# Carbohydrate Feeding During Prolonged Cycling Exercise Improves Exercise Capacity but Does Not Alter Muscle Fuel Selection

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A thesis submitted in partial fulfilment of the requirements of Liverpool John Moores University for the degree of Doctor of Philosophy

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# Abstract

The aim of this thesis was to determine the dose-response relationship of CHO feeding during exercise on muscle fuel selection and exercise capacity under conditions considered best nutritional practice. **Study 1** demonstrated that after a 36 h CHO loading protocol and high CHO pre-exercise meal, trained male cyclists are capable of completing 180-min of submaximal exercise at lactate threshold (LT) and that fatigue during the subsequent exercise capacity test typically occurred within <10 minutes. However, important methodological considerations from this exercise protocol suggest that at least one full familiarisation of the complete exercise protocol should be included in subsequent studies. **Study 2 and 3**, utilising this protocol, reported that in conditions of high pre-exercise CHO availability (similar to Study 1), CHO feeding (comprising a mixture of solids, gels and fluids) during exercise improves cycling exercise capacity in a dose dependent manner such that 90 $g \cdot h^{-1}$ > 45 $g \cdot h^{-1}$ > 0 $g \cdot h^{-1}$ CHO. Such ergogenic effects were found to be independent of whole muscle glycogen, fibre-specific glycogen and intramuscular triglyceride (IMTG) sparing and in turn were suggested to be due to a combination of liver glycogen sparing, increased plasma glucose availability and greater maintenance of CHO oxidation. In addition, we report for the first time that 180-min of submaximal exercise reduces IMTG content in both the central and peripheral regions of type I and IIa fibres independent of CHO feeding and this was reflective of decreased LD number in both fibre types whereas reductions in LD size was exclusive to type I fibres. **Study 4** aimed to subsequently quantify muscle glycogen utilisation in specific subcellular storage pools, as measured using transmission electron microscopy. However, due to the associated laboratory restrictions of the COVID-19 world pandemic, it was not possible to complete this analysis. Having assessed the effects of CHO feeding on fuel selection and exercise capacity in the laboratory setting, **Study 5** used a case-study design to demonstrate the practical delivery of a periodised approach to CHO feeding for a professional World Tour cyclist during a Grand Tour. This approach was successful in supporting a winning performance and provides the first report in the literature to document the practical application of such a periodised approach in professional cycling. In summary, given that Grand Tours in professional road cycling can be won or lost with a matter of seconds, the findings within this thesis are of practical significance for road cyclists in that the chosen CHO strategy may improve the capacity to "hold a wheel" or "mount attacks" during the closing periods of race defining mountain stages.

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# Declaration

I declare that the work in this thesis, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own and was carried out in accordance with the regulations of Liverpool John Moores University. Additionally, all attempts have been made to ensure that the work is original, does not, to the best of my knowledge, breach any copyright laws and has not been taken from the work of others, apart from the works that have been fully acknowledged within the text. Moreover, no portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

# Presentations

**Presentations of the work listed within this thesis are as follows:**

1. Fell, J.M., Ellis, D.G., Moran, J., Hearris, M., Strauss, J.A., Cocks, M.S., Louis, J., Shepherd, S.O. & Morton, J.P. (2018). Carbohydrate feeding strategies typical of pro-tour cycling improve exercise capacity in trained cyclists in a dose-dependent manner. Presented at the European College of Sport Science Conference, Dublin, Ireland, July 2018 (oral communication).

2. Fell, J.M., Ellis, D.G., Moran, J., Hearris, M., Strauss, J.A., Cocks, M.S., Louis, J., Shepherd, S.O. & Morton, J.P. (2018). Exogenous CHO ingestion during prolonged steady state exercise alters intramuscular substrate utilisation in trained male cyclists. Presented at the European College of Sport Science Conference, Prague, Czech Republic, July 2019 (oral communication). Awarded third place in the Young Investigator Gatorade Sports Science Institute (GSSI) Nutrition Award session.

3. Fell, J.M. (2019). Science in sport: Nutrition. Invited oral presentation at the European Athletics High Performance Conference, Warsaw, Poland, March 2019.

# Publications

**Publications of the work listed within this thesis are as follows:**

1. Hearris, M.A., Hammond, K.M., **Fell, J.M.** and Morton, J.M. (2018). Regulation of muscle glycogen metabolism during exericse. Implications for endurance performance and training adaptation. *Nutrients* **10,** 1–21.

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# List of abbreviations

| | |
|---|---|
| **ADP:** | adenosine diphosphate |
| **AMP**: | adenosine monophosphate |
| **AMPK**: | AMP-activated protein kinase |
| **ANOVA**: | analysis of variance |
| **ATGL:** | adipose triglyceride lipase |
| **ATP**: | adenosine triphosphate |
| **AU**: | arbitrary units |
| **β-OHB:** | β-hydroxybutyrate |
| **BM:** | body mass |
| **$Ca^{2+}$**: | calcium |
| **CHO**: | carbohydrate |
| **$CO_2$:** | carbon dioxide |
| **CPT-1**: | carnitine palmitoyl transferase 1 |
| **CV:** | coefficient of variation |
| **DAG:** | diacylglycerol |
| **DEXA:** | dual energy X-ray absorptiometry |
| **DLW:** | doubly labelled water |
| **dw**: | dry weight |
| **EA:** | energy availability |
| **EE:** | energy expenditure |
| **EI:** | energy intake |
| **FAT/CD36:** | fatty acid translocase |
| **FFA**: | free fatty acid |
| **FFM:** | fat free mass |
| **g**: | gram |
| **G-1-P:** | glucose-1-phosphate |
| **G-6-P:** | glucose-6-phosphate |
| **GAM:** | goat anti-mouse |
| **GE:** | gross efficiency |
| **GI:** | gastrointestinal |
| **GLUT-4:** | glucose transporter-4 |
| **h**: | hour |
| **$H^+$:** | hydrogen |
| **HCl:** | hydrochloric acid |
| **HR**: | heart rate |
| **HSL:** | hormone sensitive lipase |
| **IF:** | immunofluorescence |
| **Ig:** | immunoglobin |
| **IMF:** | intermyofibrillar |
| **IMTG:** | intramuscular triglyceride |
| **INTRA:** | intramyofibrillar |
| **Kcal:** | kilocalories |

| | |
|---|---|
| **Kg**: | kilograms |
| **kJ:** | kilojoules |
| **km:** | kilometres |
| **KOH:** | potassium hydroxide |
| **KCl:** | potassium chloride |
| **LBM:** | lean body mass |
| **LCFA:** | long chain fatty acid |
| **LD:** | lipid droplets |
| **LT:** | lactate threshold |
| **LTP:** | lactate turnpoint |
| **MCFA:** | medium chain fatty acid |
| **mg:** | milligrams |
| **MHC:** | myosin heavy chain |
| **ml:** | millilitres |
| **min**: | minute |
| **mmol**: | millimole |
| **MTC**: | multiple transportable carbohydrate |
| **NEFA**: | non-esterified fatty acid |
| **nm**: | nanometre |
| **OD:** | optical density |
| **PAS:** | periodic acid-Schiff |
| **PBS:** | phosphate buffer solution |
| **PDH:** | pyruvate dehydrogenase |
| **PFK:** | phosphofructokinase |
| **PGC-1α**: | peroxisome proliferator-activated receptor [gamma] coactivator-1α |
| **Pi:** | inorganic phosphate |
| **PPO:** | peak power output |
| **RER:** | respiratory exchange ratio |
| **RFPM:** | remote food photographic method |
| **RPE**: | rating of perceived exertion |
| **RPM:** | revolutions per minute |
| **SGLT1**: | sodium-dependent glucose transporter 1 |
| **s:** | seconds |
| **SS:** | subsarcolemmal |
| **TAG:** | triglyceride |
| **TEM:** | transmission electron microscopy |
| **UCI:** | Union Cycliste Internationale |
| **µg:** | microgramme |
| **µl:** | microlitre |
| **µm:** | micrometre |
| **$\dot{V}CO_2$:** | carbon dioxide production |
| **$\dot{V}O_2$:** | oxygen consumption |
| **$\dot{V}O_{2max}$:** | maximal oxygen uptake |
| **W:** | watt |

| | |
|---|---|
| **WGA:** | wheat germ agglutinin |
| ***W*max:** | watt max |
| < | less than |
| > | greater than |

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# Chapter One:

# General Introduction

*This Chapter provides a brief introduction to the field of carbohydrate metabolism and exercise so as to provide a clear rationale to the Aims and Objectives of this thesis.*

## 1.1 Background

The study of carbohydrate (CHO) metabolism in relation to the field of sport and exercise is an area of investigation that is now over 100 years old. Almost a century ago, Krogh and Lindhard (1920) reported the efficiency of CHO as a fuel source during exercise and demonstrated that fatigue occurs earlier when subjects consume a low CHO diet (as compared with a high CHO diet) in the days preceding an exercise bout undertaken at a fixed workload. Levine *et al.* (1924) also observed that runners who completed the 1923 Boston marathon exhibited levels of hypoglycemia (<4 $mmol \cdot L^{-1}$) immediately post-marathon, thus suggesting that low CHO availability may be linked to fatigue. These early studies provided the initial evidence that CHO was an important fuel source for sustaining exercise performance. Nonetheless, much of the foundation of our understanding of CHO metabolism was developed by Scandinavian researchers in the late 1960s with the introduction of the muscle biopsy technique (Bergström and Hultman 1966a; Bergström and Hultman, 1966b; Bergström *et al.*, 1967; Hermansen *et al.*, 1967). These researchers provided the platform for modern day sports nutrition practice in a series of fascinating studies that collectively recognised that 1) skeletal muscle glycogen is depleted during exercise in an intensity dependent manner, 2) high CHO diets increase muscle glycogen storage and subsequently improve exercise capacity and finally 3) muscle glycogen storage is acutely enhanced following prior glycogen depletion (i.e., the super-compensation effect), the magnitude of which is dependent on high CHO availability (Bergström and Hultman, 1966b). This body of work remains some of the most highly cited papers in the field and is referenced accordingly in the most recent sport nutrition guidelines (Thomas *et al.*, 2016). As such, elite endurance athletes are now advised to consume 6-12 $g \cdot kg^{-1}$ of CHO per day depending on the demands of training and competition (Thomas *et al.*, 2016), whilst also taking into account that glycogen is now considered a regulator of training adaptation (Impey *et al.*, 2018; Philip *et al.*, 2012).

In addition to muscle glycogen, experimental work conducted in the 1980s and 1990s later demonstrated the role of CHO feeding during endurance-based exercise in extending the capacity to sustain exercise at a given intensity (Bosch *et al.*, 1994; Coyle *et al.*, 1983; Coyle and Coggan, 1984; Coyle *et al.*, 1986; Fielding *et al.*, 1985; Hargreaves *et al.*, 1984; Ivy *et al.*, 1979; Jeukendrup *et* al., 1997; Mitchell *et al.*, 1988; Neufer *et al.*, 1987; Tsinztas *et al.*, 1995; Wright *et al.*, 1991). The mechanisms underpinning such an ergogenic effect is often cited as a combination of muscle (Björkman, Shalin, Hagenfeldt & Wahren, 1984; Stellingwerff *et al.*,

2007) and liver glycogen sparing (Gonzalez *et al.,* 2015), maintenance of plasma glucose and elevated CHO oxidation rates (Coyle *et al.,* 1986) and direct effects upon the central nervous system (CNS) (Carter, Jeukendrup & Jones, 2004). However, although muscle glycogen sparing is often highlighted as a contributing factor underpinning improved performance, experimental evidence supporting this hypothesis is, at best, equivocal. For example, whilst several researchers have observed a decrease in glycogen utilisation during steady state submaximal exercise as a result of CHO feeding (Bosch et al., 1996; Björkman *et al.,* 1984; Erickson *et al.,* 1987; Hargreaves *et al.,* 1984; Tsintzas *et al.,* 1995; Tsintzas *et al.,* 1996), such an effect is not always consistent within the literature (Arkinstall *et al.,* 2001a; Arkinstall *et al.,* 2001b; Bosch et al., 1994; Coyle *et al.,* 1986; De Bock *et al.,* 2007; Flynn *et al.,* 1987; Hargreaves & Briggs, 1988; McConell *et al.,* 1999; Mitchell *et al.,* 1989). Such contrasting findings may be related to a number of methodological factors such as exercise protocol (i.e., exercise modality, exercise intensity and duration), absolute CHO availability (i.e., pre-exercise muscle glycogen concentration, CHO dose and feeding schedule), participant training status and moreover, biochemical method of glycogen determination (for review of the following factors see Tsintzas and Williams, 1998).

Indeed, the analysis of muscle glycogen concentration within many studies is often limited to the biochemical assessment within whole muscle homogenate (see Table 2.6). Such an approach is limited given that glycogen sparing as a result of CHO feeding has, in fact, been demonstrated to be muscle fibre specific (Coyle *et al.,* 1986; De Bock *et al.,* 2007; Mitchell *et al.,* 1989; Stellingwerff *et al.,* 2007a; Tsinztas *et al*., 1995). The requirement to assess fibre type specific utilisation of muscle glycogen is also apparent given the distinct muscle fibre recruitment patterns that arise from alterations in exercise intensity and duration (Gollnick *et al.,* 1974) as well as the different muscle recruitment patterns between cycling and running exercise modalities (Millet *et al.,* 2009). Beyond the quantification of muscle fibre-specific glycogen utilisation, the need to examine the subcellular utilisation of glycogen is also warranted given that glycogen granules are stored within distinct subcellular pools within muscle fibres i.e. the subsarcolemmal, intra- and intermyofibrillar pools (Friden *et al.,* 1985; Friden *et al.,* 1989; Marchand *et al.,* 2002; Marchand *et al.,* 2007; Nielsen *et al.,* 2009; Nielsen *et al.,* 2010a; Nielsen *et al.,* 2010b; Nielsen *et al.,* 2011; Nielsen *et al.,* 2014; Nielsen *et al.,* 2015). In this regard, it is now accepted that glycogen utilisation during exercise is also dependent on the subcellular location within specific muscle fibre types, thus adding an

additional layer of complexity to truly understand glycogen metabolism during exercise (Ørtenblad & Nielsen, 2015). For example, recent evidence suggests that depletion of the intramyofibrillar (INTRA) storage pool can directly modulate skeletal muscle contractile function and is strongly associated with the onset of fatigue (Gejl *et al.,* 2014; Ørtenblad *et al.,* 2011). Moreover, a local supercompensation of subsarcolemmal glycogen prior to exercise can mediate a sparing of INTRA glycogen utilisation during the initial hour of exercise, thereby postponing exhaustion (Jensen *et al.,* 2020). Nonetheless, no researchers have yet examined the effects of CHO feeding during exercise on the fibre type-specific utilisation of subcellular glycogen pools.

Although CHO feeding during exercise is now an established practice within sport nutrition, the optimal dose (Smith *et al.*, 2013; King *et al.,* 2018, 2019), CHO blend (Currell and Jeukendrup, 2008; Rowlands *et al.,* 2015), feeding frequency (Mears *et al.*, 2020) and format (Pfeiffer *et al.,* 2010a; Pfeiffer *et al.,* 2010b; Guillochon and Rowlands, 2017) are not yet established. The most recent American College of Sports Medicine (ACSM) sport nutrition guidelines recommends CHO intake at a rate of 30-60 $g \cdot h^{-1}$ (from single sources such as glucose or maltodextrin) during 1-2.5 h of endurance exercise whereas 60-90 $g \cdot h^{-1}$ of multi-transportable CHO (glucose:fructose blends) are recommended when exercise duration is >2.5 h. At the latter ingestion rates, it is now widely accepted that the addition of fructose to the CHO blend achieves higher peak exogenous CHO oxidation rates (up to 1.8 $g \cdot min^{-1}$) when compared with matched dose single source formulations where peak exogenous CHO oxidation rates are limited to approximately 1 $g \cdot min^{-1}$ (Jeukendrup, 2014). Nonetheless, the practical application of such experimental data is often limited, owing to the fact that such studies typically provide CHO in the form of a beverage only and that exercise was not commenced in conditions associated with best nutritional practice i.e. after CHO loading and consumption of a pre-exercise meal. Such research designs clearly contrast with the common practices adopted by elite endurance athletes whereby a mixture of fluids, gels and solids are consumed as an in-race fuelling strategy and races are commenced with optimised muscle and liver glycogen stores (Burke and Hawley, 2018; Burke *et al.*, 2018; Ebert *et al.*, 2007; García-Rovés *et al.*, 1998; Heikura *et al.*, 2019; Muros *et al.*, 2019; Pfeiffer *et al.*, 2012; Rehrer *et al.*, 2010; Ross *et al.,* 2014; Sànchez-Muñoz *et al.,* 2016; Saris *et al.,* 1989; Stellingwerff, 2012). With this in mind, there is a definitive need to further evaluate the optimal dose of CHO feeding required to induce the greatest ergogenic response during exercise.

