The impact of high-carbohydrate and high-fat diets in combination with nitrate on O_2 uptake kinetics and performance during high-intensity exercise

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature: …………………………………………………..

Abstract

This study examined the impact of a high carbohydrate (HCHO) and high fat (HFAT) diet in combination with nitrate supplementation on oxygen uptake kinetics and performance during severe-intensity exercise. Ten healthy and physically active males were assigned in a double-blind, randomized, crossover design to consume HCHO (74% carbohydrate, 16% protein, 10% fat), HFAT (9% carbohydrate, 19% protein, 72% fat), along with 8 mmol of nitrate (NIT) or placebo (PL) over 4 days preceding the completion of a time-to-exhaustion trial (TTE) in the severe-intensity exercise domain. Cycling baseline RER in HCHO was significantly higher (~17%) compared to HFAT ($P<0.05$) and fat oxidation rates were 4-fold higher in HFAT ($P<0.05$) whereas CHO oxidation rates were 2-fold higher in HCHO ($P<0.05$). Resting plasma $[NO_3^-]$ increased significantly (~812%) in NIT (24.45±7.45 µM) compared to PL (2.68±0.54 µM) in HCHO, and by (~1023%) in NIT (30.33±7.88 µM) compared to PL (2.70±0.77 µM) in HFAT ($P<0.05$). Resting plasma $[NO_2^-]$ increased by ~88% in NIT (697±343 nM) compared to PL (370±121 nM) in HCHO ($P<0.05$) and by ~47% in NIT (521±221 nM) compared to PL (353±247 nM) in HFAT ($P>0.05$). Cycling baseline $\dot{V}O_2$ was significantly lower in HCHO+PL (994±107 ml/min) when compared to HFAT+NIT (1037±122 ml/min, $P<0.05$) and HFAT+PL (1072±134 ml/min, $P<0.05$). $\dot{V}O_2$ cycling baseline in HCHO+NIT (1008±145 ml/min) was significantly lower compared to HFAT+PL (1072±134 ml/min, $P<0.05$). There were no significant differences in $\dot{V}O_2$ peak between conditions ($P>0.05$). $\dot{V}O_2$ was higher across the rest-to-exercise transition in HFAT resulting in earlier attainment of $\dot{V}O_2$ peak and shorter TTE. TTE was shorter in HFAT+NIT (154±40 s) and HFAT+PL (159±39 s) compared to HCHO+NIT (174±35 s, $P<0.05$) and HCHO+PL (186±39 s, $P<0.05$). These findings suggest that 4 days of a HFAT diet elevates $\dot{V}O_2$ and impairs performance relative to HCHO during severe-intensity exercise. Additionally, the macronutrient content of diet impacts on the ability to convert $[NO_3^-]$ to $[NO_2^-]$, with this being more favourable in the HCHO compared to the HFAT diet.

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Abbreviations

Δ	difference
τp-	time constant
acetyl CoA	acetyl coenzyme A
ADP	adenosine diphosphate
ATP	adenosine triphosphate
A_p	amplitude
BRJ	beetroot juice
Ca^{2+}	calcium
CNS	central nervous system
ETC	electron transport chain
$FAD/FADH_2$	flavin adenine dinucleotide
GET	gas exchange threshold
GLUT-2	glucose transporter type 2
GLUT-4	glucose transporter type 4
H^+	hydrogen ion
HCHO	high carbohydrate diet
HFAT	high fat diet
HR	heart rate
K^+	potassium
KCl	potassium chloride
KNO_3	potassium nitrate
MRT	mean response time
$NAD^+/NADH$	nicotinamide adenine dinucleotide

NaNO$_3$	sodium nitrate
NaOH	sodium hydroxide
NIT	nitrate supplementation
NO	nitric oxide
NO$_2^-$	nitrite
NO$_3^-$	nitrate
PCr	phosphocreatine
PDH	pyruvate dehydrogenase
P$_i$	inorganic phosphate
PL	placebo
PO$_2$	partial pressure of oxygen
RER	respiratory exchange ratio
RPM	revolutions per minute
TD$_p$	time delay
TTE	time to exhaustion
$\dot{V}CO_2$	carbon dioxide output
$\dot{V}E$	ventilation
$\dot{V}O_2$ max	maximal oxygen uptake
$\dot{V}O_2$	pulmonary oxygen uptake
W$_{peak}$	maximal work rate attained during ramp incremental test
WR	work rate
ZnSO$_4$	zinc sulphate

Chapter 1: Brief Literature review

Physiological functions of nitric oxide in the human body

Nitric oxide (NO) is a gaseous physiological signalling molecule that was originally discovered as an 'endothelium-derived relaxing factor' in the 1980's. As a consequence of its importance in neuroscience, physiology, and immunology, NO was awarded "Molecule of the Year" in 1992, subsequently, the research conducted by Ignarro, Furchgott and Murad into its function led to them being awarded the Nobel Prize in 1998 for discovering the role of NO as a cardiovascular signaling molecule. Currently, NO is known for its capacity to modulate an array of physiological processes in skeletal muscle at a vascular and metabolic level in relation to both health and exercise (for reviews see Jones, 2014; Zafeiridis, 2014). Specifically, NO has been shown to improve regulation of blood flow (Stamler and Meissner, 2001), oxygen (O_2) cost and delivery (Wylie et al., 2013b; Larsen et al., 2007; Bailey et al., 2009), neurotransmission (Garthwaite, 2008), mitochondrial respiration (Ghafourifar and Cadenas, 2005) and biogenesis (Nisoli et al., 2003; Vaughan et al., 2016), sarcoplasmic reticulum calcium (Ca^{2+}) handling (Hernandez et al., 2012), and skeletal muscle glucose uptake (Wylie et al., 2013a) as well as muscle efficiency (Larsen et al., 2007), fatigue resistance (Bailey et al., 2009; 2010) and performance (Cermak et al., 2012; Lansley et al., 2011b). Therefore, the clear role of NO on muscle bioenergetics invites an opportunity for further examination in the field of applied sport and health.

Pathways to generate nitric oxide

NO is biosynthesised via enzymatic and non-enzymatic endogenous and exogenous pathways (Lundberg and Weitzberg, 2009; Zafeiridis, 2014). In the enzymatic endogenous pathway, NO is synthesised through the oxidation of L-arginine catalysed by NO synthase enzymes (NOS), whilst the non-enzymatic pathway involves the reduction of inorganic nitrate to NO via the "nitrate (NO_3^-) – nitrite (NO_2^-) -NO" pathway. This latter pathway functions independently of NOS and the availability of O_2, therefore complementing the L-arginine-NOS-NO pathway in which NOS activity can be compromised (Lundberg and Weitzberg, 2009). Additionally, it has been recently found that NO can be synthesised exogenously via the consumption of dietary NO_3^-. A diet particularly high in NO_3^-

(>4 mmol nitrate/100 g fresh weight) includes green leafy vegetables such as spinach, rocket, lettuce and beetroot (Hord et al., 2009; Gilchrist et al., 2010). Following the consumption of dietary NO_3^-, NO_3^- is absorbed from the upper part of the small intestine into the plasma reaching a peak concentration in ~ 1-2h (Jones, 2014; Zaferidis, 2014). A portion (~25%) of this NO_3^- is taken up by the salivary glands and concentrated in saliva and undergoes metabolic conversion to NO_2^- in the oral cavity (Lundberg and Weitzberg, 2009). In the human body, NO_3^- reduction to NO_2^- is heavily dependent on a symbiotic relationship with the oral bacteria on the dorsal surface of the tongue (Govoni et al., 2008). Small portions of swallowed NO_2^- are reduced to NO in the acidic environment of the stomach, but a significant amount of NO_2^- enters the systemic circulation with peak plasma $[NO_2^-]$ being achieved in ~2-3 hours, decreasing back to baseline in ~24 hours (Lundberg and Govoni, 2004; Webb et al., 2008). A number of enzymes including deoxyhaemoglobin (Cosby et al., 2003) and xanthine oxidase (Li et al., 2004) facilitate the conversion of NO_2^- to NO in the human body. This exogenous process is also facilitated in conditions of low O_2 availability (Vanhatalo et al., 2011) and low pH (Modin et al., 2001) that are not only present in certain pathologies but also in skeletal muscle during exercise.

Ergogenic potential of dietary nitrate

Following the pioneering study of Larsen et al. (2007) that found enhanced exercise efficiency following consumption of sodium nitrate, a plethora of studies investigated the effects of acute (<2 days) and chronic (2-15 days) dietary NO_3^- supplementation on exercise metabolism, capacity and performance. Indeed, NO_3^- supplementation was administered in healthy, non-athletic population and in athletes, at both moderate and high-intensity exercise and across multiple modes of exercise including running (Peacock et al., 2012; Lansley et al., 2011a), swimming (Pinna et al., 2014), cycling (Wilkerson et al., 2015; Cermak et al., 2012; Lansley et al., 2011b), team sports (Wylie et al., 2013a; Thompson et al., 2016), crossfit (Kramer et al., 2016), cross-country skiing (Sandbakk et al., 2015), rowing (Bond et al., 2012) and kayaking (Peeling et al., 2014). Subsequently, dietary NO_3^- supplementation especially via consumption of beetroot juice (BRJ) has gained popularity as a potential ergogenic aid in exercise and sports performance (Flueck et al., 2016; McMahon et al., 2016). Indeed, one of the biggest ergogenic effect of NO_3^- supplementation is reduced O_2 cost of exercise

