins p27, HIPK1 and HMBOX1 that regulate exercise-induced cardiac growth whilst also protecting against adverse cardiac remodelling and dysfunction after ischaemic injury (Liu *et al.* 2015).

Ci-miRNA-222 has been found to be a post-transcriptional regulator of SOD2 (Dubois-Deruy *et al.* 2017). SOD2 is a SOD isoform located in the mitochondrial matrix and has been found to be increased after acute exercise (Hollander *et al.* 2001; Gomez-Cabrera *et al.* 2005). Whilst aerobic exercise results in a 1-3 fold increase in oxygen free radicals during muscle contraction only a small proportion is attributable to the mitochondrial uncoupling (Sakellariou, Jackson and Vasilaki 2013); Sakellariou, Jackson and Vasilaki 2013; Ji, Kang and Zhang 2016). As there is a moderate increase in ci-miRNA-222 1Hr after LI, it can be speculated that ci-miRNA-222 is upregulated to increase SOD2 levels and diminish possible increases in mitochondrial ROS formation during state 4 (resting) respiration both during the rest intervals during the LI protocol and post-exercise with a subsequent increase in GE from visit 1 to visit 3. Whilst numerous studies have investigated the changes in ROS production during both aerobic and anaerobic exercise, further studies are required to examine the relationship between exercise intensity, ROS production, the time course of ci-miRNA expression and changes in GE.

Limitations of this study should be acknowledged. Spike-in RNAs were not used in the present study. Extraction of ci-miRNA from serum / plasma are typically low-yield and errors causing differences in isolation efficiency were not tested. Also, whilst this study used a ΔCt ratio as a quality control for haemolysis, previous studies have analysed plasma samples via spectrometry prior to isolation (Nielsen *et al.* 2014). It is also possible that changes in plasma volume may affect the changes seen in ci-miRNA expression via exercise induced haemoconcentration (Sawada *et al.* 2013). Indeed, differences in ci-miRNAs between different exercise interventions and changes over time similar to the present study have been reported (Wahl *et al.* 2016). As this is an initial "proof of concept" study the sample size of this pilot study was limited to 11 participants and only one visit of the CONT, LI and SI protocol was analysed. Therefore, future research needs to explore the reliability and validity of these results.

The precise mechanism and time course by which miRNAs are secreted and absorbed by recipient cells is currently unknown. Therefore, the expression levels of ci-miRNAs in an individual may not be totally governed by the intensity or duration of exercise. Furthermore, only the expression of free circulating miRNAs in the plasma were analysed in the present study. As miRNAs can also be found in extracellular microvesicles and exosomes (Hunter *et al.* 2008) that enter cells and exert their downstream effects it is possible that other miRNAs could influence the physiological changes observed.

6.6 Conclusions

In summary, we report that intensity of exercise may regulate ci-miRNAs related to angiogenesis and ROS hormesis and subsequent changes in gross efficiency. Further studies are needed to examine the effect of both intensity and duration of exercise on the relationship of ci-miRNAs and performance markers such as gross efficiency.

7 General discussion

7.1 Summary

Chapter 3 demonstrates that BGE is a valid and reliable method of estimating GE during acute short duration (4-minute) HIE with a mean CV of 8.8% at 100%$_{MAP}$, which is comparable to the mean CVs of 3.2 – 6.4% observed in previous research using CGE (Moseley and Jeukendrup 2001; Noordhof *et al.* 2010). Additionally, chapter 5 shows that BGE is a reliable measure of estimating GE during intervals of varying intensity and duration although with slightly higher mean CVs of 9.0 – 12.3% across the three different protocols (CONT, LI and SI). The SID in chapter 1 suggests that BGE may be a more sensitive method of detecting significant differences in GE during an acute interval high-intensity work rate. However, the SID of calculated BGE across all protocols in chapter 5 are higher but comparable to both Pre150 and Post150. Thus, BGE can be used as a reliable assessment of GE during HIE of varying intensity and duration.

Chapter 3 confirms the findings of de Koning et al. 2013 that suggests that CGE underestimates GE during HIE. Using the BE method, the decline in BGE during a short duration HIE at 100%$_{MAP}$ reveals a greater anaerobic contribution to exercise. This decrease in BGE suggests that an oxygen deficit and EPOC are the main mechanisms for a decrease in GE during short duration HIE as GE recovers to pre-exercise levels after a 10-minute submaximal bout at 50%$_{MAP}$.