## 1.2 Aims and Objectives

The aim of this thesis is to therefore determine the dose-response relationship of CHO feeding during endurance exercise on muscle fuel selection and exercise capacity. To this end, we aimed to utilise an experimental design that is considered representative of best nutritional practice and an exercise protocol that is relevant to elite road cyclists.

This aim will be achieved by completion of the following objectives:

a) To develop a prolonged submaximal endurance-based cycling protocol and a subsequent exercise capacity test that is reflective of endurance road cycling competition. This aim will be achieved by completion of Study 1 and will provide an exercise protocol that can be used in subsequent chapters to determine the dose-response relationship of carbohydrate feeding during exercise on muscle fuel selection and endurance cycling capacity.
b) To determine the dose-response relationship of carbohydrate feeding during endurance exercise on endurance cycling capacity (Study 2). This aim is to be achieved using a carbohydrate feeding protocol that is considered relevant to the typical feeding strategies adopted by elite endurance cyclists whereby carbohydrate is consumed through a mixture of solids, gels and fluids.
c) To determine the dose-response relationship of carbohydrate feeding during endurance exercise on muscle fuel selection (Study 3). This aim will be achieved by examining muscle glycogen and intramuscular triglyceride (IMTG) utilisation in both type I and II muscle fibres.
d) To determine the dose-response relationship of carbohydrate feeding during endurance exercise on subcellular substrate utilisation (Study 4). This aim will be achieved by examining glycogen and IMTG-containing lipid droplets (LDs) use in distinct subcellular storage pools within muscle.
e) To practically deliver a carbohydrate feeding strategy to a male professional road cyclist during a UCI World Tour multi-day cycling stage race (Study 5).

# Chapter Two:

# Literature review

*This chapter provides an overview of research findings that examine the regulation of carbohydrate storage and its subsequent metabolism during exercise. A critical review of the metabolic and performance effects of carbohydrate feeding before, during and after endurance-based exercise is also presented.*

*Elements of this chapter have resulted in the following publications:*
Hearris, M.A., Hammond, K.M., **Fell, J.M.** and Morton, J.M. (2018). Regulation of muscle glycogen metabolism during exericse. Implications for endurance performance and training adaptation. *Nutrients* **10,** 1–21.

Hammond, K.M., Hearris, M.A., **Fell, J.M.** and Morton, J.M. (2018). 'Carbohydrate metabolism during exercise'. In Zoladz, J.A., *Muscle and Exercise Physiology*, London, UK: Academic Press.

## 2.1 Background

In order for muscles to contract and provide movement, chemical energy is transformed into mechanical energy. Such energy is provided by adenosine triphosphate (ATP) which is the only energy capable of being used for human muscle contraction. This energy is mainly derived from the breakdown of carbohydrate (CHO) and fat, and during exercise the continual production of ATP is provided by a complex interaction between CHO and fat metabolism. Through the use of indirect calorimetry, stable isotope tracer and muscle biopsies it has become well established that the relative utilisation of extra- and intramuscular CHO and fat substrates during exercise can vary enormously and strongly depends on exercise intensity (Romijn *et al.*, 1993; Van Loon *et al.*, 2001), duration (Romijn *et al.*, 1993; Van Loon *et al.*, 2003; Watt *et al.*, 2002), training status (Hermansen *et al.*, 1967; Karlsson *et al.*, 1972; Henriksson, 1977; Hurley *et al.*, 1986; Kiens *et al.*, 1993; LeBlanc *et al.*, 2004; Phillips *et al.*, 1996; Turcotte *et al.*,1992) and feeding status (Arkinstall *et al.*, 2004).

The importance of carbohydrate as a fuel source for sustaining exercise performance has been evident as early as the 1920s when Krogh and Lindhard (1920) reported the efficiency of carbohydrate as a fuel source during exercise with additional observations by Levine *et al.* (1924) observing that runners who completed the 1923 Boston marathon exhibited hypoglycaemia immediately post-exercise, thus suggesting that low carbohydrate availability may be linked to fatigue. Nonetheless, the developments of the percutaneous muscle biopsy technique in the 1960s provided further insights into the impact of exercise training and diet manipulations on muscle substrate stores and endurance capacity (Bergström & Hultman, 1966; Bergström *et al.*, 1967; Hermansen *et al.*, 1967). These researchers collectively demonstrated that muscle glycogen is depleted during exercise in an intensity dependent manner, high carbohydrate diets prior to exercise increase muscle glycogen storage and subsequently improve exercise capacity and finally, muscle glycogen storage is enhanced following prior glycogen depletion (i.e., the super-compensation effect) with the magnitude of which is dependent on high carbohydrate availability. In more recent decades, the emergence of skeletal muscle lipid stores in providing a valuable contribution to ATP during moderate-intensity exercise has become more apparent and as such it is now recognised that intramuscular triglycerides (IMTG) are important energy substrate during prolonged exercise (De Bock *et al.*, 2005; De Bock *et al.*, 2007; De Bock *et al.*, 2008; Jevons *et al.*, 2020; Shepherd

*et al.,* 2013; Stellingwerff *et al.,* 2007; Van loon *et al.,* 2003; Van Loon *et al.,* 2005b; Van Proeyen *et al.,* 2011a; Van Proeyen *et al.,* 2011b; Watt *et al.,* 2002).

Since this seminal work investigating CHO metabolism, numerous investigations have further examined the role of CHO feeding during exercise as a potential ergogenic strategy to promote performance given that the substrate demands of many sports exceed the storage capacity of muscle glycogen. As such, it is now widely accepted that CHO feeding during exercise improves prolonged endurance performance and capacity (Cermak & Van Loon, 2013; Stellingwerff & Cox, 2014) through a combination of multiple mechanisms consisting of a sparing of muscle and liver glycogen utilisation (Bjorkman, Shalin, Hagenfeldt & Wahren, 1984; Gonzalez *et al.,* 2015; Stellingwerff *et al.,* 2007), maintenance of plasma glucose and elevated CHO oxidation rates (Coyle *et al.,* 1986) and direct effects upon the central nervous system (CNS) (Carter, Jeukendrup & Jones, 2004). Accordingly, it is now common practise for elite athletes to consume additional CHO during exercise to promote performance with current sport nutrition recommendations advising exogenous CHO ingestion rates of up to 90 $g \cdot h^{-1}$ of multi-transportable CHO during exercise >2.5 h in duration (Thomas *et al.,* 2016).

This chapter aims to present a critical evaluation of the effects of CHO availability on substrate metabolism and performance in order to provide a more thorough rationale for the experimental work conducted in this thesis. The reader is firstly introduced to an overview of substrate metabolism followed by an outline of carbohydrate and IMTG storage. Next, the regulatory steps in the control of both muscle glycogen and IMTG metabolism during exercise are presented. The well-documented effects of both endogenous and exogenous carbohydrate availability on endurance exercise performance are then discussed and the review closes by presenting a considered critique of previous experimental protocols typically used in the literature in relation to both exercise protocols and nutritional controls. As such, it is suggested that such experimental models are often unrepresentative of the habitual exercise and feeding strategies adopted by elite endurance athletes.

## 2.2 Substrate metabolism

During exercise, both CHO and fat are the two main fuel sources oxidised by skeletal muscle during prolonged endurance-type exercise. Typically, as exercise intensity increases there is a greater relative contribution from CHO sources, such that muscle glycogen and glucose uptake

predominate. In contrast, there is a reduction in fat oxidation due to a reduction in both plasma FFA and IMTG oxidation (Romijn *et al.*, 1993; Van Loon *et al.*, 2001; Figure 2.1).

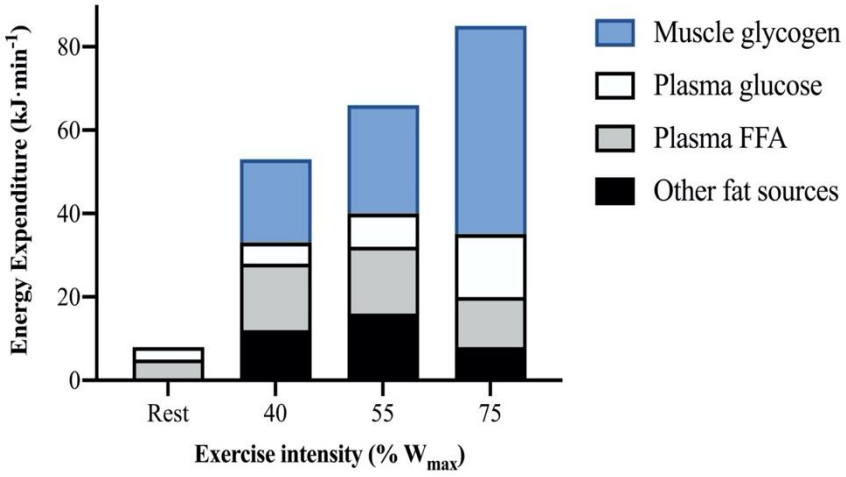


**Figure 2.1** Effects of exercise intensity on substrate utilisation during exercise (redrawn from Van Loon *et al.*, 2001).

Conversely, prolonged moderate-intensity exercise lasting several hours is characterised by a shift towards increased fat oxidation with a reciprocal reduction in CHO oxidation (Romijn *et al.*, 1993; Figure. 2.2). This shift in oxidation rates is accompanied by an increased reliance of plasma FFA towards energy expenditure and a reduced contribution of both muscle glycogen and IMTG (Romijn *et al.*, 1993; Figure 2.2).

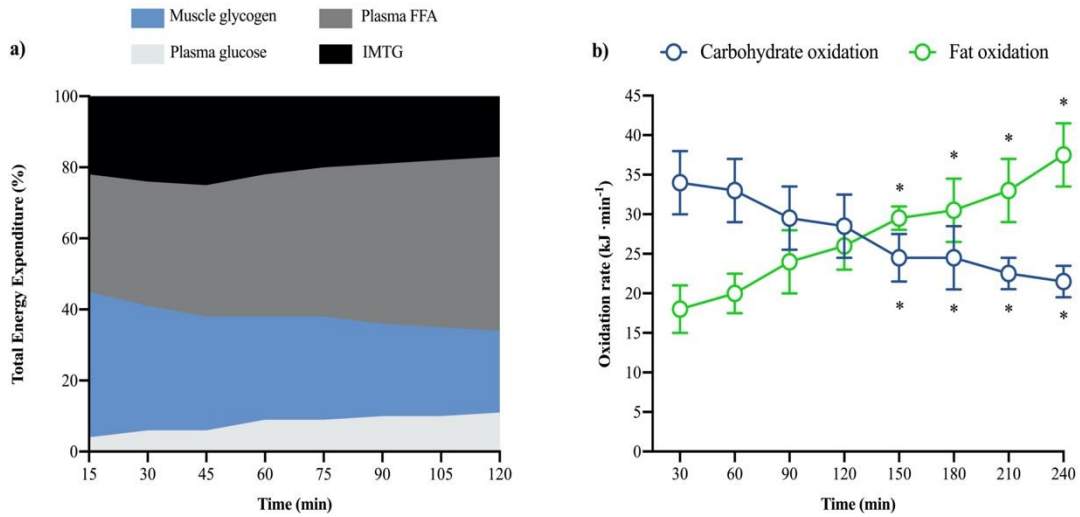


**Figure 2.2** Effects of exercise duration on substrate utilisation during a) 120-min of exercise at 65% $\dot{V}O_{2max}$ (redrawn from Romijn *et al.*, 1993) and b) 240-min of exercise at 57% $\dot{V}O_{2max}$ (redrawn from Watt *et al.*, 2002). IMTG: Intramuscular triglycerides; FFA: Free fatty acids.

Moreover, other overriding factors such as training status (Hermansen *et al.*, 1967; Karlsson *et al.*, 1972; Henriksson, 1977; Hurley *et al.*, 1986; Kiens *et al.*, 1993; LeBlanc *et al.*, 2004; Phillips *et al.*, 1996; Turcotte *et al.*,1992) and nutritional status (Arkinstall *et al.*, 2004, Figure 2.3) are also well known to influence the relative contribution of CHO and fat sources to energy provision. Indeed, one of the profound metabolic adaptations to endurance training is a reduction in CHO utilisation with a concomitant increase in fat oxidation for the same pre-training absolute exercise workload (Henriksson *et al.*, 1977). In addition, muscle glycogen availability can also regulate fuel metabolism over and above that of exercise intensity. For example, Arkinstall *et al.* (2004) reported high muscle glycogen utilisation rates at 45% $\dot{V}O_{2max}$ that was commenced with high glycogen concentrations as opposed to exercise at 70% $\dot{V}O_{2max}$ with low glycogen concentrations, despite the higher exercising intensity (see Figure 2.3).

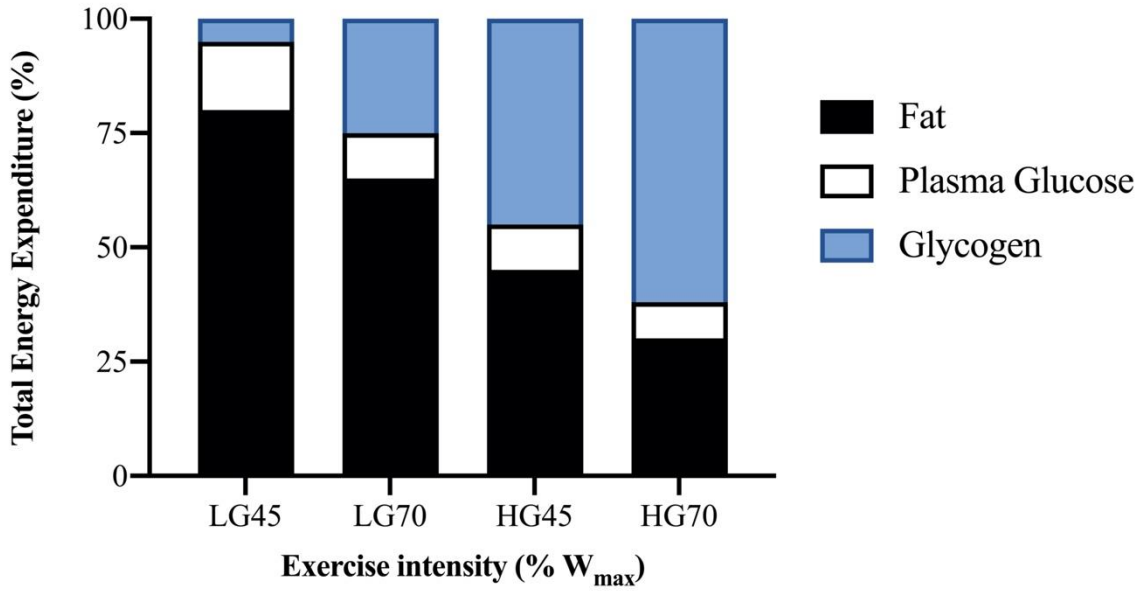


**Figure 2.3** Relative contribution of glycogen, glucose and fat oxidation to 60-min of exercise undertaken at 45 or 70% $\dot{V}O_{2max}$ with low (LG) or high pre-exercise glycogen (HG) concentrations (redrawn from Arkinstall *et al.*, 2004).

### 2.2.1 Overview of carbohydrate storage and structure

Glycogen, the primary storage form of glucose, is stored mainly in the liver (~100 g) and muscle (~350-700 g) and provides a rapid and accessible form of energy given it serves as an important energy reserve for ATP production. In addition to glycogen, there is ~5 g of glucose also circulating in the blood. These endogenous CHO stores are relatively small, representing less than 5% of the total energy storage. Specifically, within skeletal muscle, the relative

storage capacity of whole muscle homogenate glycogen concentrations can vary depending on training status, level of depletion and dietary CHO intake (Areta & Hopkins, 2018) (Figure 2.4). Although it is often viewed as a homogenous substrate, glycogen is comprised of complex individual granules that are constructed from a tiered assembly of glucose units, which forms a branched structure via 1-4- and 1-6-α-glycosidic bonds. The many branching points formed by the α-1-6 bonds (approximately every 8-12 glucose units) on the glycogen molecule provide multiple sites for the addition of glucose residues during glycogen synthesis (glycogenesis), or glycogen breakdown during exercise (through glycogenolysis).

Glycogen granules are formed on the protein glycogenin which is located at the core of glycogen molecules (Smythe & Cohen, 1991). These glycogen granules can be as large as 42 nm in diameter and constructed up to 12 tiers consisting of up to 55,000 glucosyl units (Graham *et al.*, 2010), although the majority of glycogen granules within skeletal muscle are typically reported to be 25 nm in diameter and comprise of approximately 8 tiers (Marchand *et al.*, 2002). The branched structure of glycogen allows for the dense compartmentalization of glucose and an enhanced surface area for phosphorylase mediated degradation, permitting rapid mobilization in response to elevated energy requirements, such as the onset of exercise (Melendez-Hevia *et al.*, 1993).