in moderate (Bailey et al., 2009; 2010; Vanhatalo et al., 2010; Larsen et al., 2007) and high-intensity exercise (Bailey et al., 2009; 2010). The mechanism responsible for this phenomenon has been investigated by Bailey et al. (2010) and Larsen et al. (2011) who reported that the reduced $\dot{V}O_2$ cost of exercise may be associated with improved efficiency of muscle contraction and mitochondrial oxidative phosphorylation, respectively. Bailey et al. (2010) reported that increased exercise tolerance at the same work rate can be attributed to the lower O_2 cost as a result of lower adenosine triphosphate (ATP) cost of muscle contraction for the same force production. Using 31P-magnetic resonance spectroscopy, the authors found that six days of BRJ supplementation improved muscle contraction efficiency through reduced ATP turnover rates from phosphocreatine (PCr) hydrolysis and oxidative phosphorylation with no compensatory increase in energy contribution from anaerobic glycolysis. Consequently, important contributors to muscle fatigue development such as the intramuscular accumulation of adenosine diphosphate (ADP) and inorganic phosphate (Pi), and the extent of PCr depletion were blunted following NO_3^- supplementation in this study. Bailey et al. (2010) proposed that the altered energy cost of muscle force production could be attributed to reduced Ca^{2+} ATPase and actin-myosin ATPase activity, which are enzymes responsible for Ca^{2+} pumping from sarcoplasmic reticulum and interaction between actin and myosin, respectively (Ishii et al., 1998; Evangelista et al., 2010). The possibility that NO_3^- supplementation enhances mitochondrial efficiency has been investigated by Larsen et al. (2011). These authors reported that NO_3^- supplementation improved mitochondrial efficiency by increasing the mitochondrial P/O ratio (ATP produced per amount of the oxygen used) as a result of reduced proton leakage and uncoupled respiration.

More recently, it has been suggested that the physiological effects of NO_3^- may be muscle fibre type specific. Ferguson et al. (2013) and Hernandez et al. (2012) reported that NO_3^- supplementation preferentially improved perfusion and Ca^{2+} handling in type II compared to type I muscle fibres in rodents, respectively. Jones et al. (2016) presented evidence that NO_3^- supplementation may promote the reduction of NO_2^- to NO in type II muscle fibres in humans, hereby improving local perfusion and fibre contractility. In turn, this would be expected to speed up $\dot{V}O_2$

kinetics (Breese et al., 2013; Bailey et al., 2015) and to reduce the degradation of PCr and glycogen and the accumulation of metabolites associated with fatigue development (ADP, H^+, P_i, K^+), ultimately with positive effects on exercise tolerance/performance. Indeed it has been consistently shown that ingestion of inorganic NO_3^- improves performance time-to-exhaustion during severe-intensity exercise by 15-25% (McMahon et al., 2016; Bailey et al., 2009; 2010; Lansley et al., 2011a), high-intensity intermittent exercise performance by 1-5% (Thompson et al., 2016; Wylie et al., 2013a), and time-trial performance by 1-2% (Cermak et al., 2012; Lansley et al., 2011b), although the latter has not been consistently shown in highly trained athletes (Christensen et al., 2013; Wilkerson et al., 2012; Bescós et al., 2012; Peacock et al., 2012; Sandbakk et al., 2015).

Alternatively, NO_3^- has been recently shown to positively impact on carbohydrate (CHO) metabolism (Vaughan et al., 2016; Larsen et al., 2011; Wylie et al., 2013a). Whilst the Larsen et al. (2011) study found that NO_3^- increases the respiratory exchange ratio (RER) during submaximal exercise, Wylie et al. (2013a) found lower plasma glucose during high intensity intermittent exercise. Interestingly, both studies indicate that nitrate supplementation may enhance glucose handling and CHO utilisation, a fuel that is more efficient in terms of O_2 consumption required per a given ATP turnover in comparison to fat (i.e. ~5.5-8% more ATP/L of O_2 consumed) (Burke et al., 2017; Brouwer, 1957). More recently, Vaughan et al. (2016) treated murine myocytes with different concentrations of BRJ and found that NO_3^- promotes more complete CHO oxidation, improved insulin sensitivity and increased the expression of the glucose transporter type 4 (GLUT-4), a protein responsible for the transport of glucose from blood to cells. Additionally, they found that NO_3^- increased oxidative metabolism concurrently with elevated metabolic gene expression, leading to increased mitochondrial biogenesis.

Potential interactions between dietary nitrate and diet

Considering the aforementioned, this would suggest that NO_3^- supplementation may promote CHO metabolism (Vaughan et al., 2016; Wylie et al., 2013a, Beals et al., 2017). Recently, several studies have focused their attention on substrate flux as a determinant of $\dot{V}O_2$ kinetics and exercise metabolism (Raper et al., 2014;

Leckie and Kowalchuk, 2013; Lima-Silva et al., 2011; 2013). Indeed, similar to the positive effects of NO_3^- supplementation, the availability of CHO has been shown to impact on several metabolic and functional determinants of exercise performance via enhanced $\dot{V}O_2$ kinetics in moderate (Raper et al., 2014), heavy (Leckie and Kowalchuk, 2013) and extreme-intensity exercise (Lima-Silva et al., 2011); and preferential effects on CHO oxidation and RER, blood lactate and glucose (Raper et al., 2014; Leckie and Kowalchuk, 2013). In contrast, increased availability of fat has been shown to have the opposite effect on these same variables (Raper et al., 2014; Leckie and Kowalchuk, 2013). Although dietary intake has been recently identified as a key determinant of microbiome composition and diversity, the influence of diet in combination with NO_3^- on exercise metabolism and performance is currently unclear (Donovan, 2017; Beals et al., 2017). However, it might be proposed that changing the macronutrient composition of the diet through manipulation of CHO and fat intake could influence the NO_3^--NO_2^--NO pathway and therefore impact on exercise metabolism and performance. Therefore, the aim of the present study is to investigate the impact of high-carbohydrate and high-fat diets in combination with NO_3^- on O_2 uptake kinetics and performance during severe-intensity exercise.

Chapter 2: Experimental study

The impact of high-carbohydrate and high-fat diets in combination with nitrate on O_2 uptake kinetics and performance during high-intensity exercise

Introduction

One of the fundamental tenets of exercise physiology is a highly predictable oxygen (O_2) cost for a given sub-maximal work rate (Jones and Poole, 2005). It is now well known that upon the initiation of exercise in the moderate exercise intensity domain, below gas exchange threshold (GET), pulmonary oxygen uptake ($\dot{V}O_2$) reflects and closely matches muscle O_2 consumption, achieving a steady state in 2-3 min in healthy humans (Jones and Poole, 2005). However, at higher exercise intensities, $\dot{V}O_2$ kinetics become more complex owing to the development of the "slow component" which elevates the O_2 cost above that found in the moderate domain (~10ml $O_2.Min^{-1}.W^{-1}$). This is associated with disruptions in metabolic homeostasis as reflected by an accumulation of metabolites (H^+, ADP, P_i) and ionic imbalance (K^+ efflux), the factors that are associated with fatigue processes (Allen et al., 2008; Burnley and Jones, 2007). Additionally, as exercise increases above GET and continues to proceed from the heavy to the severe-intensity exercise domain, stores of intramuscular glycogen and intramuscular PCr that are the main but finite energy substrates are utilized (Egan and Zierath, 2013). In sports performance, this eventually leads to a termination of exercise or a reduction in intensity of exercise in order for a body to replenish intramuscular PCr stores and restore homeostasis (Chidnok et al., 2013; Skiba et al., 2014).

Skeletal muscle displays remarkable adaptability, enabling substantial modifications to its metabolic and functional characteristics in response to external stimuli that includes mechanical loading and nutrient availability (Hawley et al., 2011; Maughan et al., 1997). Considering this, there has been a plethora of research that has investigated how to extend the capacity of athletes to perform at high exercise intensities, using training and/or specific dietary interventions (Burke et al., 2016; Burke, 2015; Morton and Close, 2015; Maughan and Poole, 1981). Generally, the results from those studies favour a diet rich in CHO or using

nutritional strategies such as "CHO-loading", ingestion of CHO before and/or during exercise (Burke et al., 2016; Burke et al., 2011) and CHO mouth rinsing (Carter et al., 2004; Burke and Maughan, 2015), all of which have been shown to positively impact on exercise metabolism and $\dot{V}O_2$ kinetics and therefore exercise capacity and performance (Raper et al., 2014; Leckie and Kowalchuk, 2013; Lima-Silva et al., 2011; 2013; Burke, 2015; Burke et al. 2017). The observed effects on metabolism have been attributed to the increased availability and sparing of glycogen stored in muscles and liver (Jeukendrup, 2004; Jeukendrup, 2007), increased CHO oxidation and RER (Raper et al., 2014) as well as having a positive impact on the central nervous system (CNS) (Burke and Maughan, 2015; Jeukendrup, 2007). Additionally, Stellingwerff et al. (2006) found that a diet high in CHO upregulates the pyruvate dehydrogenase complex (PDH), a rate limiting enzyme complex involved in the irreversible conversion of pyruvate to acetyl coenzyme (acetyl CoA) that regulates entry of CHO from the glycolytic pathway into the tricarboxylic acid (TCA) cycle and therefore has been identified to have a potential to affect the phase II and III of $\dot{V}O_2$ kinetics (Raper et al., 2014). Indeed, HCHO diet has been associated with shorter time constant (τ_p) and attenuated $\dot{V}O_2$ amplitude during moderate (Raper et al., 2014), heavy (Leckie and Kowalchuk, 2013) and extreme-intensity exercise (Lima Silva et al., 2011) when compared to HFAT, however this has not been examined in severe-intensity exercise (Leckie and Kowalchuk, 2013). Considering the limited availability of CHO in the human body, diets with a high fat and low CHO content have recently received attention, representing a potential alternative source of fuel to CHO (Volek et al., 2014). Nevertheless, despite the positive effects on increased breakdown, transport and oxidation of lipids in skeletal muscle (Burke et al., 2016; Yeo et al., 2011; Raper et al., 2014) that have been observed in research studies investigating high fat diets (>70% of energy consumed as fat) on exercise performance and exercise metabolism, studies conducted to date have failed to find clear evidence of benefits to performance (for review see Burke et al., 2016 and Burke, 2015) even in elite endurance athletes (Burke et al., 2017).