Chapter 4 demonstrates that CGE decreases to a greater extent after LCIE_SI than LCIE. This is in contrast to the previous findings of Almquist et al. (2019) where CGE was not significantly different after prolonged cycling (4 hours) at 50%$_{MAP}$ with sprint intervals compared to prolonged cycling at 50%$_{MAP}$. However, Almquist et al. (2019) used CGE to estimate GE after 3 x 30s sprints at the end of hours 1-3. As discussed in chapter 3, CGE overestimates the anaerobic contribution at the end of HIE and therefore is not representative of GE at the end of HIE or SIE. Furthermore, the study protocols employed by Almquist et al. (2019) are not reflective of what may be expected during a dynamic racing scenario. Therefore, chapter 5 was designed to incorporate more regular sprint intervals with a shorter duration typical of a

professional criterium road race (Ebert, 2006) and utilised BGE to better estimate the influence of the sprint intervals on GE.

After LCIE and LCIE_SI neither CGE or NIRS parameters recover to pre-exercise levels after either protocol. Whilst this suggests that duration of exercise has a greater effect than intensity with regards GE recovery it is possible that if participants were monitored for a greater time period post-exercise then a difference in the rate of recovery between protocols may become evident. The decrease in CGE after LCIE is in agreement with previous studies (Passfield and Doust 2000; Hopker, O'Grady and Pageaux 2016; Almquist *et al.* 2019) with NIRS parameters significantly lower post-exercise compared to pre-exercise. High levels of ROS have been observed during prolonged cycling exercise (Sahlin *et al.* 2010). Hopker, O'Grady and Pageaux (2016) suggest that there is potentially a decoupling of oxidative phosphorylation at the mitochondria which could be a mechanism of producing ATP to reduce ROS generation and thus oxidative damage within the cell (Brand, 2000).

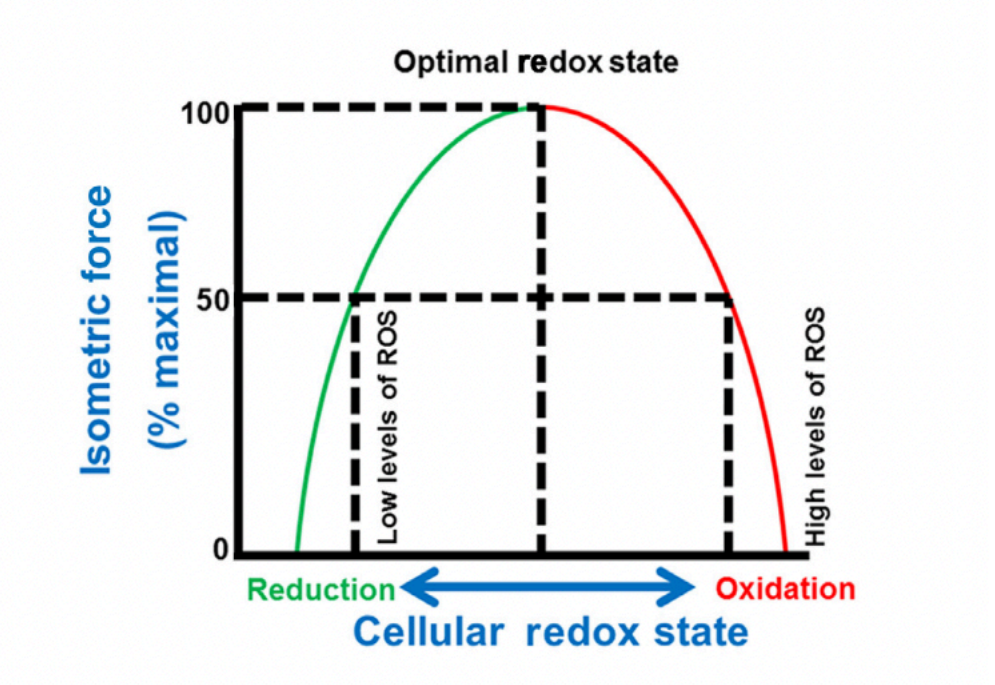

Figure 7.1 *The relationship between cellular redox state and skeletal muscle force production. Movement away from the optimal redox state results in a decrease in muscle force production. Taken from* Powers and Jackson (2008)