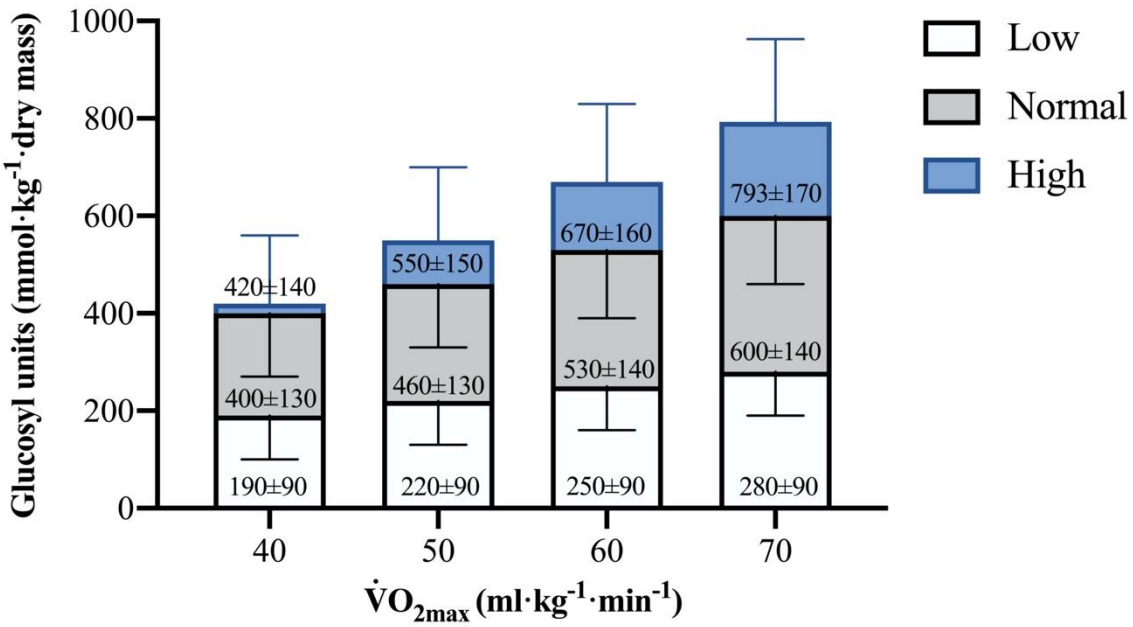


**Figure 2.4** Variations in muscle glycogen storage of the vastus lateralis according to $\dot{V}O_{2max}$ training status in conditions of low, normal and high carbohydrate availability (redrawn from Areta & Hopkins, 2018).

Beyond the structure of glycogen, the location of glycogen within the muscle is important and it has become apparent that glycogen is distributed across muscle fibres as well as being located in specific intracellular locations (Figure 2.5). Through the use of histochemical methodology, semi-quantification of muscle glycogen has reported that resting glycogen content is not markedly different between type I and II fibres (Essén & Henriksson, 1974; Essén *et al.*, 1975; Stellingwerff *et al.*, 2007), although biochemical quantification, which provides a more quantitative measure, suggests type II fibres may contain 50-100 $mmol \cdot kg^{-1}$ dw more glycogen than type I (Tsintzas *et al.*, 1995, 1996). In addition to fibre specific location of glycogen, it is now recognised that glycogen exists as individual particles located in distinct subcellular locations or compartments within human muscle fibres as it has been examined both qualitatively (Fridén *et al.*, 1985; Fridén 1989; Oberholzer *et al.*, 1976; Sjöström *et al.*, 1982) and semi-quantitively by transmission electron microscopy (TEM) (Marchand *et al.*, 2007; Nielsen *et al.*, 2011). Muscle glycogen is heterogeneously distributed between three separated compartments: below the sarcolemma (subsarcolemmal glycogen), between the myofibrils (intermyofibrillar glycogen) and within the myofibrils (intramyofibrillar glycogen) (Marchand *et al.*, 2002). Stereological glycogen quantification of TEM images has demonstrated that highly trained endurance athletes generally deposit 8-11% as subsarcolemmal glycogen, 77-84% as intermyofibrillar glycogen and 3-13% as intramyofibrillar glycogen. Furthermore, it appears that type I fibres contain 82% more intra-myofibrillar and 31% more subsarcolemmal glycogen than type II fibres, with type II fibres containing 11% more inter-myofibrillar glycogen than type I fibres (Gejl *et al.*, 2014; Nielsen *et al.*, 2011; Nielsen *et al.*, 2012; Nielsen & Ørtenblad, 2013; Ørtenblad *et al.*, 2013). However, these fibre type differences do not appear to be present in untrained muscle and may therefore represent training-induced adaptations within skeletal muscle.

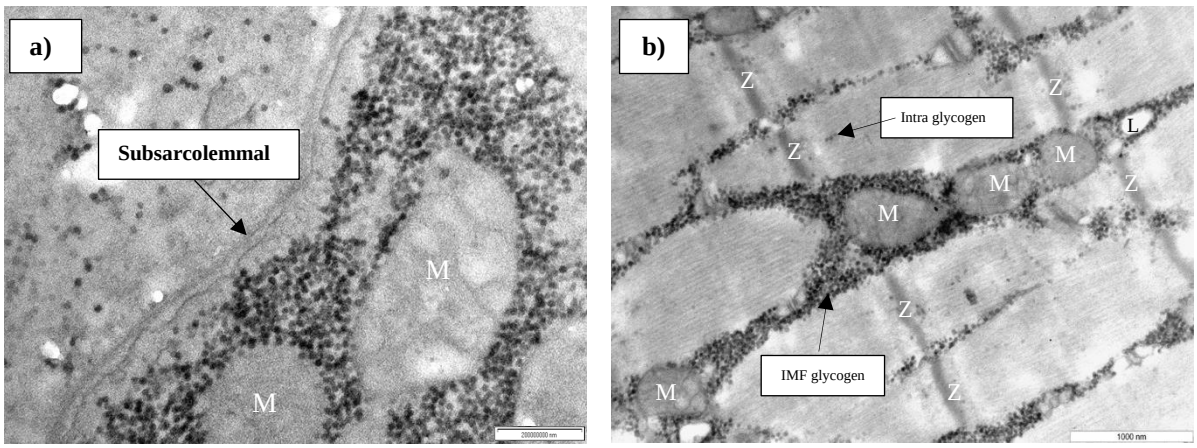


**Figure 2.5** Electron micrograph of human vastus lateralis displaying compartmental glycogen distribution. a) Subsarcolemmal; b) Myofibrillar. Image taken at Liverpool John Moores University, microscopy laboratory by J.M. Fell. M: Mitchondria; Z: Z-discs; L: Lipid droplet; IMF: Intermyofibrillar (between myofibrils); Intra: Intramyofibrillar (within myofibrils).

### 2.2.2 Regulation of carbohydrate metabolism

There are a number of key biochemical processes that are important in regulating CHO metabolism. Muscle glycogenolysis is one of these important processes and it is the breakdown of glycogen into glucose molecules, more specifically glucose-1-phosphate (G-1-P), which is then converted to glucose-6-phosphate (G-6-P) before going through glycolysis. Glycolysis is the breakdown of glucose through a series of reactions to form pyruvic acid. Muscle glycogenolysis is under the control of the key enzyme glycogen phosphorylase which in turn can be regulated via hormonal and/or allosteric control. During exercise, adrenaline is secreted from the adrenal medulla and binds to a receptor on the muscle cell membrane. This attachment causes an activation of the G-protein, which in turn activated adenylate cyclase converting ATP to cAMP. cAMP then activated the inactive protein kinase A (PKA) resulting in active PKA which in turn activates phosphorylase kinase. Activation of phosphorylase kinase converts glycogen phosphorylase from its inactive *b* form to its more active *a* form, which is the enzyme response for cleaving a glucose molecule from glycogen. The activity of glycogen phosphorylase can also be regulated via allosteric regulation via changes in $Ca^{2+}$ flux, ADP and AMP accumulation. An overview of the regulation of glycogen phosphorylase activity is provided in Figure 2.6.

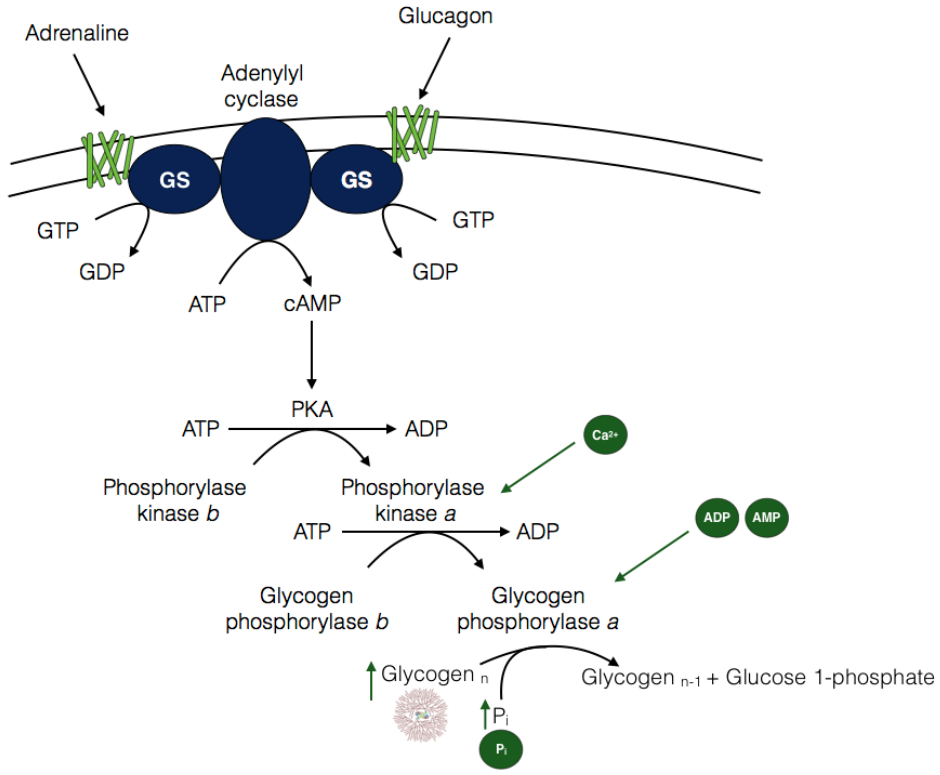


**Figure 2.6** Regulation of glycogen phosphorylase activity. Positive allosteric effectors are shown in green. ADP, Adenosine diphosphate; AMP, Adenosine monophosphate; ATP, Adenosine triphosphate; $Ca^{2+}$, calcium; cAMP, Cyclic adenosine monophosphate; GS, G protein; GDP, Guanosine diphosphate; GTP, Guanosine triphosphate; PKA, Pi, Inorganic phosphate; Protein kinase A.

An overview of key steps in the regulation of CHO metabolism is provided in Figure 2.7. During exercise, there are a number of potential sites of control that can regulate the interaction of CHO and lipid metabolism during exercise. These include availability of intra-muscular and extra-muscular substrate (controlled by diet and the action of key hormones such as the catecholamines and insulin), the abundance of transport proteins involved in transporting substrates across both the plasma and mitochondrial membranes and of course, the activity of the key regulatory enzymes involved in the metabolic pathways. The activity of regulatory enzymes can be modified acutely through covalent modification (i.e., phosphorylation and dephosphorylation largely under hormonal control) and/or allosteric regulation via important signalling molecules that are produced in the muscle as a result of contraction e.g. ADP, AMP, IMP, Pi, $Ca^{2+}$, $H^{+}$ etc. Enzyme activity can also be modified through substrate activation or product inhibition such that increasing the substrate concentration increases catalysis whereas increased product concentration may inhibit the reaction. Finally, enzyme activity can be

regulated long term through increasing the muscle cell's content of the actual enzyme protein (i.e., more of the enzyme is actually present) as would occur with endurance training. Clearly, muscle cells possess a highly co-ordinated and regulatory network of signalling and feedback pathways which function to ensure ATP demand is matched by ATP synthesis. From a physiological perspective, key factors such as exercise intensity, duration, nutritional status, training status etc can all regulate substrate utilisation during exercise, largely through influencing the potential regulatory control points discussed above.

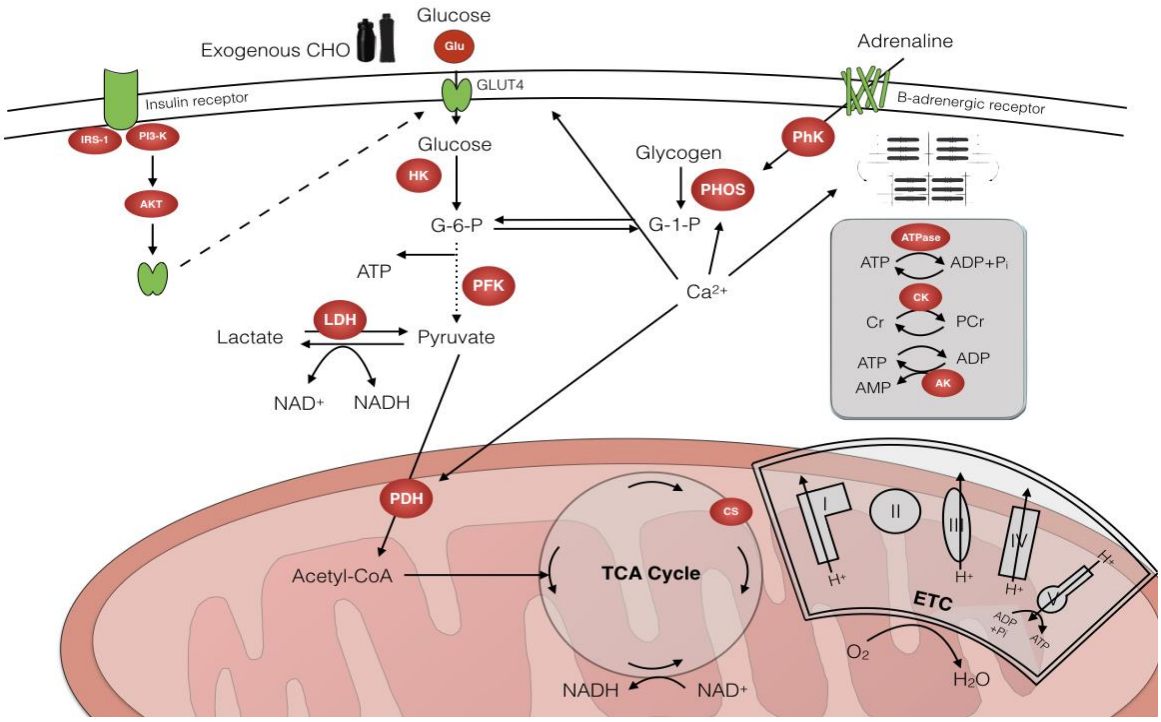


**Figure 2.7** Overview of carbohydrate metabolism and main control points. Key regulatory enzymes are well recognized as phosphorylase (PHOS), hexokinase (HK), phosphofructokinase (PFK), lactate dehydrogenase (LDH) and pyruvate dehydrogenase (PDH). Additionally, the rate of muscle glucose uptake can also determine the flux through glycolysis. ADP: Adenosine diphosphate; AK: Adenylate kinase; Akt: Protein kinase B; AMP: Adenosine monophosphate; ATP: Adenosine triphosphate; $Ca^{2+}$: Calcium; CHO: Carbohydrate; CK: Creatine kinase; Cr: Creatine; CS: Citrate synthase; ETC: Electron transport chain; G-1-P: Glucose-1-phosphate; G-6-P: Glucose-6-phosphate; Glu: Gluose; GLUT4: Glucose transporter 4: $H^+$: Hydrogen ion; $H_2O$: water; IRS-1: Insulin receptor substrate 1; HK: Hexokinase; LDH: Lactate dehydrogenase; $O_2$, Oxygen: NAD: Nicotinamide adenine dinucleotide; TCA cycle: Tricarboxylic acid cycle; $P_i$: phosphate; PCr: Phosphocreatine; PFK: Phosphofructokinase; PhK: Phosphorylase kinase; Phos: Glycogen phosphorylase; PI3-K: Phosphoinositide 3-kinase. Taken from Hearris *et al.* (2018).