The dietary supplement that has received significant attention from both health and performance perspectives in the last decade is inorganic nitrate (NO_3^-). NO_3^- that can be found in green leafy vegetables and beetroot or can be ingested in the form of sodium ($NaNO_3$) or potassium nitrate (KNO_3), has been found to be a

precursor of NO that is an important physiological signalling molecule with a capacity to modulate skeletal muscle function at vascular and metabolic levels (Jones 2014). Indeed, both acute (< 2 days) and chronic (2-15 days) ingestion of inorganic NO_3^- has been shown to positively impact on an array of physiological and performance outcomes associated with muscle efficiency and fatigue resistance at both moderate and high exercise intensities (McMahon et al., 2016; Zaferidis, 2014; Bailey et al., 2009; 2010; Larsen et al., 2007; 2011). Those include improved regulation of blood flow and increased oxygen delivery to muscles, muscle contractility, mitochondrial respiration, cell signalling and neurotransmission as well as Ca^{2+} handling (for review see Jones, 2014 or Zaferidis, 2014). More recently, nitrate supplementation has been associated with increased RER (Larsen et al., 2011), improved glucose handling (Wylie et al. 2013a), more complete CHO oxidation and alterations in expression of GLUT 4 transporter (Vaughan et al. 2016). Additionally, the efficacy of NO_3^- has been recently linked with the type II muscle fibres where degradation of PCr and glycogen occurs at higher rate than in type I fibres owing to lower O_2 availability, O_2 tension (PO_2) and pH (Jones et al. 2016). Indeed it has been consistently shown that ingestion of inorganic NO_3^- speeds up phase II $\dot{V}O_2$ kinetics (Breese et al., 2013; Bailey et al., 2015), reduces $\dot{V}O_2$ amplitude (Bailey et al. 2009; 2010) and increases time-to-exhaustion at severe-intensity, constant work rate exercise by 15-25% (McMahon et al., 2016; Bailey et al., 2009; 2010; Lansley et al., 2011a), high-intensity intermittent exercise performance by 1-5% (Thompson et al., 2016; Wylie et al., 2013a), and time-trial performance by 1-2% (Cermak et al., 2012; Lansley et al., 2011b).

Collectively, both the macronutrient content of the diet and NO_3^- supplementation have the potential to impact on muscle efficiency and metabolism during high-intensity exercise. Therefore, the aim of study was to investigate the impact of HCHO and HFAT diets in combination with and without NO_3^- on physiological responses and time-to-exhaustion (TTE) during severe-intensity exercise. In total, there were four different experimental conditions: 1) high carbohydrate diet with nitrate (HCHO+NIT); 2) high carbohydrate diet with placebo (HCHO+PL) (>70% of energy consumed as CHO); 3) high fat diet with nitrate (HFAT+NIT) and 4) high fat diet with placebo (HFAT+PL) (>70% of energy consumed as fat). We hypothesised that a HCHO+PL diet would evoke favourable changes in TTE

during severe-intensity exercise and physiology as a result of improved $\dot{V}O_2$ kinetics, increased RER, CHO oxidation and blood lactate when compared to HFAT+PL. Furthermore, we hypothesised that the addition of dietary NO_3^- to HCHO would accentuate these changes relative to HFAT+NIT.

Methods

Subjects. 10 healthy male subjects (mean± SD, age 26±7 years, weight 78.9±9.6 kg, height 178±6 cm, $\dot{V}O_2$ max 49.3±5.1 ml/kg/min, W_{peak} 331±31 W) volunteered to participate in this study that had received approval from the Sport and Health Sciences (University of Exeter) research ethics committee and was in accordance with the standards set by the World Medical Association (Declaration of Helsinki). All subjects were recreationally active, non-smokers and had no known history of respiratory, cardiovascular, metabolic or musculoskeletal disease and were not taking any medications that might have affected the physiological variables under investigation. Prior to any testing, all subjects filled out a Physical Activity Readiness Questionnaire and were informed of the protocol, risks and discomfort associated with the procedure and potential benefits, both verbally and in writing, and gave their written consent.

Study design. Subjects reported to the laboratory on 6 separate occasions. Subjects were asked to arrive at the laboratory in a rested, fasted but fully hydrated state without any prior strenuous exercise in the 24 hours preceding each laboratory visit. Participants were asked to refrain from consuming caffeine and alcohol for 24 h before each visit. Subjects were also asked to refrain from using anti-bacterial mouthwash and chewing gum throughout the study in order to preserve commensal oral bacteria that has been shown to be responsible for the reduction of NO_3^- to NO_2^- (Govoni et al., 2008).

Diet and Nitrate Supplementation. In total, there were four different experimental conditions: 1) high carbohydrate diet with nitrate (HCHO+NIT); 2) high carbohydrate diet with placebo (HCHO+PL); 3) high fat diet with nitrate (HFAT+NIT) and 4) high fat diet with placebo (HFAT+PL). We estimated energy intake (EI) based on gender and level of activity of the participants. To achieve maximal compliance to the designed diets, all participants were given food with the macronutrient composition of 74% CHO, 16% protein , 10% fat for HCHO

diet (EI 2557 kcal) and 9% CHO, 19% protein, 72% fat for HFAT (EI 2558 kcal). If subjects did not consume the given food, they were asked to bring any remaining food back for later dietary analysis. The NO_3^- supplementation interventions protocol was based on previous studies (Wylie et al., 2013b). 8 mmol of NO_3^- in the form of potassium nitrate (KNO_3) was given to the participants in two servings (4 mmol) that they were asked to consume with their morning and evening meal. Potassium chloride (KCl) was used as a placebo (PL) in this study. In the morning of the 4th day of each diet, subjects were asked to report to the laboratory and a standardized isoenergetic breakfast (60 g oats+ 330 ml skimmed milk + 35 g honey = 426 kcals in HCHO diet or 125 g coyo (coconut yoghurt) with 25 g peanut butter = 423 kcals in HFAT) with the final dose of nitrate (NIT, 8 mmol) or placebo (PL) was consumed 2 hours prior to the exercise. All participants completed all 4 conditions in a balanced, randomised double blinded and placebo-controlled cross-over design separated by a minimum of a 4 day washout period, during which participants were asked to consume their habitual mixed diet.

Exercise tests. All exercise tests were performed using an electronically-braked cycle ergometer (Lode, Excalibur Sport, Groningen, Netherland). In *visit 1*, subjects completed a ramp incremental test to exhaustion for the determination of the maximal oxygen uptake ($\dot{V}O_2$ max) and maximal power output which were used to normalise the fixed resistance for the severe-intensity time-to-exhaustion trial (TTE) we used in the study. The ramp incremental test protocol consisted of 3 min of unloaded baseline cycling at 20 W followed by a linear increase of power output at the rate of 30 W/min until volitional exhaustion. Subjects were asked to maintain their preferred cadence (70 to 90 rpm) for as long as possible, and when cadence fell >10 rpm below the chosen pedal rate for >5 s despite strong verbal encouragement, the test was terminated and peak power output was recorded. The pedal rate along with the saddle and handlebars positions were recorded and reproduced in the subsequent tests. *Visit 2* was used to familiarize the participant with the experimental set up and the intensity of the severe-intensity TTE used in visits 3-6. The test began with 10 min in a seated position and 5 min of unloaded baseline pedalling at 20 W followed by an abrupt increase to the fixed power output corresponding to participants' ramp peak power – 20 W. Subjects were instructed to maintain the cadence used in preliminary testing for as long as

possible during the test. The test was terminated when cadence fell >10 rpm below preferred cadence for more than 5 s. Strong verbal encouragement was provided throughout the test, and time to exhaustion was recorded to the nearest second. Subjects were not informed of the power output, the expected time to exhaustion, or their performance in any of the tests until the entire study had been completed. This exercise protocol was replicated in experimental visits 3-6.

Measurements

Pulmonary gas exchange and heart rate. During all tests, pulmonary gas exchange and ventilation were continuously measured using a portable metabolic cart (Jaeger Oxycon Pro, Hoechberg, Germany). A turbine digital transducer measured inspired and expired airflow while an electrochemical cell O_2 analyser and an infrared CO_2 analyser simultaneously measured expired gases. These analysers were calibrated before each test with gases of known concentration, and the turbine volume transducer was calibrated using a 3-liter syringe (Hans Rudolph, Kansas City, MO). Subjects wore a nose clip and breathed through a low dead space (90 ml) and low resistance mouthpiece and impeller turbine assembly (Jaeger Triple V). The inspired and expired gas volume and gas concentrations were continuously sampled (100 HZ) via a capillary line connected to the mouthpiece and displayed breath-by-breath. The volume and concentration signals were time-aligned by accounting for delay in capillary gas transit and analyser rise time relative to the volume signal. Rates of oxygen uptake ($\dot{V}O_2$), carbon dioxide output ($\dot{V}CO_2$) and minute ventilation ($\dot{V}E$) were calculated using standard formulae (Beaver et al. 1973) and displayed breath-by-breath. Heart rate (HR) was measured during all tests using short-range radiotelemetry (Garmin FR70, Garmin Ltd., Olathe, KS).