As LCIE_SI incorporated 15 x 10s sprint intervals it is possible that the mechanism behind the reduction of BGE is similar to those suggested by Vanhatalo et al. (2011) who suggested that the decreases in efficiency seen after a 3 minute all out test are secondary to a greater cumulative effect of the slow kinetics of the initially recruited type 2 muscle fibres, reduced contractile efficiency (due to the accumulation of metabolites such as H+, Pi, ADP and ROS or greater depletion of substrates such as muscle phosphocreatine) and EPOC. Indeed, it has been shown that ROS exerts a biphasic influence on force production (see figure 7.1) (Reid *et al.* 1992; Reid, Khawli and Moody 1993; Reid 2001; Reid *et al.* 2005). This could potentially explain why both SI_{av} and SI_{max} progressively decrease over time in LCIE_SI. This may have been a factor in the progressive decrease in sprint interval average, SI maximal power and BGE during LCIE_SI. However, NIRS parameters in both protocols were decreased during and after exercise compared to pre-exercise, there was no significant difference in TSI and HHb between protocols. This would suggest that changes in oxygenation (and thus mitochondrial uncoupling) at the level of skeletal muscle isn't the main determinant in the reduction in CGE after LCIE_SI or LCIE. An alternate explanation is that the reductions in CGE seen after LCIE and LCIE_SI are due to an increased or progressive recruitment of type 2 muscle fibres in an attempt to offset fatigue induced reductions in muscle force generation capacity of type 1 fibres.

Whilst a reduction in efficiency (i.e. an increased O_2 cost per unit of external work delivered) has been observed during both a 3-minute all out test and a 3-minute high-intensity continuous work rate (CWR) bout, an increase in integrated iEMG was only observed during CWR (Vanhatalo *et al.* 2011). Indeed, Krustrup, Söderlund, Mohr, González-Alonso, *et al.* (2004) demonstrate a progressive recruitment of type 1 and type 2 muscle fibres during high-intensity CWR (80%$\dot{V}O2_{max}$) with the lower efficiency of type 2 fibres recruited later in HIE CWR potentially an important factor in delayed onset of the $\dot{V}O2$ slow component during HIE. Conversely, Noordhof et al. (2015) found during time-trials of varying distance that there was a small but not significant mean increase in iEMG of the gluteus maximus, vastus medialis, vastus lateralis and biceps femoris but there was a mean effect of time. More recently, Almquist *et al.* (2019) showed a gradual recruitment of muscle motor units over time during prolonged (240 minutes) low-intensity cycling at 50%$\dot{V}O2_{max\ x}$ using iEMG in the vastus

lateralis and vastus medialis with the authors speculating that changes in motor-unit recruitment may reflect a decreasing efficiency of already recruited fibres. A limitation of this thesis is that muscle motor unit recruitment was not assessed and so the effect of additional muscle motor unit recruitment on GE during exercise of varying intensity or duration is unknown.

The LCIE_SI protocol was designed to mirror the duration and number of sprint intervals in a typical professional criterium road race (Ebert *et al.* 2006) the practical implication would be for cyclists and coaches to model how many high-intensity or sprint-intensity efforts can be regularly achieved over the duration of a typical road race without a loss in performance (i.e. SIav, SImax and GE)

Chapter 5 demonstrates despite varying the interval intensity and duration in acute exercise GE recovers to pre-exercise levels after an AR5 and 5-minute submaximal bout at 150W. The recovery in NIRs parameters suggest that despite an incurred oxygen deficit during each protocol, this is rapidly repaid during the recovery phase. This contrasts with Groot et al. (2017) where GE did not recover to pre-exercise levels after time-trials of 2,000m and 20,000m where participants recovered at 55%$_{MAP.}$ Therefore, it would appear that for acute 40-minute continuous (CONT) or repeated intervals of varying intensity and duration (LI and SI), an oxygen deficit and recovery related processes are the main cause of decreases in CGE. This contrasts with the findings of chapter 4 where the primary mechanism of decreases in CGE after prolonged exercise may be due to increases of less efficient type 2 muscle fibres.

Chapter 6 aims to investigate possible mechanisms for the changes in GE seen after acute exercise of varying interval intensity and duration using the participants and protocols used in chapter 5. GE has been shown to be affected by both acute exercise (Koning *et al.* 2013; Noordhof *et al.* 2015a; Hopker, O'Grady and Pageaux 2016; Groot *et al.* 2018; Almquist *et al.* 2019) and chronic training ((Hopker, Coleman and Passfield 2009; Hopker *et al.* 2010)). The findings in chapters 3 to 6 provide further evidence that GE is affected by both the intensity and duration of exercise. Therefore, it is likely that acute cellular regulatory processes link acute exercise and chronic training responses(Chen *et al.* 2005; Weber *et al.* 2010; Torma *et al.* 2020b).