### 2.2.3 Effects of exercise intensity and duration

Exercise intensity and duration are two major factors regulating substrate utilisation during exercise and it is well established that as exercise intensity progresses from moderate (i.e., 65% $\dot{V}O_{2max}$) to high intensity (85% $\dot{V}O_{2max}$), muscle glycogenolysis and glucose uptake increases such that CHO metabolism predominates. Concomitantly, there appears to be reduction in whole body lipid oxidation due to a reduction in both plasma FFA and intramuscular triglyceride oxidation. Indeed, maximal rates of lipid oxidation are considered to occur around 65% $\dot{V}O_{2max}$ though this is dependent on a number of other factors such as training status, gender and diet (Achten & Jeukendrup, 2004) (see Figure 2.1).

As stated previously, the breakdown of muscle glycogen to glucose 1-phosphate is under the control of glycogen phosphorylase, and this reaction requires both glycogen and Pi as substrates. Phosphorylase, in turn, exists as a more active *a* form (which is under the control of phosphorylation by phosphorylase kinase) and also as a more inactive *b* form (which exists in a dephosphorylated form due to the action of protein phosphatase 1). Given that phosphorylase can be transformed via covalent modification (i.e., phosphorylation by phosphorylase kinase) mediated through adrenaline, it would be reasonable to expect that greater phosphorylase transformation from *b* to *a* may be one mechanism to explain increased glycogenolysis evident with increasing exercise intensity. This would also be logical given that sarcoplasmic $Ca^{2+}$ levels would be increased with high-intensity exercise (given the need for more rapid cross-bridge cycling) and that $Ca^{2+}$ is a potent positive allosteric regulator of phosphorylase kinase through binding to the calmodulin subunit. However, the percentage of phosphorylase in the more active *a* form does not appear to be increased with exercise intensity and in actual fact, is decreased after only 10 minutes of high intensity exercise, which may be related to the reduced pH associated with intense exercise (Howlett *et al.,* 1998). Whereas this mechanism of transformation (mediated by $Ca^{2+}$ signalling) may be in operation within seconds of the onset of contraction (Parolin *et al.,* 1999), it appears that *post-transformational* mechanisms are in operation during more prolonged periods of high-intensity exercise given that glycogenolysis still occurs despite reduced transformation. In this regard, vital signals related to the energy status of the cell play a more prominent role. Indeed, as exercise intensity progresses from moderate to high-intensity exercise, the rate of ATP hydrolysis increases so much so that there is a greater accumulation of ADP, AMP and Pi. In this way, the increased accumulation of Pi as a result of increased ATP hydrolysis can increase glycogenolysis as it

provides increased substrate required for the reaction. Furthermore, greater accumulations of free ADP and AMP can also subsequently fine tune the activity of phosphorylase *a* through allosteric regulation (Howlett *et al.,* 1998). Finally, although it is well documented that phosphorylase is under the hormonal control of adrenaline, infusion of adrenaline to levels beyond that of endogenous production during high-intensity exercise (85% $\dot{V}O_{2max}$) does not augment glycogenolysis (Chesley *et al.,* 1995), likely due to already sufficient activation of phosphorylase through the local mechanisms discussed above.

In addition to muscle glycogen, the contribution of plasma glucose to ATP production also increases with exercise intensity. The most likely explanation for this is due to increased muscle blood flow (and hence substrate delivery) in addition to increased muscle fibre recruitment (Rose & Richter, 2005). Although glucose uptake is also regulated by GLUT4 content, GLUT4 is unlikely to play a role in this situation given that GLUT4 translocation to the plasma membrane is not increased with exercise intensity (Kraniou *et al.,* 2006). Once glucose is transported into the cytosol, it is phosphorylated to glucose 6-phosphate under the control of hexokinase. Evidence suggested that hexokinase activity is also not limiting given that patients with type 2 diabetes (who have reduced maximal hexokinase activity) display normal patterns of exercise-induced glucose uptake likely due to normal perfusion and GLUT-4 translocation (Martin *et al.,* 1995). In contrast, during intense exercise at near maximal or supra-maximal intensity, glucose phosphorylation may be rate limiting to glucose utilisation given that high rates of glucose 6-phosphate secondary to muscle glycogen breakdown can directly inhibit hexokinase activity (Katz *et al.,* 1986). Once glucose enters the glycolytic pathway, the rate limiting enzyme to glycolysis is considered as phosphofructokinase (PFK). PFK is allosterically activated by ADP, AMP and Pi and this mechanism is likely to explain high rates of glycolysis during intense exercise even in the face of metabolic acidosis when PFK could be inhibited.

In contrast to exercise intensity, prolonged steady state exercise lasting several hours is characterized by a shift towards increased lipid oxidation and reduced carbohydrate oxidation rates. This shift in oxidation rates is accompanied by an increased contribution of plasma FFA towards energy expenditure and a decreased reliance on both muscle glycogen and IMTGs. Studies examining the regulatory mechanisms underpinning this shift in substrate utilisation have suggested that a reduction in muscle glycogen availability (due to progressive glycogen depletion) and hence a reduced glycolytic flux down-regulate PDH activity thereby leading to

reduced CHO oxidation. In addition, progressive increases in plasma FFA availability (due to continual lipolysis in adipose tissue) stimulate lipid oxidation. The down-regulation of PDH activity as exercise duration progresses may be due to reduced pyruvate flux therefore reducing substrate production required for the PDH reaction (Watt *et al.,* 2002). In addition, more recent data demonstrate an up-regulation of PDH kinase activity during exercise which would therefore directly inhibit PDH activity (Watt *et al.,* 2004). Taken together, these data are consistent with the many observations that increasing or decreasing substrate availability is one of the most potent regulators of fuel utilisation patterns during exercise and this concept is discussed in the next section.

### 2.2.4 Effects of substrate availability

Modifying substrate availability through dietary manipulation (such as CHO loading regimens, pre-exercise meals or providing enhanced substrate availability during exercise) has been consistently shown to alter metabolic regulation during endurance exercise through various control points. Increasing muscle glycogen concentration enhances glycogenolysis during exercise (Hargreaves *et al.,* 1995) by enhancing phosphorylase activity given that glycogen is a substrate for phosphorylase. The enhanced glycogenolysis with elevated glycogen stores does not appear to affect muscle glucose uptake (Hargreaves *et al.,* 1995; Arkinstall *et al.,* 2004). In addition to glycogenolysis, muscle glycogen also appears to be a potent regulator of PDH activity (and thus CHO oxidation) during exercise. Indeed, commencing exercise with reduced muscle glycogen attenuates the exercise-induced increase in PDH activity and vice versa (Kiilerich *et al.,* 2010), likely due to reduced glycolytic flux as well as increased resting content of PDK4 (the kinase responsible for deactivating PDH) when glycogen concentration is low. PDH regulation appears particularly sensitive to nutritional status even at rest. In fact, just 3 days of a low CHO (but increased fat diet) up-regulates PDH kinase activity and down regulates PDH activity (Peters *et al.,* 1998).

Although the effects of exercise intensity on substrate utilisation were discussed previously, interestingly muscle glycogen availability seems to influence fuel metabolism over and above that of exercise intensity. As such, Arkinstall *et al*. (2004) observed that glycogen utilisation was enhanced during exercise at 45% $\dot{V}O_{2max}$ that was commenced with high glycogen concentrations (591 mmol·kg$^{-1}$ dw) as opposed to exercise at 70% $\dot{V}O_{2max}$ commenced with low glycogen concentrations (223 mmol·kg$^{-1}$ dw), despite the higher exercising intensity. In contrast to glycogen utilisation and CHO oxidation rates, lipid oxidation was highest when

exercise was commenced with reduced glycogen stores (see Figure 2.3). The shift towards fat oxidation when pre-exercise muscle glycogen is low is likely mediated by a number of contributing factors. Firstly, reduced glycogen availability is associated with increased plasma FFA availability as well as adrenaline concentrations thus favouring conditions for augmented lipid oxidation and lipolysis, respectively, compared with conditions of high glycogen concentration (Arkinstall *et al*., 2004). However, when a pre-exercise meal is ingested and glucose infused during glycogen depleted exercise such that minimal differences exist between plasma FFA and adrenaline, lipid oxidation is still augmented (Roepstorff *et al*., 2005). In such circumstances, available evidence points to regulation within the muscle cell itself and more specifically, a carnitine mediated increase in lipid oxidation. Indeed, these researchers observed lower PDH activity, acetyl CoA and acetyl carnitine content and increased free carnitine concentrations during exercise when glycogen depleted compared with glycogen loaded conditions. Interestingly, ACC phosphorylation increased, and malonyl CoA decreased similarly in both conditions despite higher AMPK activity when glycogen was reduced. Such data provide further evidence that malonyl CoA is not involved in regulating lipid metabolism during exercise but provide further support for a critical role of carnitine in regulating the interaction between CHO and lipid utilisation (Wall *et al.*, 2011).

When compared with exercise after overnight fasting, ingestion of carbohydrate rich meals within the hours before exercise (as well as carbohydrate ingestion during exercise) has been shown to enhance endurance performance (Wright *et al.*, 1991). Consequently, it is common practice for athletes to adopt such dietary approaches to competition. However, it is now well documented that pre- and during exercise CHO ingestion is one of the most potent ways to alter the pattern of CHO utilisation during exercise through a number of control points. One of the main responses to CHO feeding is to attenuate plasma FFA availability and lipid oxidation whilst simultaneously increasing CHO oxidation rates. The reduced plasma FFA availability is due to an attenuation of lipolysis that is regulated by increased circulating insulin concentrations caused by CHO feeding. The anti-lipolytic effect of insulin is mediated through its ability to activate the enzyme phosphodiesterase which degrades cAMP and thereby attenuates activation of protein kinase A and eventually hormone sensitive lipase (HSL).

Convincing data confirming that lipolysis limits fat oxidation following CHO feeding is provided by Horowitz *et al.* (1997). In this study, male subjects completed 60 min of exercise at 45% $\dot{V}O_{2max}$ in fasted conditions or 1 h after consuming 0.8 $g \cdot kg^{-1}$ of glucose (to induce a

high insulin response), 0.8 g·kg$^{-1}$ fructose (to induce a low insulin response) or an additional glucose trial during which intralipid and heparin were infused so as to maintain plasma FFA availability in the face of high insulin. In accordance with the insulin response, lipolysis (as indicated by rate of appearance of glycerol) was reduced with CHO feeding and plasma FFA availability was reduced in these conditions. In addition, rates of lipolysis exceeded lipid oxidation rates during fasted exercise, whereas in the CHO conditions, rates of lipolysis appeared to equal lipid oxidation rates thus implying that lipolysis limits fat oxidation. However, when intralipid and heparin was infused during an additional glucose trial, lipid oxidation rates were enhanced by 30% (4.0 µmol·kg$^{-1}$·min$^{-1}$) compared with the glucose only trial (3.1 µmol·kg$^{-1}$·min$^{-1}$) but were still not restored to levels occurring during fasted exercise (6.1 µmol·kg$^{-1}$·min$^{-1}$). Taken together, whilst these data suggest that only small elevations in insulin can attenuate lipolysis (i.e., 10-30 µU/ml), they also demonstrate a limitation within the muscle cell itself during CHO fed conditions. In accordance with reduced lipid oxidation following CHO feeding, CHO oxidation was increased due to increased glucose uptake (and oxidation) as well as muscle glycogenolysis. The enhanced rates of glycogenolysis was suggested to be due to increased allosteric activation of phosphorylase given that AMP and Pi production is greater during conditions of reduced plasma FFA availability, as is the case with CHO feeding.

In an effort to ascertain the source of limitation to lipid oxidation within the muscle following CHO feeding, Coyle *et al.* (1997) infused octanoate (a MCFA) or palmitate (a LCFA) during 40 min of exercise at 50% $\dot{V}O_{2max}$ after an overnight fast or 60 minutes after ingesting 1.4 g·kg$^{-1}$ of glucose. As expected, plasma FFA and lipid oxidation was higher in the fasted trials whilst CHO oxidation was lower in this condition compared with the glucose trials. However, the major finding of this study was that the percentage of palmitate oxidized during the glucose trial was reduced compared with fasting (70 v 86%, respectively) whereas octanoate was unaffected (99 v 98%, respectively). These data therefore suggest that LCFA uptake into the mitochondria is reduced with CHO feeding and when taken in the context of previous sections in this chapter, it becomes increasingly apparent that any condition which accelerates glycolytic flux (e.g. increased intensity, muscle glycogen, glucose feeding) can regulate intramuscular lipid metabolism, which again points to a carnitine mediated limitation. Furthermore, more recent data has demonstrated that the increased insulin and decreased adrenaline levels which accompany glucose ingestion during exercise appears to result in an

attenuation of intra-muscular HSL activity (Watt *et al.*, 2004), thus highlighting an additional point of control.

### 2.2.5 Effects of training status

Endurance training results in a number of profound physiological and metabolic adaptations which function to reduce the degree of perturbations to homeostasis for a given exercise intensity and ultimately, delay the onset of fatigue. Adaptations to endurance training are most recognised functionally by an increase in maximal oxygen uptake as well as a rightward shift in the lactate threshold. From a metabolic perspective, the most prominent adaptation is an increase in the size and number of mitochondria (i.e., mitochondrial biogenesis) which essentially permits a closer matching between ATP requirements and production via oxidative metabolism. The adaptive response of muscle mitochondria is also accompanied by increases in capillary density, substrate transport proteins and increased activity of the enzymes involved in the main metabolic pathways. In addition, endurance training increases the capacity for skeletal muscle to store glycogen (see Figure 2.4) and triglycerides thereby increasing substrate availability. In relation to substrate utilisation during exercise following endurance training, the most notable response is a reduction in CHO utilisation with a concomitant increase in lipid oxidation (Henriksson, 1977).

For a given exercise intensity, glycogen utilisation is reduced with exercise training (Karlsson *et al.*, 1974), an effect that is confined locally to the actual muscles that were trained (Saltin *et al.*, 1976). The reduced glycogenolysis observed after training was not due to any change in phosphorylation transformation but rather, allosteric mechanisms (Chesley *et al.*, 1996; Le Blanc *et al.,* 2004). Indeed, exercise in the trained state is associated with reduced content of ADP, AMP and Pi thereby providing a mechanism leading to reduced phosphorylase activity. Le Blanc *et al*. (2004) also observed reduced pyruvate and lactate production during exercise undertaken in the trained state as well as reduced PDH activity. As a result of the reduced CHO flux, it is therefore likely that the attenuated pyruvate production (in addition to reduced ADP accumulation) may have attenuated PDH activity.

In addition to training-induced reductions in muscle glycogenolysis, several investigators have observed that training reduces exercise-induced liver glycogenolysis, as demonstrated by the rate of appearance of glucose in the circulation. There is some evidence (although this is not

consistent within the literature) that endurance training also increases gluconeogenesis skeletal muscle following training (Bergman *et al*., 2000). In accordance with reduced rates of glucose production, muscle glucose uptake is reduced when exercise is undertaken at the same absolute workload following a period of endurance training (Bergman *et al*., 1999). This reduction in CHO metabolism when exercising at the same absolute workload is recognised as one of the main metabolic adaptations underpinning improved performance that occurs with prolonged endurance training.

### 2.2.6 Overview of intramuscular triglyceride storage

Fat is mainly stored as triacylglycerol (TAG) in subcutaneous and deep visceral adipose tissue. Smaller quantities of TAG are present as lipid droplets inside muscle fibres, commonly known as intramuscular triglycerides (IMTG). In contrast to the previously stated limited storage capacity of endogenous CHO stores, lipid stores have a considerably larger storage capacity with the average non-obese male (~70 kg) storing between 9 and 15 kg (equating to an energy store of 80,000 to 140,000 kcal). IMTG contents has been shown to be quite variable, ranging between 2 and 10 $mmol \cdot kg^{-1}$ wet weight (ww), equating to a mixed muscle TAG content of approximately 0.2 kg and an energy storage of 1,850 kcal (Van Loon, 2004; Watt *et al.*, 2002; Wendling *et al.*, 1996). IMTG content is predominantly influenced by energy intake, dietary status and training status. Indeed, a number of cross-sectional reports demonstrate that one of most prominent metabolic adaptations to a period of endurance training is greater IMTG storage (reviewed in, Shaw *et al.*, 2010) which appears in parallel to greater glycogen storage in the endurance trained state as seen in Figure 2.4. Moreover, electron microscopy analysis of skeletal muscle tissue has shown that IMTG-containing lipid droplets (LD) are located in close proximity to the mitochondria (Hoppeler, 1999; Shaw *et al.*, 2008) (see Figure 2.8), which is believed to enable fatty acids (FAs) liberated from IMTG to be efficiently shuttled to the mitochondria to produce energy which can be used as an oxidative substrate source during exercise in trained individuals. In addition, the location of IMTG within the muscle has been revealed by several studies to have an approximate threefold greater storage of lipids in type I vs. type II muscle fibres (Essén *et al.*, 1975; Koopman *et al.*, 2001; Malenfant *et al.*, 2001; Van Loon *et al.*, 2003a; Van Loon *et al.*, 2003b), with the use of immunofluorescence microscopy further identifying that IMTG is deposited in greater abundance in subsarcolemmal regions of the individual muscle fibres (Shaw *et al.*, 2008; Stellingwerff *et al.*, 2007; Van Loon *et al.*, 2004).