Blood sampling. A cannula (Insyte-W ™, Bescton-Dickinson, Madrid, Spain) was inserted in an antecubital vein. Venous blood samples were drawn into lithium-heparin tubes before exercise and at exhaustion. From these samples 200 µl was immediately analysed for blood lactate (La^-) and glucose concentrations (YSI 2300, Yellow Springs Instruments, Yellow Springs, OH). The remaining sample was centrifuged at 4,000 rpm and $4°C$ for 10min, within 1 min of collection. Plasma was subsequently extracted to Eppendorfs and one of the three samples was immediately analysed for K^+ and Na^+ (9180 Electrolyte Analyzer, F. Hoffman-La

Roche, Basel, Switzerland) and other two were immediately frozen at -80∘C for later analysis of [NO$_2^-$] via chemiluminescence as described previously (Bailey et al., 2009; Bateman et al., 2002).

All equipment and surfaces were regularly rinsed with deionized water to remove any residual [NO$_3^-$] and [NO$_2^-$] before analysis. Before analysing the sample [NO$_2^-$], the samples were thawed at room temperature and deproteinised using the procedures of Higuchi and Motomizu (1999). The deproteinised plasma samples were then refluxed in 0.3 M sodium iodide and glacial acetic acid at room temperature and analysed for [NO$_2^-$] using a NO analyser (Sievers, 280i, Analytix, Durham, UK). Prior to determination of [NO$_3^-$], samples were deproteinised using sodium hydroxide (NaOH) and zinc sulphate (ZnSO$_4$) precipitation. 500µl of 0.18M NaOH and 300 µl of 5% ZnSO$_4$ were added to 100 µl of sample, vortexed and were left to stand at room temperature for 10 min. Thereafter, samples were centrifuged at 4000 rpm and 4°C for 10 min.

Data analysis. The breath-by-breath pulmonary gas exchange data were collected continuously during the ramp incremental test and averaged over 10 s periods. $\dot{V}O_2$ max was determined as the highest mean $\dot{V}O_2$ during any 30 s period. The values from this test were used to determine power outputs representing high-intensity exercise for each individual (i.e. 20 W was deducted from the ramp peak power).

The breath-by-breath $\dot{V}O_2$ data from each severe-intensity exercise test were initially examined to exclude errant breaths caused by coughing and swallowing and values that were lying more than four SD from the local mean were removed. The first 20 s of data after exercise onset were deleted to eliminate the cardiodynamic phase (phase I) data from the model fit. A nonlinear least-square algorithm was used to fit the data, as described in the following equation:

$$\dot{V}O_2\ (t) = \dot{V}O_{2\ baseline} + A_p(1-e^{-(t-TD_p)/\tau_p})$$

where $\dot{V}O_2$ (*t*) represents the absolute $\dot{V}O_2$ at a given time *t*; $\dot{V}O_{2baseline}$ represents the mean $\dot{V}O_2$ in the baseline period (the mean $\dot{V}O_2$ measured over the final 90s of unloaded cycling period); A$_p$, TD$_p$, and τ_p represent the amplitude, time delay, and time constant, respectively, describing the increase in $\dot{V}O_2$ above baseline

when exercise had commenced. The end-exercise $\dot{V}O_2$ was defined as the mean $\dot{V}O_2$ measured over the final 30 s of exercise. The mean response time (MRT) was calculated by fitting a single exponential curve to the data with no time delay from the onset to the end of exercise.

An iterative process was used to minimise the sum of the squared errors between the fitted function and the observed values. As the asymptotic value (A_P) of the exponential term describing the $\dot{V}O_2$ response to severe-intensity exercise may represent a higher value than is actually reached at the end of exercise, the $\dot{V}O_2$ amplitude at the end of exercise was defined as A´. 10 s averages were used to calculate resting and cycling baseline $\dot{V}O_2$, $\dot{V}CO_2$, $\dot{V}E$ over the last 5min and 3min of the 10min resting and 5 min cycling baseline stages, respectively. 10 s averages were also used to calculate exercise $\dot{V}O_2$, $\dot{V}CO_2$ and $\dot{V}E$ throughout the TTE. Oxygen uptake 1 min ($\dot{V}O_{2\ 1MIN}$) and 2 min ($\dot{V}O_{2\ 2MIN}$) into TTE were defined as the average $\dot{V}O_2$ at three time points with 10s averages (50 s, 1 min, 1 min 10s) and (1min 50s, 2 min, 2 min 10s), respectively. Average $\dot{V}E_{1MIN}$, $\dot{V}E_{2MIN}$ and $\dot{V}CO_{2\ 1MIN}$, $\dot{V}CO_{2\ 2MIN}$ were also calculated using the aforementioned time parameters and these values were used to calculate ventilatory equivalent for oxygen ($\dot{V}E/\dot{V}O_2$) and carbon dioxide ($\dot{V}E/\dot{V}CO_2$).

The carbohydrate and fat oxidation rates were calculated for rest, cycling baseline and exercise according to the following equations (Raper et al., 2014).

Carbohydrate (CHO) oxidation rate (g/min) = 4.585 ($\dot{V}CO_{2p}$)-3.226($\dot{V}O_{2p}$)

Fat oxidation rate (g/min) =1.695 ($\dot{V}O_{2p}$)-1.701($\dot{V}CO_{2p}$)

HR analysis. HR data were collected continuously during all experimental visits. Resting, cycling baseline and exercise HR were averaged over 30 s periods. Maximal HR was identified as the highest HR achieved during the exercise.

Statistical analysis. One-way repeated measures ANOVA was used to assess the difference in pulmonary gas-exchange variables, HR variables, time-to-exhaustion and the change in blood and plasma variables during the exercise between conditions. Two-way repeated measures ANOVAs (time x condition) were used to assess differences in blood and plasma variables between conditions. Significant interactions and main effects were followed up using LSD post hoc tests. Statistical analyses were performed using SPSS (v. 23, SPSS, Chicago, IL). Statistical significance was accepted at $P<0.05$. Results are presented as mean ± SD unless stated otherwise.

Results

Subject characteristics, diet and plasma nitrate [NO_3^-] and nitrite [NO_2^-]

All subjects reported consuming all prescribed food and servings of each supplement at the correct times and maintained their habitual level of exercise prior to each experimental visit. Despite no significant difference between calorific value of HCHO and HFAT diets (2559±4 kcal vs 2558±5 kcal, $P>0.05$), respectively, body mass was significantly lower in both HFAT+NIT (77.7±9.6 kg) and HFAT+PL (77.8±9.7 kg) compared to HCHO+NIT (78.9±9.6 kg) and HCHO+PL (78.7± 9.5 kg) ($P<0.05$). There were no significant differences in body mass between NIT and PL conditions within the HCHO or the HFAT diets ($P>0.05$). There was a significant interaction effect ($P<0.05$) and a main effect by condition ($P<0.05$),   but not by time ($P>0.05$) on plasma [NO_3^-]. Resting plasma [NO_3^-] increased significantly (~812%) in NIT (24.45±7.45 µM) compared to PL (2.68±0.54 µM) in HCHO, and in NIT (~1023%) (30.33±7.88 µM) compared to PL (2.70±0.77 µM) in HFAT ($P<0.05$). Resting plasma [NO_3^-] in NIT was significantly higher (~24%) in HFAT compared to HCHO ($P<0.05$) and there was no significant difference in resting plasma [NO_3^-] in PL between HCHO and HFAT ($P>0.05$). There was a significant effect of time, ($P<0.05$), condition ($P<0.05$) and there was a trend for an interaction effect on plasma [NO_2^-] ($P=0.089$). Resting plasma [NO_2^-] increased significantly (~88%) in NIT (697±343 nM) compared to PL (370±120 nM) in HCHO ($P<0.05$); however there was no significant increase in resting plasma [NO_2^-] (~47%) between NIT (521±221 nM) and PL (353±247 nM) in HFAT ($P>0.05$).

Substrate oxidation, blood metabolites and plasma [K⁺]

Calculated RER, CHO and fat oxidation rates for rest, cycling baseline and TTE are presented in Table 1. RER was significantly higher in HCHO+NIT and HCHO+PL compared to HFAT+NIT and HFAT+PL at rest, cycling baseline and during the TTE ($P<0.05$). However, there were no significant differences in RER between NIT and PL within the HCHO or HFAT diets at rest, cycling baseline and during TTE (Table 1; Figure 1D, $P>0.05$).

CHO oxidation was significantly higher in HCHO+NIT and HCHO+PL compared to HFAT+NIT and HFAT+PL at rest, cycling baseline and during the TTE (Table 1; $P<0.05$). There were no significant differences in CHO oxidation between NIT and PL conditions within the HCHO or HFAT diets at rest, cycling baseline and during TTE (Table 1; $P>0.05$).

Fat oxidation was significantly higher in HFAT+NIT and HFAT+PL compared to HCHO+NIT and HCHO+PL at rest and cycling baseline. There were no significant differences in fat oxidation between NIT and PL conditions within the HCHO or HFAT diets at rest and cycling baseline (Table 1; $P>0.05$).

Table 1. Summary of substrate oxidation variables at rest, cycling baseline and during TTE in response to the prescribed diets.

	HCHO+NIT	HCHO+PL	HFAT+NIT	HFAT+PL
RER				
Rest	0.97±0.08‡°	1.00±0.11‡°	0.86±0.14	0.88±0.07
Baseline	0.94±0.11‡°	0.95±0.08‡°	0.82±0.06	0.81±0.06
Exercise	1.21±0.09‡°	1.23±0.10‡°	1.08±0.15	1.09±0.08
CHO oxidation (g/min)				
Rest	0.45±0.15‡°	0.50±0.21‡°	0.28±0.28	0.29±0.15
Baseline	1.05±0.48‡°	1.12±0.33‡°	0.56±0.28	0.51±0.31
Exercise	7.26±1.75‡°	7.63±1.64‡°	5.63±2.49	5.70±1.63
FAT oxidation (g/min)				
Rest	0.01±0.04	0.01±0.06	0.07±0.09*†	0.07±0.04*†
Baseline	0.11±0.21	0.08±0.13	0.31±0.11*†	0.34±0.14*†

RER- respiratory exchange ratio, CHO oxidation- carbohydrate oxidation, HCHO+NIT- high carbohydrate diet with nitrate, HCHO+PL- high carbohydrate diet with placebo, HFAT+NIT- high fat diet with nitrate, HFAT+PL- high fat diet with placebo, * significantly different from HCHO+NIT, † significantly different from HCHO+PL, ‡ significantly different from HFAT+NIT, ° significantly different from HFAT+B ($P<0.05$).