Chapter 6 describes a study which aims to investigate the effect of different acute exercise protocols (CONT, LI and SI) on ci-miRNA expression. Ci-miRNAs that have been found to regulate the activation and/or repression of specific cell signalling pathways and thus the transcription and translation of proteins, enzymes and genes that influence exercise adaptation (Chen *et al.* 2005; Weber *et al.* 2010; Torma *et al.* 2020b) Ci-miRNA-21 and -222 have been shown to be higher in endurance athletes compared to strength athletes (Wardle *et al.* 2015) and upregulated after acute exercise and training (Baggish *et al.* 2011; Horak *et al.* 2018). Potential mRNA targets of ci-miRNA- 21 and -222 include SOD proteins involved in redox metabolism (Dubois-Deruy *et al.* 2017; Zhang *et al.* 2012b) and angiogenesis (Weber *et al.* 2010; Liu *et al.* 2011) which may influence metabolic and mechanical efficiency.

In chapter 6, there was a decrease in ci-miRNA-21 immediately post-exercise in CONT and ci-miRNA-21 was negatively correlated with the CGE change between visits 3 and 1 in SI. The expression of ci-miRNA-21 appears to increase in response to both continuous shear stress within the vasculature (Nicoli *et al.* 2010) and short-term hypoxia (Wahl. 2016) with resultant increases in angiogenesis via the indirect upregulating of the pro-angiogenic protein vascular endothelial growth factor (VEGF) and hypoxia inducible factor-1 (HIF-1α) expression (Liu *et al.* 2011) as well as increasing endothelial nitric oxide synthase activity (Weber *et al.* 2010; Traub and Berk 1998). An increase in angiogenesis in continuous exercise may be advantageous in offsetting the fatigue of type 1 muscle fibres and the additional recruitment of less efficient type 2 muscle fibres that may occur during prolonged duration exercise (Almquist *et al.* 2019) and longer time-trials. Contrastingly, the increase in angiogenesis stimulated by SI may help to increase oxygenation of mitochondria and maintain ROS hormesis. The increase in GE at visit 3 compared to visit 1 after SI would indicate a beneficial adaptive response that may partly explain increases in GE after chronic training (Hopker, Coleman and Passfield 2009; Hopker *et al.* 2010).

Chapter 6 also demonstrated that ci-miRNA-222 had a negative correlation with the CGE change between visits 3 and 1 in LI. One of the validated targets of ci-miRNA-21 and ci-miRNA-222 are the antioxidant enzymes superoxide dismutase (SOD) that exist both within the mitochondrial (SOD2 and ci-miRNA-222) and extracellular matrix (SOD3 and ci-miRNA-21) as components of the body's antioxidant defence system.

Strenuous single bouts of acute moderate to intense endurance exercise are known to induce the overproduction of ROS (Powers and Jackson 2008; Gomes, Silva and Oliveira 2012; Powers, Radak and Ji 2016) with subsequent oxidative damage potentially causing elevated lipid perioxidation, DNA oxidative damage (Mastaloudis et al 2004), immunosuppression (Neilsen et al. 2004), muscle damage and contractile dysfunction (Powers and Jackson 2008).

It has been suggested that regular exercise generates mitochondrial ROS that is necessary for the activation of primary signalling pathways associated with muscle adaptation (He *et al.* 2016) including the enhancement of mitochondrial biogenesis (see figure 6.2)