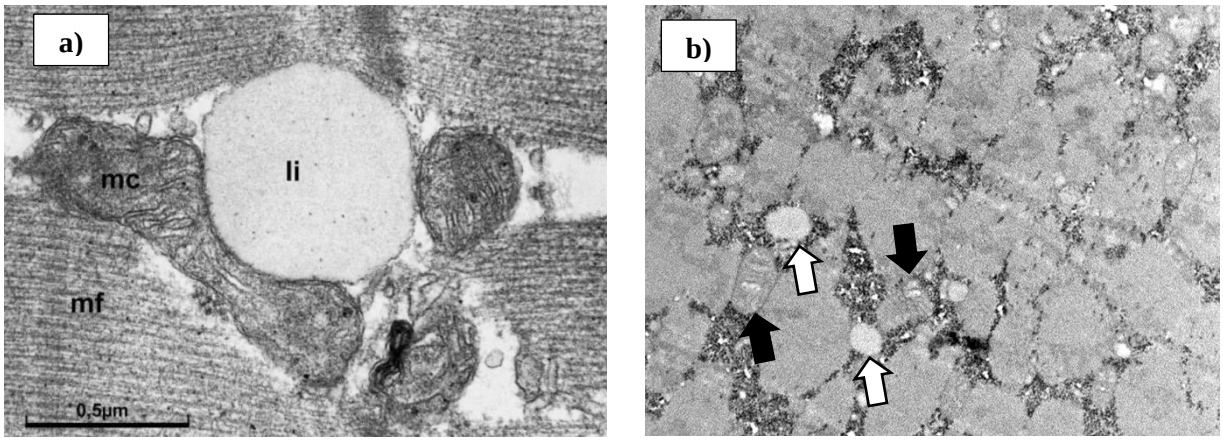


**Figure 2.8** a) Electron micrograph of a longitudinal section of skeletal muscle tissue. In the centre, on the level of the z-line, is the intermyofibrillar mitochondria with a lipid droplet immediately adjacent, (taken from Hoppeler, 1999). b) Transmission electron microscopy image showing the presence of lipid droplets in human skeletal muscle. The white arrow highlights lipid droplets in close proximity to mitochondria; The black arrow highlights the mitochondria (Image 2 taken at Liverpool John Moores University, microscopy laboratory by J.M. Fell). Li: Lipid droplet; MC: Mitochondria; MF: Myofilaments.

### 2.2.7 Intramuscular triglyceride as a substrate source during exercise

As mentioned in previous sections, both CHO and fat provide the two most important energy substrates during exercise and total fat oxidation is known to increase approximately ten-fold compared to resting values during moderate-intensity exercise in trained individuals (Romijn *et al.*, 1993; Van Loon *et al.*, 2001), and it is therefore plausible that the IMTG pool provides a readily available source of FAs for mitochondrial β-oxidation, especially considering the close location of LDs to the mitochondria, as previously described (Hoppeler, 1999; Shaw *et al.*, 2008). A number of early studies employed indirect calorimetry measures coupled with the infusion of a $^{13}$C-labelled FA tracer (Romijn *et al.*, 1993; Van Loon *et al.*, 2001; Van Loon *et al.*, 2003; Van Loon *et al.*, 2005b; Watt *et al.*, 2002a) to measure total fat and plasma FFA oxidation and subsequently calculate the contribution of other fat sources to total fat oxidation from the difference between the two. Stable isotope methodology has been employed to demonstrate that the rate of non-plasma FA oxidation (i.e., IMTG and lipoprotein-derived TG) is greatest during exercise between 40 and 65% $\dot{V}O_{2max}$ and accounts for ~30 – 50% of total fat oxidation (Romijn *et al.*, 1993; Van Loon *et al.*, 2001). In addition, the use of $^{1}$H magnetic resonance spectroscopy ($^{1}$H-MRS) permits a more direct quantification of both IMTG and extramyocellular lipid (EMCL) content. The technique has consistently reported that IMTG concentration is significantly reduced (~20-40%) in response to moderate-intensity exercise

(Van Loon *et al*., 2004) and therefore contributes to the convincing evidence underpinning IMTG as a viable energy reserve during exercise.

In spite of both of these methodologies highlighting the contribution of IMTG stores to total fat oxidation during exercise, direct evidence obtained through the measurement of net changes in IMTG concentration using the biochemical extraction technique in skeletal muscle tissue are less clear. Indeed, a significant net reduction in IMTG concentration following exercise is reported in a number of studies (Essén-Gustavsson & Tesch, 1990; Phillips *et al.*, 1996a; Sacchetti *et al.*, 2002; Watt et al., 2002a), while others do not observe such decreases (Bergman *et al.*, 1999; Kiens *et al.*, 1993; Kiens and Richter, 1998; Wendling *et al.*, 1996). These inconsistent findings may be attributed to methodological issues, given the large between biopsy variability (20-26%) associated with this technique (Van Loon, 2004; Watt *et al.*, 2002b).

Nonetheless, $^{1}$H-MRS or biochemical TAG extraction only permits the quantification of whole mixed muscle TAG concentration and is therefore limited in its application for identifying fibre-specific and subcellular distribution of IMTG-containing LDs. In this regard, the recent developments of immunofluorescence microscopy have enabled the ability to discriminate between IMTG content in type I and II muscle fibres. In accordance, this method has been successfully employed to demonstrate that 2-h of moderate-intensity cycling induces a >60% net decline of the intramuscular lipid pool specific to type I fibres (Van Loon *et al.*, 2003). Further studies have since demonstrated that IMTG concentration is significantly depleted in type I fibres following both endurance-type exercise (De Bock *et al.*, 2005; De Bock *et al.*, 2007; De Bock *et al.*, 2008; Jevons *et al.*, 2020; Shepherd *et al.*, 2013; Stellingwerff *et al.*, 2007; Van Loon *et al.*, 2005b; Van Proeyen *et al.*, 2011a; Van Proeyen *et al.*, 2011b) and resistance exercise (Koopman *et al.*, 2006). Interestingly, mixed-muscle IMTG content determined through immunofluorescence microscopy correlates well with values obtained using biochemical TAG extraction (Stellingwerff *et al.*, 2007b). However, this relationship disappears when used to quantify IMTG utilisation during exercise (Stellingwerff *et al.*, 2007a), as the rate of IMTG utilisation is higher in type I than in type II fibres. More recently, exercise-induced changes in the volume fraction of LD have also been examined through TEM. The greater magnification and resolving power of TEM enabled identification of LDs in two distinct subcellular compartments of skeletal muscle (subsarcolemmal and intermyofibrillar regions). Koh *et al.* (2017) reported that LD utilisation during cross-country skiing was

exclusive to the intermyofibrillar region with no changes in subsarcolemmal LD volume fraction. Moreover, the authors also reported that LD stores were reduced by 58 and 51% in both type I and II fibres, respectively. This LD use was exclusive to arm muscles and this finding may be due to the highly oxidative nature of type II fibres specifically in the arms of cross-country skiers compared to untrained individuals.

It is also worth noting that immunofluorescence microscopy only provides a semi-quantitative analysis of IMTG content, as the efficacy of the relevant staining procedure and image acquisition settings strongly determines the area fraction stained (i.e., the commonly reported unit of IMTG concentration). Thus, strict analytical controls should be implemented to minimise this potential variability. When accurately controlled, however, the use of immunofluorescence microscopy demonstrates the importance of considering fibre-specific responses in order to accurately reflect the contribution of IMTG oxidation during exercise. Collectively, the aforementioned studies demonstrate that IMTG does indeed provide a readily available energy substrate during endurance-based exercise in trained individuals.

### 2.2.8 Intramuscular triglyceride hydrolysis

The hydrolysis of IMTG occurs in three consecutive steps and is dependent on the interaction of at least three different lipases in order to sequentially cleave a FA from the glycerol backbone. Lipolysis is initially catalysed by adipose triglyceride lipase (ATGL) which converts TAG to diacylglycerol (DAG). Hormone-sensitive lipase (HSL) is the predominant lipase responsible for hydrolysing DAG to monoacylglycerol (MAG), after which MAG lipase liberates the final FA (see Figure 2.9). HSL is a cytosolic protein that is found in both adipose tissue and skeletal muscle with a greater abundance within adipose tissue. Nonetheless, HSL expression in skeletal muscle is greater in type I fibres compared to type II fibres (Langfort *et al.*, 1999; Peters *et al.*, 1998) which would correlate with the fibre-specific capacity for IMTG oxidation. Moreover, skeletal muscle HSL activity has also been found to increase in response to exercise at varying intensities (Watt *et al.*, 2003a; Watt *et al.*, 2003b), although this does not necessarily result in significant IMTG hydrolysis (Watt *et al.*, 2004). It seems that HSL is required for complete TAG hydrolysis since HSL-null mice demonstrate DAG accumulation (Haemmerle *et al.*, 2002). In skeletal muscle, the hydrolase activity of HSL accounts ~60% of total neutral hydrolase activity at rest, whereas during exercise increased HSL activity has been reported to account for almost all of the total lipase activity (Watt *et al.*, 2004), indicating the

importance of HSL activation in the lipolytic process. In general, HSL activation is a hormonal- and contraction-mediated process. Firstly, adrenaline concentrations are increased during exercise which activates β-adrenergic receptors leading to protein kinase A (PKA) stimulation and serine phosphorylation ($S^{563}$ and $S^{660}$) of HSL (Watt *et al.*, 2006). Upon phosphorylation, cytosolic HSL is redistributed to LDs, a process which appears to be crucial for complete hydrolase activity of HSL (Prats *et al.*, 2006; Wang *et al.*, 2009), and has been shown to occur in response to moderate intensity exercise in human skeletal muscle (Whytock *et al.*, 2018) and also within isolated rat soleus muscle following adrenaline incubation or electrically-stimulated muscle contraction (Prats *et al.*, 2006). In addition to Prats *et al.* (2006) reporting the translocations of HSL to LDs, they further reported a concomitant reduction in IMTG measured by Bodipy-493/503 staining. However, dissociations between HSL activity and the change in IMTG content during exercise in humans have been observed (Roepstorff *et al.*, 2004; Watt *et al.*, 2004). The mismatch between HSL activation and IMTG breakdown in humans may signify the importance of other lipases, such as ATGL, in skeletal muscle lipolysis of IMTG.

ATGL, first detected in adipose tissue is also expressed in skeletal muscle (Zimmermann *et al.*, 2004), and, like HSL, appears to be most abundant in type I compared to type II muscle fibres (Jocken *et al.*, 2008). Importantly, overexpression of ATGL in non-adipocyte cells enhances the degradation of TAG (Smirnova *et al.*, 2006), whereas ATGL knockout mice demonstrate reduced TAG lipolysis leading to IMTG accumulation (Haemmerle *et al.*, 2006), underlining the importance of ATGL to the liberation of FA stores in intracellular LDs.

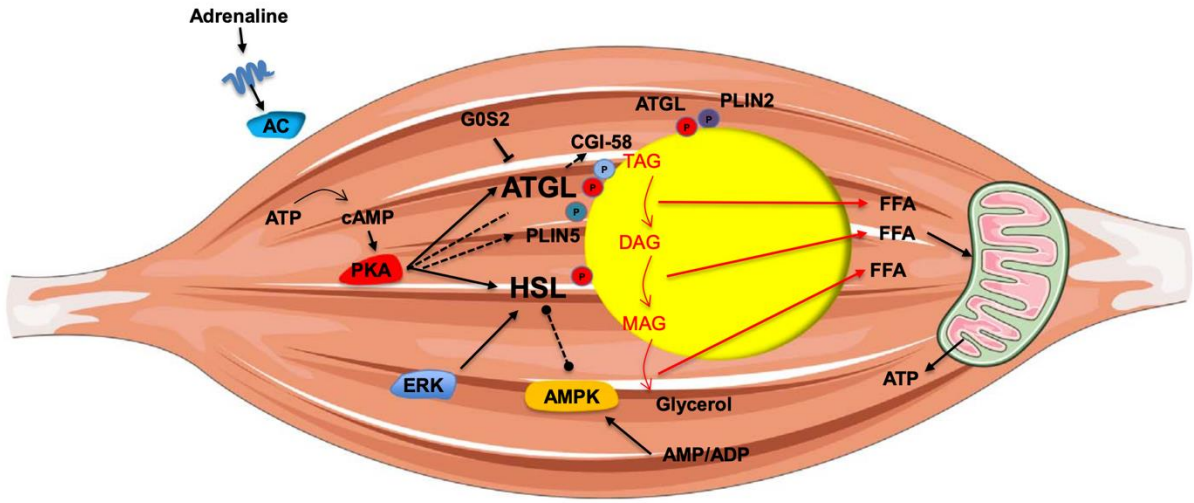


**Figure 2.9** Schematic representation of the key proteins controlling IMTG lipolysis during exercise. Solid line denotes known events. Dotted line represents likely events based on studies in other tissues. Dotted line with circles represents a likely inhibitory function (taken from Watt & Cheng, 2017). IMTG: Intramuscular triglyceride; AC: Adenylate cyclase; AMPK: AMP activated protein kinase; ATGL: Adipose triglyceride lipase; CGI-58: Comparative gene identification 58; DAG: diacylglycerol; ERK: Extracellular regulated kinase; G0S2; GO/GI switch gene 2; HSL: Hormone sensitive lipase; PKA: Protein kinase A; PLIN2: Perilipin 2; PLIN5: Perilipin 5.

### 2.2.9 Effects of carbohydrate feeding upon intramuscular triglyceride utilisation

In addition to the accumulating evidence demonstrating that IMTG stores can significantly contribute to energy provision, the effect of CHO feeding upon this energy substrate is poorly understood given the paucity of studies within this area, the conflicting findings between studies and the important complicated methodological differences between studies (see Table 2.1). In this regard, De Bock *et al.* (2005) initially reported an attenuated use of IMTG in type I muscle fibres when CHO was consumed before (~150 g) and during 2 h moderate intensity cycling (1 g·kg·h$^{-1}$), an effect attributed to the anti-lipolytic action of insulin (and decreased circulating adrenaline concentrations) and down regulation of hormone sensitive lipase (HSL) (Watt *et al.*, 2004). In contrast, Stellingwerff *et al.* (2007a) observed no effect of CHO feeding (0.7 g·kg·h$^{-1}$) during 3 h of exercise upon the net utilisation of IMTG stores in either type I or II muscle fibres. These authors (Stellingwerff *et al.*, 2007a) further concluded that the previously proposed stimulating effects of reduced plasma FFA availability on IMTG mobilisation and/or oxidation (Van Loon *et al.*, 2003; Watt *et al.*, 2001) were offset by the inhibitory effects of CHO feeding on IMTG hydrolysis. Most likely as a result of the greater

associated glycolytic flux with CHO intake, concomitantly higher circulating insulin, and decreased epinephrine levels, all of which have been associated with reduced HSL activity in muscle (Watt *et al.*, 2004).

Such discrepancies in IMTG use between these aforementioned studies may be explained by the difference in CHO feeding protocols between studies. As such, although Stellingwerff *et al.* (2007a) provided ~0.7 $g \cdot kg \cdot h^{-1}$ (~50 $g \cdot h^{-1}$) of glucose during 3 h of exercise in the absence of a pre-exercise meal (i.e., a total of ~150 g CHO), the protocols adopted by De Bock *et al.* (2005) provided 1 $g \cdot kg \cdot h^{-1}$ of maltodextrin during 2 h of exercise in conditions where CHO was also fed before exercise (~2.5 $g \cdot kg^{-1}$ of CHO ) in the CHO fed trial (thus providing a total of ~300 g of CHO in the CHO fed trial) whereas the control trial was commenced after an overnight fast. When taken together, such studies clearly demonstrate the importance of isolating the effects of CHO feeding to that consumed during exercise (as opposed to pre-exercise) as well as the requirement to assess fibre type specific substrate utilisation.