There was a significant effect of time ($P<0.05$), condition ($P<0.05$) and an interaction effect ($P<0.05$) on blood [lactate]. Resting blood [lactate] was significantly higher in HCHO+NIT (0.8±0.2 mmol) and HCHO+PL (0.7± 0.2 mmol/L) compared to HFAT+NIT (0.5±0.2 mmol/L) and HFAT+PL (0.4±0.1 mmol/L) ($P<0.05$). Resting blood [lactate] was significantly higher in HCHO+NIT than in HCHO+PL ($P<0.05$) but there were no significant differences between resting blood [lactate] in HFAT+NIT and HFAT+PL ($P>0.05$).

Post-exercise blood [lactate] was significantly higher in HCHO+NIT (5.6±2.2 mmol/L) and HCHO+PL (5.3±2.0 mmol/L) compared to HFAT+NIT (4.1±1.3 mmol/L) and HFAT+PL (3.6±1.4 mmol/L) ($P<0.05$). There were no significant differences in post-exercise blood [lactate] between NIT and PL conditions within the HCHO or the HFAT diets ($P>0.05$). There were no differences in the increase from baseline to end-exercise in blood [lactate] between NIT and PL conditions within the HCHO or the HFAT diets ($P>0.05$).

There was a significant effect of time ($P<0.05$) and condition ($P<0.05$) on blood [glucose]. Resting blood [glucose] was significantly higher in HFAT+NIT (3.9±0.4 mmol/L) compared to HCHO+NIT (3.3±0.9 mmol/L) and HCHO+PL (3.3±0.8 mmol/L) ($P<0.05$). Resting blood [glucose] in HFAT was significantly higher in NIT compared to PL (3.6±0.3 mmol/L) ($P<0.05$). End-exercise blood [glucose] in HFAT+NIT (4.2±0.3 mmol/L) was significantly higher compared to HCHO+NIT (3.5±0.7 mmol/L, $P<0.05$) and HCHO+PL (3.7±0.6 mmol/L, $P<0.05$). There was a tendency for end exercise blood [glucose] to be higher in HFAT+PL (4.0±0.3 mmol/L) compared to HCHO+NIT (3.5±0.7 mmol/L, $P=0.057$). There were no other differences in blood [glucose] between NIT and PL conditions within the HCHO or HFAT at rest and during TTE ($P>0.05$).

There was a significant effect of time on plasma [K^+] such that plasma [K^+] increased in all conditions during the TTE ($P<0.05$). There was significant effect of condition ($P<0.05$) but not interaction ($P>0.05$) on plasma [K^+]. Resting plasma [K^+] was significantly higher in HFAT+PL (4.18±0.30 mmol/L) compared to NIT (3.94±0.27 mmol/L) and PL (3.93±0.31 mmol/L) in HCHO ($P<0.05$). End exercise plasma [K^+] was significantly higher in HFAT+NIT (4.97±0.31 mmol/L) and HFAT+PL (4.96±0.34 mmol/L) compared to HCHO+PL (4.69±0.24 mmol/L) ($P<0.05$).

Pulmonary Gas Exchange Kinetics

Pulmonary gas exchange and ventilatory responses to the prescribed diets for rest, cycling baseline and exercise are presented Figures 1 and 2 and in Table 2.
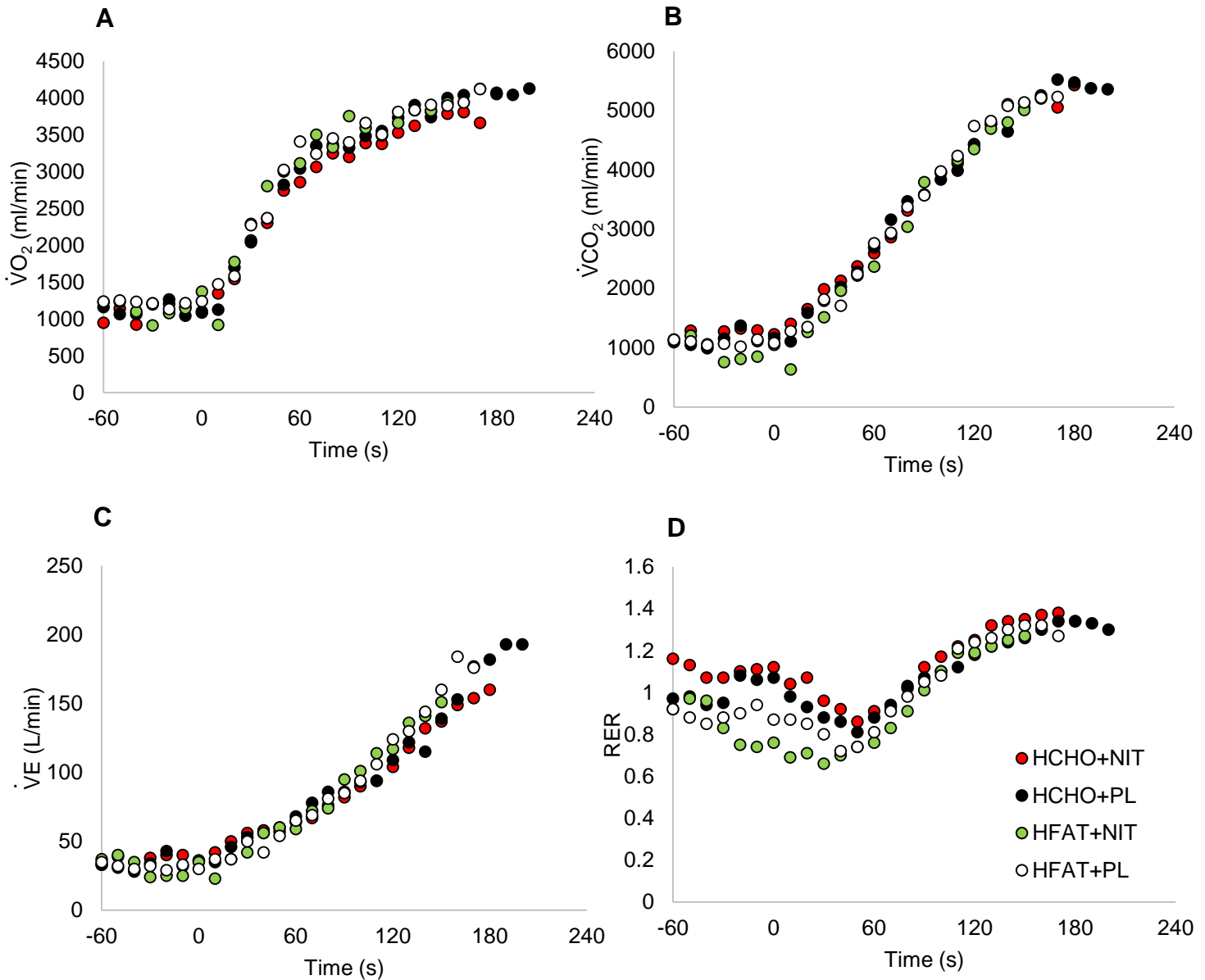
Figure 1. Pulmonary gas exchange and ventilatory responses to the prescribed diets from baseline to exhaustion in a representative subject. (A) $\dot{V}O_2$, (B) $\dot{V}CO_2$, (C) $\dot{V}E$, (D) RER;*RER-respiratory exchange ratio, $\dot{V}E$ -minute ventilation, $\dot{V}O_2$-pulmonary oxygen uptake, $\dot{V}CO_2$-carbon dioxide output, HCHO+NIT-high carbohydrate diet with nitrate, HCHO+PL- high carbohydrate diet with placebo, HFAT+NIT- high fat diet with nitrate, HFAT+PL- high fat diet with placebo