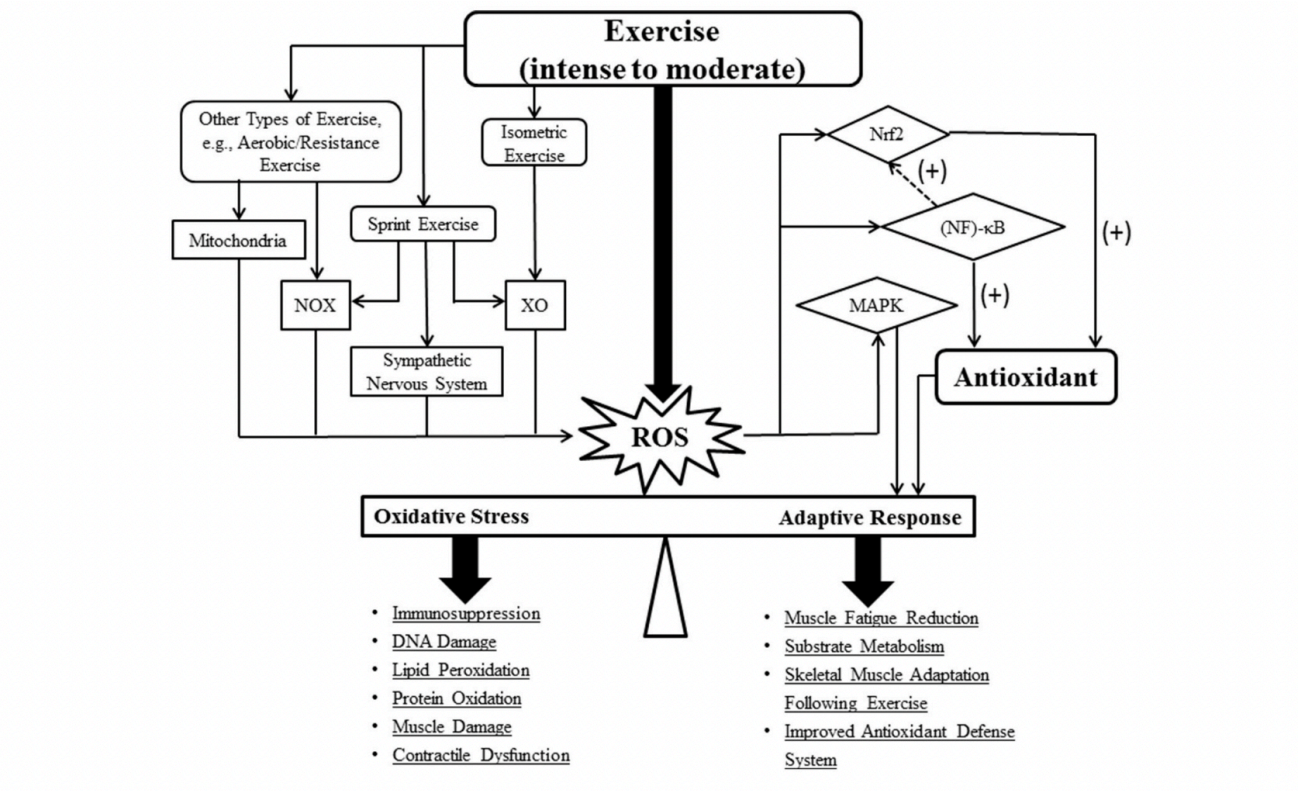

Figure 6.2 *Schematic illustrating ROS generation during different types of exercise and their associated roles in adaptive response. The dash arrow represents an indirect effect. Abbreviations: NADPH oxidase (NOX); xanthine oxidase (XO); mitogen-activated protein kinase (MAPK_ nuclear erythroid 2 p45-related factor 2 (NRf2); nuclear factor kB (NF-kB)*

Several studies have demonstrated that SODs are increased in response to exercise training (Ji, Fu and Mitchell 1992; Powers *et al.* 1994; Booth *et al.* 2015). Therefore, the decreases in fold change of ci-miRNAs-21 and -222 and subsequent increase in GE may be secondary to a triggered adaptive response mediated in part by an increase in SOD3 and SOD2 respectively. These changes in ci-miRNAs and corresponding changes in GE may be beneficial in providing a biomarker of individual adaptation in response to acute exercise and chronic training in response to different intensities and duration of exercise. However, this thesis did not measure ROS directly or downstream regulatory protein targets in relation to these changes in ci-miRNAs. Furthermore, the reliability of these changes in ci-miRNAs in relation to GE has yet to be investigated.

7.2 CONCLUSIONS

The CGE method is not reflective of $\dot{V}O2$ measured at the mouth during HIE and thus underestimates the anaerobic contribution to HIE. BGE is a valid and reliable method of estimating GE during high-intensity intervals of varying duration and intensity. Furthermore, CGE can recover to pre-exercise levels if a low enough recovery intensity is employed.

Additionally, changes in ci-miRNAs involved in angiogenesis and ROS hormesis have been observed after acute interval exercise of varying interval intensity and duration. These changes could explain the adaptive response and increase in GE seen with chronic training.

7.2 PRACTICAL APPLICATIONS

The measurement and use of BGE could be used in a variety of contexts. It could potentially be used as an index of a rider's ability to respond and recover to surges in power within a typical racing scenario or as a part of a pacing strategy during time-trials of varying duration. Additionally, ci-miRNAs may potentially be used as a marker of individualised adaptive training adaptation in response to exercise of different intensity and duration. However, further work is needed to assess the reliability of ci-miRNA changes and the associated temporal response with chronic training.

7.3 FUTURE WORK

Future directions based on this thesis could include:

1. What is the optimal work rate that would allow GE to fully recover whilst not impacting on a race scenario?
2. How does BGE change directly after acute HIE or SIE when an AR5 is not employed?
3. What is the reliability and validity of the changes in ci-miRNA-21 and -222 seen in chapter 6 and how do these changes relate to physiological exercise performance?
4. How do changes in ci-miRNA-21 and -222 after HIE and SIE relate to downstream markers pf skeletal muscle redox metabolism and angiogenesis?

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