**Table 2.1.** Fibre-specific muscle glycogen and intramuscular triglyceride metabolic responses to carbohydrate ingestion during prolonged exercise.

| Study reference | Training status of subjects ($\dot{V}O_{2max}$) | Exercise modality | Pre-exercise feeding status | Duration of exercise | Intensity of exercise | Type and amount of CHO during exercise | Fibre-specific metabolic outcome |
|---|---|---|---|---|---|---|---|
| Coyle *et al.* (1986) | Endurance-trained cyclists (~70 ml·kg·min$^{-1}$) (n = 7, male) | Constant-intensity cycling | Undertaken after 16-h fast | Time matched biopsy at: ~180-min | 71% $\dot{V}O_{2max}$ | 1. GP: ~114 g·h$^{-1}$<br>2. Placebo | **Glycogen:** A slight, but not significant, reduction in glycogen utilisation in some Type II fibres in 2 of the participants when they were fed a CHO solution. |
| De Bock *et al.* (2005) | Physically active (n = 8, male) | Constant-intensity cycling | Provided with ~6 g·kg·day$^{-1}$ CHO (3 days before) and 2 g·kg$^{-1}$ CHO pre-exercise meal during CHO trial OR ~6 g·kg·day$^{-1}$ CHO (3 days before) and Overnight fasted | 120-min | ~75% $\dot{V}O_{2max}$ | 3. MD: 75 g·h$^{-1}$<br>4. Placebo | **IMTG:** Sparing of Type I IMTG utilisation with CHO intake before and during exercise. |
| De Bock *et al.* (2007) | Physically active (n = 8, male) | Constant-intensity cycling | Provided with ~6 g·kg·day$^{-1}$ CHO (3 days before) and 2 g·kg$^{-1}$ CHO pre-exercise meal during CHO trial OR ~6 g·kg·day$^{-1}$ CHO (3 days before) and Overnight fasted | 120-min | ~75% $\dot{V}O_{2max}$ | 1. MD: 75 g·h$^{-1}$<br>2. Placebo | **Glycogen:** Sparing of Type IIa muscle glycogen utilisation with CHO intake before and during exercise. |
| Mitchell *et al.* (1989) | Highly trained cyclists (~63 ml·kg·min$^{-1}$) (n = 10, male) | 1. Constant-intensity Cycling<br>2. Intermittent Cycling | - | 105-min | 1. Constant intensity (70% $\dot{V}O_{2max}$)<br>2. Intermittent (7 × 15 min at 70% $\dot{V}O_{2max}$ with 3 min rest between) | 1. GP + F: 74 g·h$^{-1}$<br>2. Placebo | **Glycogen:** Sparing of Type II muscle glycogen utilisation with CHO intake during the intermittent trial only. |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Stellingwerff *et al.* (2007a) | Endurance-trained cyclists (59.8 ± 5.6 ml·kg·min$^{-1}$) (n = 10, male) | Constant-intensity cycling | Provided with ~1.7 g g·kg$^{-1}$ CHO meal the evening before and undertaken the trials after an overnight fast | 180-min | 63 ± 4% $\dot{V}O_{2max}$ | 1. G: 50 g·h$^{-1}$<br>2. Placebo | **IMTG:** No difference between CHO and CON trial.<br><br>**Glycogen:** Sparing of Type I and II muscle glycogen utilisation during CHO trial. |
| Yaspelkis *et al.* (1991) | Competitive cyclists (65.8 ml·kg·min$^{-1}$) (n = 12, male) | Constant-intensity cycling | Undertaken after overnight fast | 120-min in the heat | 49% $\dot{V}O_{2max}$ | 1. MD + F: 75 g·h$^{-1}$<br>2. Placebo | **Glycogen:** Substantial reduction in Type I muscle glycogen utilisation only with CHO supplementation. |
| Yaspelkis *et al.* (1993) | Competitive cyclists (n = 7, male) | Intermittent cycling | Undertaken after overnight fast | 190-min | 45 & 75% $\dot{V}O_{2max}$ | 1. GP: ~75 g·h$^{-1}$<br>2. Placebo | **Glycogen:** Substantial reduction in Type I muscle glycogen utilisation only with CHO supplementation. |
| Tsintzas *et al.* (1995) | Recreational runners (54.5 ml·kg·min$^{-1}$) (n = 7, male) | Constant-intensity running | Undertaken after overnight fast | 60-min | 72% $\dot{V}O_{2max}$ | 1. GP+F+G: 50 g·h$^{-1}$<br>2. Placebo | **Glycogen:** Substantial reduction in Type I muscle glycogen utilisation only with CHO supplementation. |
| Tsintzas *et al.* (1996) | Recreational runners (61.8 ml·kg·min$^{-1}$) (n = 8, male) | Constant-intensity running | Undertaken after overnight fast | 100-min | 76% $\dot{V}O_{2max}$ | 1. GP+F+G: 45 g·h$^{-1}$<br>2. Placebo | **Glycogen:** Substantial reduction in Type I muscle glycogen utilisation only with CHO supplementation. |

**Note:** CHO, carbohydrate; G, glucose; GP, glucose polymer; F, fructose; MD, maltodextrin.

## 2.3 Carbohydrate and performance

It is clear that endogenous CHO availability may become a limiting factor for endurance performance and capacity in the absence of CHO feeding (Bergström & Hultman, 1967; Cermak & Van Loon, 2013). In this regard, the impact of muscle glycogen availability and exogenous CHO provision before (Burke *et al.*, 2011), during (Cermak & Van Loon, 2013; Stellingwerff & Cox, 2014) and after (Burke *et al.*, 2017) endurance-type exercise performance and capacity has been a central topic in sports nutrition research.

### 2.3.1 Muscle glycogen and carbohydrate loading

Most competitive endurance events are dependent on CHO-based fuels (muscle and liver glycogen, blood glucose and liver lactate) to sustain high rates of muscle energy production (Bosch *et al.*, 1990; Bosch *et al.*, 1993; Leckey *et al*., 2016; O'Brien *et al.*, 1993; Torrens *et al.*, 2016). However, as previously alluded to, the relative storage capacity of endogenous CHO is limited and an important goal of an athlete's diet in preparation for competition is to provide the trained musculature with the substrates necessary to fuel performance. Muscle glycogen concentrations within skeletal muscle typically range between 300 – 600 mmol·kg$^{-1}$ dw (Areta & Hopkins, 2018) under habitual dietary conditions. However, these concentrations can be markedly augmented through both exercise and nutritional interventions.

Indeed, pioneering work in the late 1960s by Scandinavian researchers developed the fundamental principles of CHO loading, where it was identified that muscle glycogen concentrations could be influenced by a super-compensatory effect whereby an exhaustive exercise bout is undertaken, followed by several days of high CHO intake in order to super-compensate glycogen concentrations (Bergström & Hultman, 1966; Bergström *et al.*, 1967). In spite of these early elegant findings, a landmark study in the 1980s by Sherman *et al.* (1981) observed similar magnitudes of glycogen supercompensation with a less severe exercise protocol (i.e., without the exhaustive exercise and CHO restriction phase) incorporating several days of a combined exercise taper and moderate CHO intake (5 g·kg$^{-1}$ body mass). In more recent work, it has been found that glycogen storage can be increased in both type I and II fibres within 24 – 48-h of increased CHO provision (Bussau *et al.*, 2002). Specifically, these authors observed that 10 g·kg$^{-1}$ body mass per day of CHO intake increased glycogen concentrations from 90 mmol·kg$^{-1}$ wet weight (392 mmol·kg$^{-1}$ dw) to 180 mmol·kg$^{-1}$ wet weight (783 mmol·kg$^{-1}$ dw) within 24-h. After two more days of rest and the CHO rich diet,

glycogen levels did not increase further but remained stable. Moreover, Jensen *et al.* (2020) recently replicated the seminal study by Bergström *et al.* (1967), but this time employed sophisticated transmission electron microscopy to assess the storage of glycogen within subcellular pools within the muscle after a low and high CHO diet. The results revealed that a diet in high in CHO (8.0 $g \cdot kg^{-1} \cdot day^{-1}$) compared with low CHO (0.2 $g \cdot kg^{-1} \cdot day^{-1}$) increased IMF, INTRA and SS glycogen volume within type I fibres, and increased IMF and SS glycogen in type II fibres. The authors further concluded that the local supercompensation of SS glycogen prior to exercise can mediate a sparing of INTRA glycogen utilisation during the initial hour of exercise within type I fibres, thereby postponing exhaustion (Jensen *et al.*, 2020). However, it must be noted that professional athletes within team sports and cycling in various formats, one-days classics and multi-day cycling stage races, are often faced with congested competition schedules which often limits the time between events and removes the opportunity for a traditional taper in order to elevate muscle glycogen stores. Nonetheless, recent work by Doering *et al.* (2019) showed for the first time that back-to-back muscle glycogen supercompensation is possible following exhaustive exercise with 4-d of high CHO feeding, when ~10 $g \cdot kg^{-1}$ body mass of CHO was consumed per day. This in turn, induced muscle glycogen concentrations of 835 $mmol \cdot kg^{-1}$ dw and 848 $mmol \cdot kg^{-1}$ dw after each 4-d period.

Despite a greater reliance on muscle glycogen when pre-exercise glycogen concentrations are elevated (Bosch *et al.,* 1993; Gollnick *et al.*, 1972; Hargreaves *et al.*, 1995), the general consensus from the plethora of studies undertaken in the last 40-years is that CHO loading can improve performance and capacity when the exercise duration is greater than 90-min (Hawley *et al.,* 1997). Specifically, Hawley *et al.* (1997) further cited within their review of the literature that CHO loading can improve time trial performance by 2-3% and exercise capacity by 20%. Furthermore, in shorter, more intense exercise bouts lasting 60-90 min, the benefits of glycogen loading are not apparent (Hawley *et al.,* 1997; Madsen *et al.,* 1990; Sherman *et al.,* 1981), possibly due to muscle glycogen availability not being a limiting factor in the non-CHO-loaded trial. Nonetheless, the previous enhancements in performance seem to be mediated by the delay in the time-point at which muscle glycogen availability becomes limiting, which is dependent on the maintenance of high rates of CHO oxidation (Hawley & Leckey, 2015). In addition to providing substrate availability for ATP production, it is now recognized that glycogen availability (especially the intramyofibrillar storage pool) can directly modulate skeletal muscle contractile function. Indeed, a series of studies from Ørtenblad and colleagues

(Ørtenblad *et al.,* 2011; Gejl *et al.,* 2014) have collectively shown preferential utilisation of this storage pool during exercise in a manner that also correlates with impaired $Ca^{2+}$ release from the sarcoplasmic reticulum (SR). Indeed, a reduction in SR $Ca^{2+}$ will *per se* lead to reductions in tetanic intracellular free $Ca^{2+}$ (Ørtenblad *et al.,* 2013) and is in accordance with previous observations of faster decreases in tetanic $Ca^{2+}$ in fibres with low muscle glycogen (Chin *et al.,* 1997). The implications of these distinct pools of glycogen upon muscle function and fatigue resistance are of key interest but require further investigation before practical recommendations can be made to the elite sporting environment.

The current practical application of CHO loading is that high glycaemic CHO-based foods are encouraged over low glycaemic sources in augmenting glycogen concentrations (Burke *et al.,* 1993) and intakes of 8-12 $g \cdot kg^{-1}$ body mass per day of CHO are likely required to maximise endogenous glycogen stores (Thomas *et al.,* 2016). It must further be noted, that 1 g of glycogen is stored in muscle with 3-5 g of water (Olsson & Saltin, 1970; Sherman *et al.,* 1982) consequently leading to a 1-2% increase in body mass associated with CHO loading strategies. Therefore, low-fibre CHO foods and low-fibre diet used in conjunction with CHO loading strategies have been encouraged in weight-sensitive sports in an attempt to partially offset the additional mass of glycogen and stored water (Burke *et al.,* 2019).

### 2.3.2 Pre-exercise carbohydrate availability

When compared with overnight fasting, ingestion of CHO-rich meals within the hours before exercise has been shown to enhance endurance performance (Neufer *et al.,* 1987; Schabort *et al.,* 1999; Sherman *et al.,* 1989, 1991; Wright *et al.,* 1991). However, when CHO provision was given < 1-h prior to exercise, performance outcomes were reported as being reduced (Foster *et al.,* 1978; Hargreaves *et al.,* 1987) and neutral (Devlin *et al.,* 1986). Nevertheless, the consumption of a pre-exercise meal has been observed to further enhance muscle glycogen content by up to 10% by the provision of a 2.5 $g \cdot kg^{-1}$ CHO meal 3 h prior to exercise (Chryssanthopoulos *et al.,* 2004; Coyle *et al.,* 1985) and given liver glycogen concentrations are substantially reduced after an overnight fast, ingestion of CHO may additionally increase or replenish these reserves (Coyle *et al.,* 1985; Casey *et al.,* 2000). These metabolic effects may in turn be associated with a greater maintenance of blood glucose during the latter stages of exercise when glycogen concentrations become limiting. Consequently, it is common practice for athletes to adopt such dietary practices to competition and current nutritional

guidelines for pre-exercise carbohydrate feeding advise an intake of 1 - 4 $g \cdot kg^{-1}$ body mass, 3-4 h prior to exercise (Thomas *et al.,* 2016). This pre-exercise CHO intake should be similar to that mentioned in the previous section whereby high glycaemic index (Burke *et al.,* 1993) and low-fibre CHO-based foods should be advocated with low-moderate protein intake to minimise propensity to gastrointestinal problems and promote gastric emptying (Rehrer *et al.,* 1992).

### 2.3.4 Carbohydrate ingestion during exercise

The ergogenic potential of CHO provision during prolonged moderate- to high-intensity exercise has been well recognised within the literature. Indeed, the first report on the enhancing effects of CHO ingestion in humans was that by Gordon *et al.* (1925) who encouraged athletes to engage in a high CHO diet 24-h before the Boston Marathon and to eat "candies" from 24-km onwards during the race, based of initial observations from the previous year that runners who completed the Boston marathon, exhibited hypoglycaemia immediately post-race (Levine *et al.,* 1924). High CHO ingestion before and during the race resulted in an improvement in the runners' performances, and their blood glucose levels post-race were elevated. From these initial investigations, authors have undertaken a plethora evidence within this specific sport nutrition area using well controlled laboratory settings and experimental designs. A number reviews have been also been undertaken in this area (Cermak & Van Loon, 2013; Sawka *et al*., 2007; Jeukendrup, 2010, 2011) and a recent systematic-review on endurance-based exercise protocols found that of the 61 included published performance studies within this review, 82% of these showed statistically significant performance benefits with CHO ingestion, with 18% showing no change compared to a placebo (Stellingwerff & Cox, 2014). They further concluded, that there was a significant correlative relationship between increasing total exercise time and the subsequent percent increase in performance with CHO intake vs. placebo. Furthermore, a meta-analysis of 88 randomised, crossover studies investigating CHO ingestion during exercise demonstrated performance benefits of up to 6% (range -2 to 6%) with CHO supplementation (Vandenbogaerde & Hopkins, 2011). The underpinning mechanisms associated with these performance enhancements seem to be related to a combination of multiple mechanisms consisting of a sparing of muscle and liver glycogen utilisation (Björkman, Shalin, Hagenfeldt & Wahren, 1984; Gonzalez *et al.,* 2015; Stellingwerff *et al.,* 2007a), maintenance of plasma glucose and elevated CHO oxidation rates (Coyle *et al.,* 1986) and direct effects upon the central nervous system (CNS) (Carter, Jeukendrup & Jones, 2004).

The following sections will aim to review the effects of CHO feeding during endurance-based exercise protocols where exercise duration is less than 1 h, 1 – 2 h and ≥ 3 h so as to provide further background for the present thesis.

#### 2.3.4.1 Carbohydrate ingestion during 0 – 60 minutes of endurance-based exercise

During exercise protocols <1 h in duration, it seems the mechanisms responsible for underpinning any associated performance enhancements are not related to a metabolic perspective given sufficient muscle and liver glycogen stores are generally not a limiting factor during such short durations. However, seminal work in the area by Bergström and Hultman (1967) reported that continuous intravenous glucose infusion rates up to 3.5 $g \cdot min^{-1}$ during 60-min intermittent cycling exercise reduced muscle glycogen breakdown by ~20%. Conversely, Arkinstall *et al.* (2001) reported no effect of 64 $g \cdot h^{-1}$ upon muscle glycogen utilisation during either 60-min of continuous cycling or running exercise at 70% $\dot{V}O_{2max}$. Additionally, other work has examined the fate of glucose infused during exercise exceeding 75% $\dot{V}O_{2max}$ and Carter *et al.* (2004b) reported that despite the elevation in blood glucose concentrations and increased glucose uptake when 1 $g \cdot min^{-1}$ of glucose was directly infused into the bloodstream compared to a placebo during a ~60-min cycling time trial, there was no apparent improvement in time trial performance. In this regard, the authors concluded and speculated that a mechanism prior to glucose uptake may produce a performance enhancing effect.