$\dot{V}O_2$

There was no significant difference in resting $\dot{V}O_2$, end-exercise $\dot{V}O_2$ and $\dot{V}O_2$ peak between conditions ($P>0.05$) but baseline $\dot{V}O_2$ and $\dot{V}O_2$ $_{1MIN}$ were significantly higher in HFAT when compared to HCHO ($P<0.05$) (Figure 1A and 2; Table 2). τp was significantly shorter in HFAT+PL (44±19 s) compared to HCHO+NIT (52±21 s) ($P<0.05$) and there was a trend for HFAT+PL to be significantly shorter when compared to HCHO+PL (55±21s) ($P=0.08$). There were no significant differences in MRT, TD and amplitude between conditions ($P>0.05$) (Table 2).
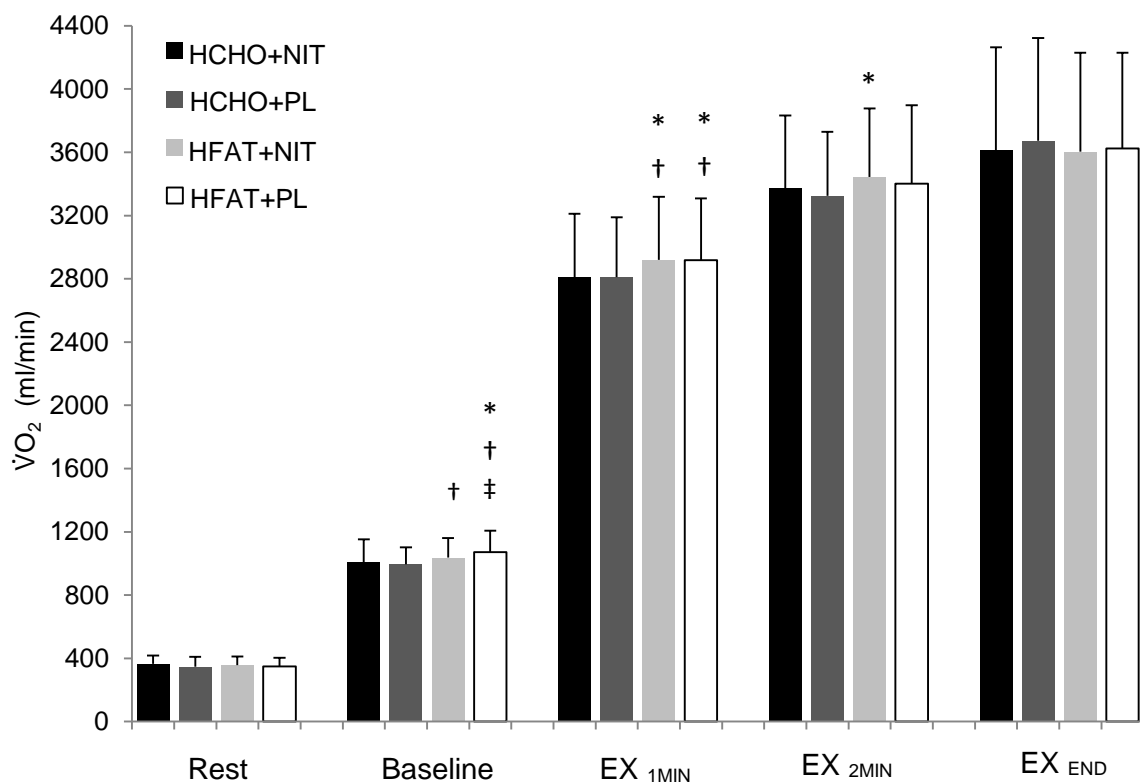
Figure 2. $\dot{V}O_2$ response to the prescribed diets for rest, cycling baseline and exercise.
EX $_{1MIN}$ and EX $_{2MIN}$- oxygen uptake 1 and 2 min into time-to-exhaustion trial (TTE), EX $_{END}$- oxygen uptake at the end of TTE; HCHO+NIT- high carbohydrate diet with nitrate, HCHO+PL- high carbohydrate diet with placebo, HFAT+NIT- high fat diet with nitrate, HFAT+PL-high fat diet with placebo, * significantly different from HCHO+NIT, † significantly different from HCHO+PL, ‡ significantly different from HFAT+NIT, [0] significantly different from HFAT+B ($P<0.05$).

$\dot{V}CO_2$

Resting $\dot{V}CO_2$ in HFAT+PL (309±66 ml/min) was significantly lower compared to HCHO+NIT (355± 65 ml/min) and HCHO+PL (353±82 ml/min) ($P<0.05$). There was a trend for resting $\dot{V}CO_2$ to be lower in HFAT+NIT compared to HCHO+PL (313±94 vs. 353±82 ml/min, $P=0.053$). Cycling baseline $\dot{V}CO_2$ was significantly lower in HFAT+NIT (851± 113ml/min) and HFAT+PL (866± 112ml/min) compared to in HCHO+NIT (938±129 ml/min) and HCHO+PL (944 ± 110 ml/min) ($P<0.05$). There were no significant differences in $\dot{V}CO_2$ cycling baseline between NIT and PL condition within the HCHO or the HFAT diet ($P>0.05$). Exercise $\dot{V}CO_2$ throughout the TTE was significantly lower in HFAT+NIT (3262±798 ml/min) and HFAT+PL (3276±646 ml/min) than in HCHO+NIT (3620±653 ml/min) and HCHO+PL (3749±619ml/min) ($P<0.05$).There were no significant differences in exercise $\dot{V}CO_2$ between NIT and PL condition within the HCHO or the HFAT diet ($P>0.05$) (Figure 1B; Table 2,).

$\dot{V}E$

There was no significant difference in resting $\dot{V}E$ across the conditions. $\dot{V}E$ was significantly higher in HCHO+PL compared to HFAT+NIT (26 ± 7 vs 24±6 L/min, $P<0.05$) at cycling baseline and throughout the TTE compared to HFAT+NIT (98±16 vs 89±21 L/min, $P<0.05$) and HFAT+PL (98±16 vs 91± 18 L/min, $P<0.05$). There were no other significant differences at cycling baseline and exercise $\dot{V}E$ ($P>0.05$) (Figure 1C; Table 2).

$\dot{V}E/\dot{V}O_2$ and $\dot{V}E/\dot{V}CO_2$

There was a trend for $\dot{V}E/\dot{V}O_2$ 1MIN in HCHO+PL (24±3 L/min) to be higher compared to HFAT+NIT (23±3 L/min) ($P=0.053$) and HFAT+PL (24±3 L/min) ($P=0.063$). There was also a trend for $\dot{V}E/\dot{V}O_2$ 1MIN in HCHO+NIT (25±2 L/min) to be higher compared to HFAT+NIT ($P=0.059$). There was a trend for $\dot{V}E/\dot{V}O_2$ 2MIN in HFAT+PL (38±7 L/min) to be higher compared to HFAT+NIT (37±6 L/min) ($P=0.056$). $\dot{V}E/\dot{V}CO_2$ 1MIN was significantly higher in HFAT+NIT (25±2 L/min) and HFAT+PL (25±2 L/min) compared to HCHO+NIT (23±2L/min) and HCHO+PL (23±3 L/min) ($P<0.05$). $\dot{V}E/\dot{V}CO_2$ 2MIN was significantly higher in HFAT+PL (29±4 L/min) compared to HCHO+NIT (26±2 L/min) and HCHO+PL (27±4 L/min)

($P<0.05$). $\dot{V}E/\dot{V}CO_2$ 2MIN was significantly higher in HFAT+NIT (28±3 L/min) compared to HCHO+NIT ($P<0.05$) (Table 2).

Heart rate

Resting HR was significantly lower in HCHO+NIT in comparison to HFAT+NIT (66±6 vs 69±6 bpm, $P<0.05$). There was no significant difference in cycling baseline HR between the conditions ($P>0.05$). Average exercise HR in TTE was significantly higher in HCHO+PL in comparison to HFAT+NIT (156±7 vs 152±8 bpm, P<0.05). HR peak was significantly higher in HCHO+PL in comparison to HFAT+PL (173±7 vs 167±8 bpm, $P<0.05$) and there was a trend towards higher HR peak in HCHO+PL compared to HFAT+NIT (168±9 bpm, $P=0.052$). There was no significant difference between the rest of the conditions in HR exercise and HR peak ($P>0.05$).

Time to Exhaustion (TTE)

TTE was longer in HCHO+NIT (174 ± 35s) compared to HFAT+NIT (154± 40 s, $P<0.05$) and HFAT+ PL (159 ± 39s, $P=0.059$). TTE was significantly longer in HCHO+PL (186 ± 39 s) compared to HFAT+NIT ($P<0.05$) and HFAT+PL ($P<0.05$). There was no significant difference in TTE between HFAT+NIT and HFAT+PL ($P>0.05$) (Figure 3).
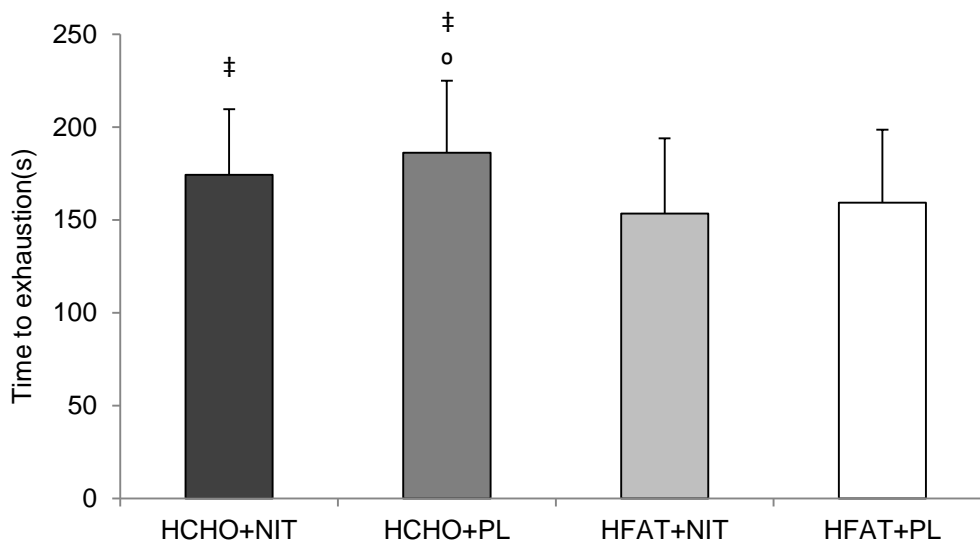
Figure 3. Time-to-exhaustion during severe-intensity exercise in response to the prescribed diets. HCHO+NIT-high carbohydrate diet with nitrate, HCHO+PL-high carbohydrate diet with placebo, HFAT+NIT-high fat diet with nitrate, HFAT+PL-high fat diet with placebo; ‡ significantly different from HFAT+NIT, [0] significantly different from HFAT+PL, ($P<0.05$).

Table 2. Pulmonary gas exchange and ventilatory responses to the prescribed diets.

	HCHO+NIT	HCHO+PL	HFAT+NIT	HFAT+PL
$\dot{V}O_2$				
Rest, ml/min	365±54	348±62	357±55	348±54
Baseline, ml/min	1008±145	994±107	1037±122†	1072±134*†‡
Exercise $\dot{V}O_{2\ 1MIN}$, ml/min	2808±403	2811±377	2920±400*†	2918±392*†
Exercise $\dot{V}O_{2\ 2MIN}$, ml/min	3371±462	3325±405	3443±435*	3402±495
Exercise End $\dot{V}O_2$, ml/min	3614±651	3672±650	3604±626	3624±607
Amplitude, ml/min	2607±565	2665±543	2563±521	2568±532
Peak $\dot{V}O_2$, ml/kg/min	46.5±6.7	47.1±7.4	46.0±6.2	46.3±7.3
MRT, s	54±9	54±11	47±9	53±10
тр, s	52±21	55±21	47±21	44±19*
$\dot{V}CO_2$				
Rest, ml/min	355±65	353±82	313±94	309±66*†
Baseline, ml/min	938±129	944±110	851±113*†	866±112*†
Exercise, ml/min	3620±653	3749±619	3262±798*†	3276±646*†
$\dot{V}E$				
Rest, L/min	11±3	11±4	11±6	11±3
Baseline, L/min	25±6	26±7‡	24±6	24±5
Exercise, L/min	94±18	98±16‡⁰	89±21	91±18
$\dot{V}E/\dot{V}O_{2\ 1MIN}$, L/min	25±2	24±3	23±3	24±3
$\dot{V}E/\dot{V}O_{2\ 2MIN}$, L/min	37±7	38±9	37±6	38±7
$\dot{V}E/\dot{V}CO_{2\ 1MIN}$, L/min	23±2	23±3	25±2*†	25±2*†
$\dot{V}E/\dot{V}CO_{2\ 2MIN}$, L/min	26±2	27±4	28±3*	29±4*†

*MRT-mean response time, тр-time constant, $\dot{V}O_{2\ 1MIN}$ and $\dot{V}O_{2\ 2MIN}$-oxygen uptake 1 and 2 min into time-to-exhaustion trial (TTE), $\dot{V}E/\dot{V}O_{2\ 1MIN}$ and $\dot{V}E/\dot{V}O_{2\ 2MIN}$- ventilatory equivalent for oxygen 1 and 2 min into TTE, respectively, $\dot{V}E/\dot{V}CO_{2\ 1MIN}$ and $\dot{V}E/\dot{V}CO_{2\ 2MIN}$- ventilatory equivalent for carbon dioxide 1 and 2 min into TTE, respectively; HCHO+NIT-high carbohydrate diet with nitrate, HCHO+PL- high carbohydrate diet with placebo, HFAT+NIT-high fat diet with nitrate, HFAT+PL-high fat diet with placebo, * significantly different from HCHO+NIT, † significantly different from HCHO+PL, ‡ significantly different from HFAT+NIT, ⁰ significantly different from HFAT+PL, ($P<0.05$).

Discussion

This is the first study to investigate the impact of HCHO and HFAT diets on O_2 uptake kinetics and performance in severe-intensity exercise. Additionally, this is the first study to examine a possible interaction effect of NO_3^- with the HCHO and HFAT diets on physiological responses and performance. The principal finding of this study was that TTE in severe-intensity exercise was higher in HCHO compared to HFAT as a result of elevated cycling baseline and exercise $\dot{V}O_2$ in HFAT diet that resulted in earlier attainment of $\dot{V}O_2$ peak. This is consistent with our first experimental hypothesis that increasing availability of carbohydrates is important when sustaining performance at high intensities. Contrary to our second hypothesis, there was no effect of NO_3^- supplementation on performance and physiological variables measured in this study, however there was a difference in the ability to convert $[NO_3^-]$ to $[NO_2^-]$, with this being more favourable in the HCHO compared to the HFAT diet.

Effectiveness of the dietary intervention

The effectiveness of the dietary intervention in the present study is supported by the following: 1) cycling baseline RER in both HCHO conditions was significantly higher (~17%) compared to the HFAT conditions; and 2) fat oxidation rates were four-fold higher in HFAT diets compared to HCHO, whereas CHO oxidation rates were two-fold greater in HCHO diets compared to HFAT. This is in accordance with previous studies, suggesting that reduced availability of CHO during HFAT and greater CHO availability in HCHO shifted preferred substrate utilization by muscle during the exercise (Raper et al., 2014; Leckie and Kowalchuk, 2013; Lima-Silva et al., 2011; 2013; Burke et al., 2016; Jeukendrup, 2003; Staudacher et al., 2001). 3) Moreover, resting plasma $[NO_3^-]$ increased by ~812% in NIT compared to PL in HCHO, and by ~1023% in NIT compared to PL in HFAT; whilst 4) resting plasma $[NO_2^-]$ increased by ~88% in HCHO and by ~47% in HFAT diet, following the 3 day supplementation period and final ingestion of NO_3^- on the morning of the experimental visit. The magnitude of increase in plasma $[NO_2^-]$ is consistent with the values attained in previous studies (~50-150%) (Bailey et al., 2009; 2010; Larsen et al., 2007; 2010; Vanhatalo et al., 2010; Lansley et al. 2011a) whilst increase in plasma $[NO_3^-]$ attained in the present study was greater

compared to the values reported in previous studies (~400-600%) (Bescós et al., 2012; Larsen et al., 2007; 2010).

Effect of dietary intervention on TTE, $\dot{V}O_2$ kinetics and blood metabolites

TTE in severe-intensity exercise was greater in HCHO compared to HFAT. Considering findings from previous studies examining substrate flux as a determinant of $\dot{V}O_2$ kinetics and performance, this can be attributed to an array of physiological factors that could contribute to lower TTE in the HFAT diet in this study. Firstly, we found that all measured variables of $\dot{V}O_2$ kinetics with the exception of $\dot{V}O_2$ cycling baseline and τp were not significantly different between HFAT and HCHO condition. Cycling baseline $\dot{V}O_2$ was ~3% higher in NIT and ~8% in PL in HFAT diet compared to HCHO diet. This translated into exercise $\dot{V}O_{2\ 1MIN}$ that was 4% higher in both NIT and PL in HFAT compared to the HCHO diets. This therefore led to earlier attainment of $\dot{V}O_2$ peak in HFAT that was not significantly different between conditions, and consequently resulted in earlier exhaustion. This is consistent with the previous findings that showed an impact of HFAT and HCHO diet on $\dot{V}O_2$ kinetics in moderate-intensity exercise and ~11% increase in $\dot{V}O_2$ cycling baseline after 6 days HFAT diet compared to HCHO diet (Raper et al. 2014). Raper et al. (2014) attributed this finding to altered efficiency associated with greater use of fat as a substrate in HFAT diet. Specifically, fat as a substrate is less economical fuel (ATP/O_2) in terms of oxidation in comparison to CHO (i.e. ~5.5-8% more ATP produced per L of oxygen consumed) which is associated with higher O_2 cost (Burke et al., 2017). Secondly, HFAT diet has been associated with impaired blood flow through impaired endothelial function that has been shown to limit microvascular blood flow and O_2 delivery into the muscle (Raper et al., 2014).

A surprising finding of our study was that the τp in HCHO was longer compared to HFAT (~52 s vs 44 s). This is in contrast with studies of Raper et al. (2014), Leckie and Kowalchuk (2013) and Lima Silva et al. (2011) that found significantly shorter τp in HCHO diet compared to HFAT in moderate (32 s vs 40 s), heavy (34 s vs 42 s) and extreme-intensity (33 s vs 48 s) exercise domains. However, considering the response of $\dot{V}O_2$ kinetics in the severe-intensity exercise domain, the possible explanation for this is that the τp was 'artificially' shortened when $\dot{V}O_2$ did not attain a true steady state but rather the response was truncated by the

attainment of $\dot{V}O_2$ max. As $\dot{V}O_2$ started and was higher throughout the transition in HFAT diet compared to HCHO, an earlier attainment of $\dot{V}O_2$ peak occurred and this could result in the тp appearing to be shorter in our study.

Although we observed a clear shift in fat utilization through RER and fat oxidation that decreased by ~14% and increased 4-fold in HFAT, respectively, HFAT diet down-regulated CHO metabolism (~50% lower CHO oxidation) that is fundamental for sustaining high-intensity exercise (Burke et al., 2017; Burke et al., 2016; Burke, 2015). Specifically, HFAT has been shown to attenuate CHO oxidation and down-regulate PDH complex that is a rate-limiting enzyme that directly impacts on delivery of CHO derived substrates (i.e. acetyl CoA, NAD/NADH and FAD/$FADH_2$) to the mitochondrial TCA and ETC, and therefore is involved in regulation of the rate at which aerobic and anaerobic metabolism occurs (Raper et al., 2014; Lima-Silva et al., 2013; Spriet and Heigenhauser, 2002). Indeed, previous studies found that as little as 1-3 days of HFAT diet or 30 min of intralipid infusion (to raise plasma free fatty acids), decreased the activity of PDH complex. A study by Putman et al. (1993) found that PDH activity was attenuated at rest and exercise in HFAT diet whereas a diet high in carbohydrates had the opposite effect (Stellingwerff et al., 2006). This could explain the findings from Lima-Silva et al. (2013) who examined the effect of LCHO and HCHO diet on energy system contribution and reported that LCHO diet has been associated with reduced aerobic energy contribution, potentially as a result of lowered CHO oxidation and PDH down-regulation, whereas HCHO has been shown to elicit an opposite effect (Lima-Silva et al., 2011; 2013; Maughan and Poole, 1981; Greenhaff, 1987a; 1987b; 1988). Therefore, as the pyruvate-acetyl CoA pathway was attenuated through down-regulation of PDH complex, this perhaps drove the metabolism to compensate via using anaerobic glycolysis where substrate availability (i.e. glycogen) was already limited due to low availability of CHO in the HFAT diet. Indeed, resting and peak exercise blood [lactate] in our study were significantly higher in HCHO compared to HFAT diet. Therefore, limited CHO stores in the participants on HFAT diets could be another contributing factor to shorter TTE observed in HFAT conditions.