To investigate this hypothesis, the same authors conducted a follow up study whereby subjects rinsed ~1.5 g CHO in 125 ml in their mouths for ~10-s every 7.5-min or an identical tasting placebo with the solution not being ingested to omit any exogenous intake effect during the ~60-min cycling TT. Fascinatingly, this CHO mouth washing method resulted in a ~3% improvement in performance and was underpinned by non-metabolic effects but a CNS-based mechanism given the bypassing of the gastrointestinal tract (Carter *et al.,* 2004a*).* This mechanism was later confirmed by Chambers *et al.* (2009) through magnetic resonance brain imaging whereby CHO mouth rinsing was found to stimulate the brain areas of the insula/frontal operculum, orbitofrontal cortex, and striatum, which are involved with brain centres responsible for reward and motor control. Similarly, individual ratings of perceived effort have been shown to be equivalent among subjects between CHO intervention and placebo trials, despite the higher power outputs being produced (Pottier *et al.,* 2010; Rollo *et al.,* 2008; Rollo *et al.,* 2010; Sinclair *et al.,* 2014). In addition to the aforementioned

performance improvement in Carter *et al.* (2004a), many other investigations have also found similar improvements with CHO mouth rinsing regardless of sweetness (Chambers *et al.,* 2009) in both running (Rollo *et al.,* 2008; Rollo *et al.,* 2010) and cycling protocols (Chambers *et al.,* 2009; Fares and Kayser, 2011; Gam *et al.,* 2013; Lane *et al.,* 2013; Pottier *et al.,* 2010). However, the magnitude of performance gain is especially apparent in the absence of a pre-exercise CHO meal (Lane *et al.,* 2013) and low pre-exercise muscle glycogen (Kasper *et al.,* 2015), although this effect is not always evident (Ali *et al.,* 2016). Moreover, CHO mouth rinse has also been observed to improve performance over CHO ingestion during a ~60-min cycling time trial (Pottier *et al.,* 2010). Nevertheless, actual CHO ingestion, during more intense (>75% $\dot{V}O_{2max}$) exercise protocols of less than 1 h have also reported improvements in performance and/or capacity (Anantaraman *et al.,* 1992; Ball *et al.,* 1995; El-Sayed *et al.,* 1997; Jeukendrup *et al.,* 1997, see Table 2.2) whilst other studies have not observed such effects (Bonen *et al.,* 1981; Desbrow *et al.,* 2004; Jeukendrup *et al.,* 2008; Powers *et al.,* 1990, see Table 2.2).

**Table 2.2** Effects of carbohydrate supplementation during exercise lasting < 60 min on exercise performance or capacity (adapted from Stellingwerff & Cox, 2014).

| Study | Training status of subjects ($\dot{V}O_{2max/peak}$) | Pre-exercise nutritional status | Exercise and performance description/duration | CHO Type: study-dependent variable | Mean CHO intake rate ($g \cdot h^{-1}$) | Performance effect vs. control trial | % Difference (compared with control unless otherwise stated) | Significant performance effect? |
|---|---|---|---|---|---|---|---|---|
| **Anantaraman et al. (1995)** | Moderately trained (51.3 ± 4.5 $ml \cdot kg \cdot min^{-1}$) (n=3; males (3) and females (2)) | Performed in a post absorptive state (≥4h) | Total cycling work done for 1-h TT at self-selected PO of ~90% $\dot{V}O_{2max}$ (201 ± 21 W) | P before and during (P/P) | 0 | 560 kJ | - | **Yes** |
| | | | | 10% GLU pre-exercise with P during (G/P) | 30 | 619 kJ | ~10.5% | |
| | | | | GLU pre- exercise with GLU during (GLU/GLU) | 120 | 599 kJ | ~7.0% | |
| **Below et al. (1995)** | Endurance-trained males (~63 $ml \cdot kg \cdot min^{-1}$) (n=8) | Overnight fast. Subjects asked to consume same diet 24 h before each trial | Cycling at 80% of $\dot{V}O_{2max}$ for 50 min followed by 10-min TT | Small volume (200 mL) | 0 | 11.3 min | - | **Yes** |
| | | | | large volume (1330 mL) | 0 | 10.5 min | ~7.1% | |
| | | | | Small volume (200 mL) of 40% (GLU+SUC); | 79 | 10.5 min | ~7.1% | |
| | | | | Large volume (1330 mL) of 6% (GLU+SUC) | 79 | 9.93 min | ~12.1% | |
| **Bonen et al. (1981)** | Moderately trained males (G: 54.1 ± 1.7 and P: 51.4 ± 0.5 $ml \cdot kg \cdot min^{-1}$) (G: n=8; P: n=8) | Fasted and muscle glycogen depleted state (fasted for 36-44 h before the study and after the initial 12-h of fasting they exercised to exhaustion) | Cycling at 80% $\dot{V}O_{2max}$ to exhaustion/group design (not crossover) | Placebo | 0 | 29.9 min | - | **No** |
| | | | | 20% GLU ingestion during exercise | 112 g total (over 30 min) | 26.1 min | -15.0% | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Desbrow et al. (2004)** | Endurance-trained males (65.1 ± 5.9 ml·kg·min$^{-1}$) (n=9) | 9.1 g·kg$^{-1}$ CHO provided 24h day before. Followed by 1.8 g·kg$^{-1}$ CHO provided 2h prior to exercise | Total cycling work done for 1-h TT at self-selected PO | Placebo | 0 | 62.4 min (282.9 W) | - | **No** |
| | | | | 6% GLU ingestion during warm-up and during exercise | ~60 | 62.3 min (283.0 W) | 0.2% | |
| **El-Sayed et al. (1997)** | Endurance-trained males (66.5 ± 2.5 ml·kg·min$^{-1}$) (n=8) | Subjects fasted for 3 h before arrival | Total cycling work done for 1-h TT at self-selected PO | Placebo | 0 | 269 W | - | **Yes** |
| | | | | 8% GLU right before the TT | ~25 | 277 W | 3.0% | |
| **Jeukendrup et al. (1997)** | Moderately trained (males: 72.9 ± 1.4 and females: 56.1 ± 11.2 ml·kg·min$^{-1}$) (n=19; males (19) and females (2)) | 1 h preceding the test, subjects were not allowed to eat. Food records from first trial provided for the day before and morning of the test. | Cycling total amount of work (J) predicted to be a 1-h TT | Placebo | 0 | 60.15 min | - | **Yes** |
| | | | | 8% GLU solution | ~75 | 58.74 min | 2.4% | |
| **Jeukendrup et al. (2008)** | Endurance-trained males (66.4 ± 6.2 ml·kg·min$^{-1}$) (n=12) | Subjects reported to the lab following a 3 h postprandial fast | Cycling 16-km TT (~25 min) | Placebo | 0 | 25.50 min | **-** | **No** |
| | | | | 8% GLU+SUC CHO solution | 35 g total (over 30 min) | 25.63 min | -0.3% | |
| **Nicholas et al. (1995)** | Trained male soccer players (56.3 ± 1.3 ml·kg·min$^{-1}$) (n=9) | Overnight fast (10 h) | 75 min of intermittent exercise (running/sprinting) followed by intermittent running to fatigue (8 min) | Placebo | 0 | 6.7 min | - | **Yes** |
| | | | | 7% GLU consumed in 2 mL/kg every 15 min | ~66 | 8.9 min | **3.3%** | |
| **Powers et al. (1990)** | | - | 85% $\dot{V}O_{2max}$ until | 210 mL every 15 min | 0 | 35.8 min | - | **No** |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Endurance-trained males (61.9 ± 3.2 ml·kg·min$^{-1}$) (n=9) | | fatigue (~40 min) | of P | | | | |
| | | | | 210 mL every 15 min of water | 0 | 40.2 min | -10.9% | |
| | | | | 210 mL every 15 min of 7% GLU | ~66 | 39.2 min | -8.7% | |
| **Sugiura and Kobayashi (1998)** | Moderately trained males (~56.2 ml·kg·min$^{-1}$) (n=8) | Overnight fast | 90 min of exercise over 6 trials of 3 continuous (65% $\dot{V}O_{2max}$; CONT) and 3 intermittent (65% $\dot{V}O_{2max}$ with 30-s sprints every 2.5 min; INT) with an all-out 40-s Wingate performance test | Halfway through trial subjects received 250 mL of Placebo | 0 | CONT: 574 W | - | **Yes** |
| | | | | | | INT: 596.3 W | - | |
| | | | | Halfway through trial subjects received 250 mL of 20% GLU | ~33 | CONT: 614 W | 6.9% | |
| | | | | | | INT: 629.5 W | 5.6% | |
| | | | | Halfway through trial subjects received 250 mL of 20% FRU | ~33 | CONT: 603.8 W | 5.2% | |
| | | | | | | INT: 598.4 W | 0.4% | |

**Note:** CHO, carbohydrate; FRU, fructose; GLU, glucose; HI, high intensity: LOW, low intensity; P, placebo; TT, time trial; SUC, sucrose.

### 2.3.4.2 Carbohydrate ingestion during 60 – 120 of minutes endurance-based exercise

When exercise protocols extend to between 60-120 min in duration, the potential mechanisms underpinning any ergogenic effects of exogenous CHO provision inherently change to more metabolic factors in accordance with the greater metabolic and physiological stress. It was previously hypothesised that CHO ingestion during prolonged endurance exercise improves performance by slowing the rate of muscle glycogen degradation (Coyle & Coggan, 1984). In regard to studies utilising 60-120 min exercise protocols, studies have yielded conflicting results in respect to muscle glycogen utilisation. Indeed, some studies have supported this original hypothesis (Erickson *et al.,* 1987; Tsintzas *et al.,* 1995; Tsintaz *et al.,* 1996; Yaspelkis *et al.,* 1993), whereas others have provided contrary results (Coyle *et al.,* 1986; De Bock *et al.,* 2007; Flynn *et al.,* 1987; Hargreaves & Briggs, 1988; McConell *et al.,* 2000; Mitchell *et al.,* 1989). Such discrepancies in findings could be explained by the difference in exercise modalities, duration, intensity, environmental conditions, and CHO feeding protocols as can be seen in Table 2.6. Interestingly, the combination of CHO intake before and during 2-h of cycling exercise at ~75% $\dot{V}O_{2max}$ in line with nutritional recommendations compared to fasted exercise has demonstrated a fibre-specific reduction in muscle glycogen breakdown exclusively in type IIa fibres when examined by histology techniques (De Bock *et al.,* 2007). Given the mixed findings of muscle glycogen sparing in the above studies, it seems that a greater maintenance of plasma glucose concentrations with CHO ingestion, thereby allowing augmented rates of CHO oxidation is a more plausible primary mechanism underpinning any associated improvements in performance, with such metabolic effects being repeatedly observed within the literature (Erickson *et al.,* 1987; Flynn *et al.,* 1987; Hargreaves & Briggs, 1988; Mitchell *et al.,* 1989; Newell *et al.,* 2018; Tsintzas *et al.,* 1995; Tsintaz *et al.,* 1996; Yaspelkis *et al.,* 1993). Maintenance of plasma glucose concentrations and higher CHO oxidation rates even during exercise between 60-120 min seems to most likely come from preservation of endogenous hepatic glycogen stores (Newell *et al.,* 2018). Specifically, Jeukendrup *et al.* (1999) showed that the ingestion of 63 $g \cdot h^{-1}$ markedly suppressed hepatic glucose output, a high ingestion rate of 175 $g \cdot h^{-1}$ completely suppressed endogenous glucose production during 120-min cycling at 50% $\dot{V}O_{2max}$.

Based of the above metabolic factors associated with exogenous CHO ingestion, it is well-established that CHO ingestion during exercise of between 1 – 2-h in duration readily improves endurance performance and capacity (Stellingwerff & Cox, 2014). As such, within the

previously mentioned systematic review by Stellingwerff & Cox (2014), the authors further reported that of the 18 studies of 1 – 2-h in duration involving either a time trial or with a pre-load, 83% of these showed a significantly positive benefit of CHO intake on performance. Despite the varying research designs used and the range of CHO doses, these improvements were observed with CHO ingestion rates as low as 16 $g \cdot h^{-1}$ (Maughan *et al.,* 1996) and as much as 74 $g \cdot h^{-1}$ (Mitchell *et al.,* 1989). However, not all studies have found such inclusive performance benefits (Davis *et al.*, 1988; Flynn *et al.*, 1987; McConell *et al.*, 2000) with Table 2.3 summarising these findings.

**Table 2.3** Effects of carbohydrate supplementation during exercise lasting 60 – 120 min on exercise performance or capacity (adapted from Stellingwerff & Cox, 2014).

| Study | Training status of subjects ($\dot{V}O_{2max/peak}$) | Pre-exercise nutritional status | Exercise and performance description/duration | CHO Type: study-dependent variable | Mean CHO intake rate ($g \cdot h^{-1}$) | Performance effect vs. contrail trial | % Difference (compared with control unless otherwise stated) | Significant performance effect? |
|---|---|---|---|---|---|---|---|---|
| **Exercise Duration lasting 1 – 2 h** | | | | | | | | |
| **Bjorkman et al. (1994)** | Male Recreationally trained for endurance (56 $ml \cdot kg \cdot min^{-1}$) (n = 8) | Each trial conducted after an overnight fast | Cycling at 68% $\dot{V}O_{2max}$ until exhaustion | Placebo | 0 | 116 min | - | **Yes** |
| | | | | 7% GLU | ~53 | 137 min | 18.1% | |
| | | | | 7% FRU | ~53 | 116 min | 0% | |
| **Davis et al. (1988a)** | Endurance-trained males (63.0 ± 6.0 $ml \cdot kg \cdot min^{-1}$) (n = 15) | Each trial conducted following 10 h fast | 2 consecutive 60-min cycling bouts at 60% $\dot{V}O_{2max}$, each followed time to complete a 270-revolution TT (3 min) immediately after and 20 min after | Water Placebo | 0 | 163.7 s | - | **No (strong positive trend)** |
| | | | | 6% GLU (LC) | ~50 | 153.9 s | 6.4% | |
| | | | | 12% GLU (HC) | ~100 | 158.7 | 3.2% | |
| **Maughan et al. (1989)** | Male Recreationally trained for endurance (53.0 $ml \cdot kg \cdot min^{-1}$) (n = 6) | Fasted state at beginning of trial | 4 repeated trials of running at 70% $\dot{V}O_{2max}$ to exhaustion | water Placebo | 0 | 70.2 min | - | **YES (LGLU vs. P); No for rest of comparisons** |
| | | | | 4% GLU (LGLU) | 29.3 | 90.8 min | 29.3% | |
| | | | | 34% GLU + 15% FRU (GLU+FRU) | 240 | 79.5 min | 12.5% | |
| | | | | 36% GLU (HGLU); | 262 | 79 min | 12.5% | |
| | | | | 34% FRU + 2% GLU (HFRU) | 229 | 65.6 min | 6.5% | |
| **McConell et al. (2000)** | Endurance-trained males (65.7 ± 1.5 $ml \cdot kg \cdot min^{-1}$) (n = 13) | 24 h prior to trial provided with standardised mixed diet (65% CHO, 19.2% Fat, 15% Pro). Performed trial after an overnight fat (10 - | Cycling at 83% $\dot{V}O_{2max}$ to exhaustion | Placebo | 0 | 69.6 min | - | **NO** |
| | | | | 6% GLU | ~81 | 68.1 min | -2.2% | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 11 h) or a 6 – 8 h fast (afternoon trials). | | | | | | |
| **Murray et al. (1987)** | Untrained males (45.1 ± 7.7 ml·kg·min$^{-1}$) (n = 10) | Overnight fast (10 h) | ~90 min of intermittent cycling at 55%–65% $\dot{V}O_{2max}$ followed by a 6-min TT sprint | Placebo | 0 | 432 s | - | **YES (6CHO and 7CHO vs P); NO for 5CHO** |
| | | | | 5% GLU (5CHO) | 24 | 400 s | 8.0% | |
| | | | | 4% SUC + 2% GLU (6CHO) | 29 | 384 s | 12.5% | |
| | | | | 5% GLU + 2% FRU (7CHO) | 34 | 375 s | 15.2% | |
| **Murray et al. (1989a)** | Untrained males (42.8 ± 1.8 ml·kg·min$^{-1}$) (n = 12) | Provided with standard breakfast and lunch providing a total of 4060 kJ and began the trial 4 h after lunch | ~115 min of intermittent cycling at 65%–80% $\dot{V}O_{2max}$ followed by a 7-min TT sprint | 6% GLU (GLU) | 38 | 424 s | 15.1% (vs. FRU) | **Yes (GLU and SUC vs. FRU)** |
| | | | | 6% SUC (SUC) | 38 | 419 s | 16.6% (vs. FRU) | |
| | | | | 6% FRU (FRU) | 38 | 488 s | - | |
| **Neufer et al. (1987)** | Endurance-trained males (~60 ml·kg·min$^{-1}$) (n = 10) | Subjects consumed a standard breakfast and lunch providing an average of 64% CHO, 26% Fat and 10% Protein. Subjects fasted for 4 h prior to the start of exercise | 45 min of cycling at 77% $\dot{V}O_{2max}$ followed by total work done during a ~15-min TT | Placebo | 0 | 159143 NM | - | **Yes** |
| | | | | Liquid CHO | 45 | 175204 NM | 10.1% | |
| | | | | Solid CHO | 45 | 176013 NM | 10.6% | |
| **Too et al. (2012)** | Endurance-trained males (58.2 ± 4.8 ml·kg·min$^{-1}$) (n = 11) | A 240-kcal snack (68% CHO, 16% fat and 16% protein) (Clif Bar, Berkeley, CA) was provided to consume 10-hr before each of their testing times. Subjects performed trial after overnight fast | 80 min of running at 80% $\dot{V}O_{2max}$ followed by a 5-km TT | Placebo | 0 | 21.6 min | - | **Yes** |
| | | | | Raisins (R) | ~65 | 20.6 min | 4.9% | |
| | | | | Chews/blocks (B) | ~65 | 20.7 min | 4.3% | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Tsintzas et al. (1993)** | Endurance-trained males (~62 ml·kg·min$^{-1}$) (n = 7) | Overnight fast (10 – 12 h) | 30-km running road race | Placebo | 0 | 131.2 min | - | **Yes** |
| | | | | 5.5% CHO (GLU polymer + GLU + FRU) | 50 (first hour only) | 128.3 min | 2.1% | |
| **Tsintzas et al. (1996a)** | Endurance-trained males (61.8 ± 2.3 ml·kg·min$^{-1}$) (n = 8) | Overnight fast (12 – 16 h) | Running at 70% $\dot{V}O_{2max}$ until exhaustion | Placebo | 0 | 104.3 min | - | **Yes** |
| | | | | 5.5% CHO (GLU+FRU) | ~41 | 132.4 min | 2.3% | |
| **Tsintzas et al. (1996b)** | Recreationally trained for endurance (males) (61.7 ± 1.8 ml·kg·min$^{-1}$) (n = 11) | Overnight fast (10 – 12 h) | Running at 70% $\dot{V}O_{2max}$ until exhaustion | Placebo | 0 | 109.6 min | - | **Yes** |
| | | | | 5.5% CHO (5CHO) | ~55 | 124.5 min | 13.6% | |
| | | | | 6.9% CHO (6CHO) | ~69 | 121.4 min | 10.8% | |
| | | | | (first hour only for CHO treatments, water thereafter) | | | | |
| **Wilber & Moffet (1992)** | Endurance-trained males (64.9 ± 4.8 ml·kg·min$^{-1}$) (n = 10) | Overnight fast (10 – 12 h) | Running at 80% $\dot{V}O_{2max}$ until exhaustion | Placebo | 0 | 92.0 min | - | **Yes** |
| | | | | 7% GLU | 41 | 115.4 min | 25.4% | |
| **Zachwieja et al. (1992)** | Endurance-trained males (~65 ml·kg·min$^{-1}$) (n = 8) | 24 h prior to trial provided with standardised mixed diet (325g CHO). Subjects commenced trial after overnight fast | 105 min of cycling at 70% $\dot{V}O_{2max}$ followed by total work done during a ~15-min TT | Placebo | 0 | 242 W | - | **Yes** |
| | | | | 4%GLU + 6%FRU (GLU + FRU) | 63 | 264 W | 9.1% | |