Alternatively to the aforementioned factors, CHO availability during severe-intensity exercise could affect skeletal muscle fatigue sites (Simmonds et al., 2010). McKenna et al. (2008) emphasised the importance of K^+ homeostasis in

muscle fatigue (Sjogaard, 1990). This is partially due to a reduction in the sarcolemma Na^+-K^+-ATPase activity (Green, 2004; Green at al., 2007) that appears to prefer CHO as a fuel to meet its energy requirement (Okamoto et al., 2001) and may be sensitive to insulin concentration (Weltan et al., 1998). Indeed, in the current study, resting and the peak plasma $[K^+]$ were higher in HFAT compared to HCHO. Therefore, as availability of CHO was compromised in HFAT, this could reduce activity of the sarcolemma Na^+-K^+-ATPase, leading to loss of membrane excitability and consequently to shorter TTE in HFAT (Green et al., 2007; 2004; Clausen, 2003).

The surprising finding of our study was that blood glucose at both rest and end exercise was significantly higher in HFAT than in HCHO. This is in contrast to previous studies that showed increased blood glucose following HCHO diets (Raper et al., 2014; Leckie and Kowalchuk, 2013; Spriet 2014). Although there is no clear explanation for this finding, there are several possibilities that could explain this result: 1) a short-term HFAT diet caused the liver output to compensate for lack of CHO by releasing hepatic glucose from liver glycogen; 2) HFAT has been shown to impair muscle blood flow/microvascular perfusion through impaired endothelial function and GLUT-2 activity, therefore delivery of blood glucose to the cells can be compromised (Raper et al., 2014; Stanimirovic et al., 2016); 3) HFAT diet has been associated with developing insulin resistance (Schrauwen, 2007), however due to the short-term nature of the diet and inconsistent findings in recreationally trained subject or athletes, this explanation is less likely.

Collectively, these results suggest that the shorter TTE in HFAT diets could be associated with decreased metabolic efficiency, impaired or attenuated microvascular blood flow and O_2 delivery, limited CHO stores, down-regulation of PDH complex and finally elevated plasma $[K^+]$ when compared to HCHO.

The potential explanation for "no effect" of nitrate supplementation on exercise

A theoretical explanation for why there was no impact of NO_3^- supplementation on the variables examined could be the KNO_3 used as an alternative option to BRJ. In the present study, BRJ needed to be avoided due to its high CHO content (14 g per Beet IT Sports Shot) as avoidance of CHO was key in HFAT diets. In contrast to the previous studies that used BRJ, the studies that used $NaNO_3$ or

KNO$_3$ failed to either produce the same or had no ergogenic effect on performance or TTE in high-intensity exercise (Bescós et al., 2011; 2012; Peacock et al., 2012; Callahan et al., 2016; Kramer et al., 2016; Flueck et al., 2016). A recent study of Flueck et al. (2016) compared the effects of different doses of NaNO$_3$ and BRJ on O$_2$ consumption in moderate and severe-intensity exercise in male athletes and found that BRJ reduced O$_2$ consumption to greater extent than NaNO$_3$. They suggested that additional compounds contained in BRJ may play an important role in nitrate metabolism. BRJ is not only rich in NO$_3^-$ but also contains a high amount of polyphenols, vitamin C and antioxidants (Wootton-Beard and Ryan, 2011), which were found to facilitate NO$_2^-$ reduction to NO in the gut (Rocha et al., 2009) as well as in the brain (Pereira et al., 2013). Therefore, BRJ might be more beneficial compared with nitrate salts in impacting exercise performance or alternatively higher amounts of KNO$_3$ could potentially elicit previously observed effects evoked by BRJ (McMahon et al., 2016).

Effect of diet on the efficacy of [NO$_3^-$] to [NO$_2^-$] conversion

Although, we found no effect of NO$_3^-$ supplementation on performance and physiological variables measured in this study, NO$_3^-$-NO$_2^-$ analysis revealed a difference in the ability to convert [NO$_3^-$] to [NO$_2^-$] between HCHO and HFAT diets. We found a smaller increase (~47%) in plasma [NO$_2^-$] in HFAT compared to HCHO (~88%) after consumption of the same amount of NO$_3^-$, suggesting that the prescribed diets have impacted on the NO$_3^-$-NO$_2^-$ conversion efficacy. To confirm this we examined the impact of NO$_3^-$ supplementation on plasma [NO$_3^-$] to see whether there was a difference in the initial resting plasma [NO$_3^-$] between HCHO and HFAT. Surprisingly, we found that plasma [NO$_3^-$] was ~24% higher in HFAT compared to HCHO. Considering that there was higher level of plasma [NO$_2^-$] in HCHO regardless of higher availability of [NO$_3^-$] to be converted to [NO$_2^-$] in HFAT, this finding could perhaps indicate that HFAT diet adversely affect the oral microbiome responsible for conversion of [NO$_3^-$] to [NO$_2^-$]. Alternatively, HCHO may have had positive effect on nitrate-reducing microbiota while HFAT had no effect. The mechanism behind this finding is currently unclear, although previous studies suggest that HFAT diet impairs NO production and bioavailability through impaired endothelial function and/or lipid and glucose metabolism (Beals et al., 2017; Stanimirovic et al., 2016.; Raper et al., 2014,). Alternatively, the oral microbiome relies on CHO as its preferred energy substrate

that is required in order to convert $[NO_3^-]$ to $[NO_2^-]$ and since CHO was restricted in the HFAT diet, this could potentially explain the impaired ability to convert $[NO_3^-]$ to $[NO_2^-]$ in HFAT (Beals et al., 2017). Further studies are required to explore the complex mechanism behind the interactions between diet and NO_3^- metabolism found in the present study.

Limitations

The extreme diets and exercise used in this study had the following disadvantages: participants that consumed HFAT reported gastro-intestinal problems, cramps, low levels of energy and in some cases headaches. One participant complained about acid indigestion symptoms in HCHO diet. Additionally, we did not measure muscle glycogen content or PDH activity. However, the methodology used in this study closely followed that of previous studies and can therefore be assumed to produce previously observed changes in substrate availability and oxidative metabolism (Raper et al., 2014; Leckie and Kowalchuk, 2013; Wylie et al., 2013b). In relation to our second hypothesis, prescribing KNO_3 as a NO_3^- supplement might not be the most effective approach considering the previous findings from nitrate-exercise focused research studies that used BRJ (Flueck et al., 2016; McMahon et al., 2016).

Implications

Recently, claims that fat adaptation can enhance sports performance have re-emerged (Noakes et al., 2014; Volek et al., 2014; Phinney, 2004; Brukner, 2013). Although the present study did not prescribe HFAT for a sufficiently long period of time to observe fat adaptation, this study suggests that CHO deprivation adversely affects performance during high-intensity exercise. However, recent findings from a 3-week HFAT study conducted on 21 elite race walkers (Burke et al., 2017) are consistent with our results in terms of impaired economy and performance, suggesting that HFAT diets might not be an effective nutritional intervention strategy in performance sport. Although there can be suggestions that fat oxidation could satisfactory replace the role of muscle glycogen oxidation during moderate-intensity exercise, in view of the small differences that typically separate winning from losing in sport, benefits of CHO outweigh those observed in HFAT. Carbohydrate is not only a versatile fuel that contributes to performance across the full range of intensities via multiple and separate pathways but it is

also a more economical fuel in comparison to fat that benefits CNS (Burke et al, 2016; Burke 2015). Therefore, the balance of evidence including the present study would seem to support the use of diets high in CHO at least in the sports ranging from sprints to marathons where there is a need to regularly perform high-intensity sessions (Seiler, 2010), recoup glycogen stores in less than 6-8 hours due to biphasic training (Burke et al., 2016), and surging or strong finish needed during competition. Additionally, if the aim is to maximise benefits from NO_3^- supplementation, one should consider diet as a potential factor that can influence its conversion efficacy to NO_2^- and subsequently to NO.

Conclusion

In conclusion, TTE during severe-intensity exercise was significantly shorter when subjects consumed the HFAT compared to HCHO diet. This was attributed to 1) elevated $\dot{V}O_2$ at cycling baseline and throughout the exercise that resulted in earlier attainment of $\dot{V}O_2$peak in HFAT; 2) limited stores of CHO to support anaerobic glycolysis; 3) down-regulation of CHO metabolism (i.e. CHO oxidation, PDH complex) and 4) elevated plasma $[K^+]$ concentration. This suggests that a diet containing CHO is important for high-intensity exercise performance in healthy, recreationally trained males. In contrast, the NO_3^- supplementation we used in this study did not elicit the favourable changes hypothesised. This may be potentially due to the ingestion of KNO_3 rather than BRJ. Additionally, there was higher level of plasma $[NO_2^-]$ in HCHO diet regardless of higher initial availability of plasma $[NO_3^-]$ in HFAT that suggests that the ability to convert $[NO_3^-]$ to $[NO_2^-]$ was impaired in HFAT or alternatively improved in HCHO. This indicates that NO_3^--NO_2^- conversion was altered potentially via changes in the oral microbiome. Subsequently, future research studies should examine the efficacy of various sources of nitrate on exercise metabolism and investigate the possible impact of diet on the NO_3^--NO_2^- pathway that may have significant positive implications not only in exercise and sports performance but also in general health.

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