**Note:** CHO, carbohydrate; FRU, fructose; GLU, glucose; HI, high intensity: LOW, low intensity; P, placebo; TT, time trial; SUC, sucrose; NM, no measurement; GI, gastrointestinal; HFCS, high-fructose corn syrup.

### 2.3.4.3 Carbohydrate ingestion during > 120 minutes of endurance-based exercise

In addition to the previous two sections detailing the mechanistic and performance effects of CHO provision during endurance exercise up to 120-min, there is a number of studies which have also examined such effects in exercise protocols beyond 120-min in duration. These findings have much greater translation potential given professional endurance events require performance in excess of 120 and 180 min in duration coupled with the significantly challenging metabolic demands of such events. Nonetheless, the apparent primary mechanisms delaying the onset of fatigue with CHO provision during such prolonged endurance durations seems again to be related to a greater maintenance of plasma glucose (Coyle *et al.,* 1986) and a liver glycogen sparing effect (Gonzalez *et al.,* 2015), with the magnitude of performance improvement also increasing with the extension in exercise duration (Stellingwerff & Cox, 2014). In this regard, it was initially thought that exogenous CHO intake during such prolonged exercise would attenuate the decline of endogenous CHO stores when glycogen becomes more limiting. Indeed, the first study to examine the effects of CHO ingestion on muscle glycogen use during exercise directly, reported that feeding subjects 43 $g \cdot h^{-1}$ of sucrose every hour during 4-h of intermittent cycling decreased glycogen concentration in the vastus lateralis by 20% less than during the placebo trial (Hargreaves *et al.,*, 1984). However, closer examination of the data suggested that the glycogen sparing effect seemed to be an artefact of higher pre-exercise glycogen concentrations in the placebo trial given four subjects within this trial had significantly higher pre-exercise muscle glycogen contents. In contrast, Coyle *et al.* (1986) reported no difference in muscle glycogen utilisation rates when ~114 $g \cdot h^{-1}$ of CHO was ingested compared to a placebo. This equivocal net muscle glycogen breakdown pre- to post-exercise between nutritional conditions has been further shown in exercise protocols > 120-min (Bosch *et al.,* 1994; Fielding *et al.,* 1985) whilst others have reported contrasting sparing effects (Bosch *et al.,* 1996; Yaspelkis *et al.,* 1993) as detailed in Table 2.6. However, the lack of observed change in the prior studies may be due to methodological factors. As such, the first study to examine fibre-specific muscle glycogen content with exogenous CHO provision during endurance-based exercise beyond > 120-min, observed a sparing effect in both type I and II fibres (Stellingwerff *et al.,* 2007a) (see Table 2.1). However, this is the only study to date to investigate such effects during 180-min of prolonged moderate intensity exercise. Interestingly, the authors also alluded to a time-dependent sparing of muscle glycogen as the result of reduced utilisation during the initial stages of exercise with CHO ingestion when measured by stable isotope tracer methodology.

In spite of the contrasting findings in relation to muscle glycogen sparing, the ingestion of glucose or sucrose at a rate of 102 $g \cdot h^{-1}$ during 180-min of cycling exercise at 50% $W_{max}$ was found to completely preserve post-exercise liver but not muscle glycogen concentrations when measured by $^{13}C$ magnetic resonance spectroscopy (Gonzalez *et al.*, 2015). Such data suggest that liver glycogen sparing may be a more potent mechanism underpinning the ergogenic potential of exogenous CHO provision compared to muscle. Additionally, one immediate benefit from the supply of exogenous CHO during exercise is the greater maintenance of plasma glucose concentrations in turn, providing exogenous substrate to the contracting muscle allowing high CHO oxidation rates. Coyle *et al.* (1986) demonstrated that after ~3-h of exercise at 71% $\dot{V}O_{2peak,}$ plasma glucose concentrations decreased to ~2 $mmol \cdot L^{-1}$ in the placebo trial which coincided with the point of fatigue. In contrast, the ingestion of CHO maintained plasma glucose concentrations between 4.2 – 5.2 $mmol \cdot L^{-1}$ even when subjects continued exercising for an extra hour in the CHO condition. Similarly, CHO oxidation rates were maintained at a higher rate with CHO ingestion, with an average RER at exhaustion of 0.86 whereas in the placebo trial, CHO oxidation rates decreased, and RER values decreased from 0.85 to 0.80 at exhaustion. Fascinatingly, the point which corresponded to fatigue within the CHO trial showed no indication that the rate of CHO oxidation was insufficient, and thus it might be expected that the subjects could have continued exercising. In accordance, a subsequent study by Coggan and Coyle (1987) further elucidated the impact of maintaining plasma glucose concentrations, as they attributed the significant reduction in time to exhaustion reported within the placebo trial compared to the glucose ingestion and glucose infusion conditions to the decline in plasma glucose concentrations and the consequent fall in CHO oxidation. Moreover, only the glucose infusion trial was able to maintain euglycemia and resulted in significantly longer time to fatigue compared to glucose ingestion. However, not all studies have reported the same impact of reduced plasma glucose levels on subsequent performance or capacity (Claassen *et al.*, 2005; Felig *et al.*, 1982). Felig *et al.* (1982) reported that glucose ingestion rates of 40 or 80 $g \cdot h^{-1}$ prevented hypoglycaemia but did not significantly increase time to fatigue.

Collectively, the precise mechanisms and combination of mechanisms underpinning the ergogenic effects of CHO ingestion during prolonged endurance exercise are not fully elucidated. However, it is likely that muscle and liver glycogen sparing, and/or the maintenance of plasma glucose concentrations are involved, thereby allowing high CHO oxidation rates during the latter stages of exercise when muscle glycogen stores are compromised. The

contribution of these factors may depend on the variables of the exercise bout (i.e., duration, intensity and type of exercise), the training status and pre-exercise nutritional status of the subjects and the specifics of the CHO feeding protocol employed (type, blend and dose of CHO).

Studies investigating the performance and/or capacity effects of exogenous CHO ingestion during exercise longer than 120-min in duration have unsurprisingly observed positive improvements as summarised in Table 2.4. Indeed, Stellingwerff & Cox (2014) reported a significant correlative relationship between increasing total exercise time and the subsequent percent increase in performance with CHO compared to a placebo. Predictably, performance would be more sensitive to change as endogenous CHO stores become limited and there are reductions in fuel availability.

**Table 2.4** Effects of carbohydrate supplementation during exercise lasting > 120 min on exercise performance or capacity (adapted from Stellingwerff & Cox, 2014).

| Study | Training status of subjects ($\dot{V}O_{2max/peak}$) | Pre-exercise nutritional status | Exercise and Performance description/duration | CHO Type: study-dependent variable | Mean CHO intake rate ($g \cdot h^{-1}$) | Performance effect vs. control | % Difference (compared with control unless otherwise stated) | Significant performance effect? |
|---|---|---|---|---|---|---|---|---|
| **Exercise Duration lasting 1 – 2 h** | | | | | | | | |
| **Angus et al. (2000)** | Endurance-trained males (~65 $ml \cdot kg \cdot min^{-1}$) (n = 8) | Food provided for 24-hours before experimental trial (71% CHO, 15% PRO, 14% fat). Morning of the trial, subjects arrived 2 h after a breakfast – 66% CHO – 2 muesli bars, 250 ml orange juice & 300ml water | Time to complete 35 kJ/kg on cycle ergometer (100-km TT) | Placebo | 0 | 178 min | - | **Yes** |
| | | | | 6% CHO (GLU+SUC) | 60 | 166 min | 7.2% | |
| **Carter et al. (2003)** | Endurance-trained males (59.5 ± 1.6 $ml \cdot kg \cdot min^{-1}$) (n = 8) | Overnight fast | Cycling at 60% (LOW) and 73% (HI) $\dot{V}O_{2max}$ to exhaustion | Placebo | 0 | 123.1 min | - | **Yes** |
| | | | | 6% CHO (GLU) | ~65 | 145.6 min | 18.3% | |
| **Coggan & Coyle (1987)** | Endurance-trained males (~66.0 $ml \cdot kg \cdot min^{-1}$) (n = 7) | Overnight fast (14 – 16 h) | Cycling at 73% $\dot{V}O_{2max}$ to exhaustion (170 min), followed by 20-min recovery and again cycling at 73% $\dot{V}O_{2max}$ to exhaustion | Placebo | na (taken between exercise bouts) | 10 min | - | **Yes** |
| | | | | 3 g/kg GLU (210 g GLU) consumed during 20 min recovery | | 26 min | 160% | |
| **Coyle et al. (1983)** | Endurance-trained males (~58 $ml \cdot kg \cdot min^{-1}$) (n = 10) | Overnight fast (12-h) | Cycling at 74% $\dot{V}O_{2max}$ until exhaustion | Placebo | 0 | 134 min | - | **Yes** |
| | | | | 7% GLU | ~51 | 157 min | 17.2% | |
| **Coyle et al. (1986)** | Endurance-trained males (~65 $ml \cdot kg \cdot min^{-1}$) (n = 7) | Overnight fast (16-h) | Cycling at 71% $\dot{V}O_{2max}$ until exhaustion | Placebo | 0 | 3.02 h | - | **Yes** |
| | | | | GLU polymer | ~114 | 4.02 h | 33.1% | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | (GLU) | | | | |
| **Davis et al. (1988b)** | Endurance-trained males (63.8 ± 1.3 ml·kg·min$^{-1}$) (n = 19) | Overnight fast (10 h) | Cycling at 75% $\dot{V}O_{2max}$ for 130 min, followed by 30-min rest, followed by 30-min TT at 75% $\dot{V}O_{2max}$ | Placebo | 0 | 34.3 min | - | **YES (HCHO vs. P)** |
| | | | | 2.5% CHO (LCHO) | 23 | 31.9 min | 7.5% | |
| | | | | 6% CHO (HCHO) as SUC+GLU | 56 | 31.3 min | 9.6% | |
| **Fielding et al. (1985)** | Recreationally trained for endurance (males) (63.8 ± 1.3 ml·kg·min$^{-1}$) (n = 9) | Overnight fast | 240-min cycling followed by a sprint at 100% $\dot{V}O_{2max}$ to exhaustion | Placebo | 0 | 81 s | - | **Yes** |
| | | | | 5% CHO solution (SUC) | 21.5 | 121 s | 49.4% | |
| **Hargreaves et al. (1984)** | Endurance-trained males (~62 ml·kg·min$^{-1}$) (n = 10) | Overnight fast | 4 h of intermittent intensity cycling followed by a sprint at 100% $\dot{V}O_{2max}$ to exhaustion | Placebo | 0 | 87 s | - | **Yes** |
| | | | | Candy bar (C) having 43 g SUC, 6 g fat, and 3 g protein | 43 | 127 s | 46% | |
| **Ivy et al. (1983)** | Healthy males (60.4 ± 6.5 ml·kg·min$^{-1}$) (n = 10) | 2-day controlled diet prior to trial. Overnight fast (12-h) | Walking at 45% $\dot{V}O_{2max}$ until exhaustion | Placebo | 0 | 268 min | - | **Yes** |
| | | | | 20% GLU polymer | 24 - 29 | 299 min | 11.6% | |
| **Madsen et al. (1996)** | Endurance-trained males (63.1 ± 1.5 ml·kg·min$^{-1}$) (n = 9) | 4-h fast | 100-km cycling TT | Placebo | - | 160 min | - | **No** |
| | | | | 5% CHO solution (CHO) | 66 | 160 min | 0% | |
| **McConell et al. (1996)** | Endurance-trained males (~69 ml·kg·min$^{-1}$) (n = 8) | Overnight fast or at least 6-h post prandial for afternoon trials | 2 h of cycling at 70% $\dot{V}O_{2max}$ followed by total work done over a 15-min TT | Placebo | 0 | 242 kJ | - | **Yes** |
| | | | | 7% CHO solution (CHO) | 79 | 268 kJ | **10.7%** | |
| **Wright et al. (1991)** | Endurance-trained males (~60 ml·kg·min$^{-1}$) (n = 9) | 10 hours before reporting to the lab the subjects consumed two granola bars (55% | Cycling at 70% $\dot{V}O_{2max}$ until exhaustion | Placebo after 3 h pre-exercise fast | 0 | 237 min | - | **Yes** |
| | | | | 8% CHO (5%GLU + 3%SUC) after a 3 | ~40 | 289 min | 21.9% (vs Placebo 3 h fast) | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | CHO, 30% fat and 15% protein). Pre-exercise feeding was 333g CHO for "CHO before trial" and "CHO before & During exercise" (so 2 out of the 4 trials) | | h pre-exercise fast | | | | |
| | | | | Placebo after 10 h pre-exercise fast | 0 | 201 min | - | |
| | | | | 8% CHO (5%GLU + 3%SUC) after a 10 h pre-exercise fast | ~40 | 266 min | 32.3% (vs Placebo 10 h fast) | |
| **GLU:FRU CHO combinations vs. GLU alone** | | | | | | | | |
| **Currell & Jeukendrup (2008)** | Endurance-trained males (64.7 ± 3.9 ml·kg·min$^{-1}$) (n = 8) | Overnight fast | Cycling at 55% $W_{max}$ (~ 65% $\dot{V}O_{2max}$) for 2 h followed by a total work done in 1-h TT | Placebo | 0 | 4022 s | - | **Yes** |
| | | | | GLU | 108 | 3641 s | 8.1% (vs P) | |
| | | | | GLU:FRU (2:1 ratio) | 108 | 3367 s | 19.5% and 10.5% (vs. P and GLU) | |
| **Langenfield et al. (1994)** | Endurance-trained males | Two days of prescribed diet followed by a feeding 3 – 4 h prior to exercise | 128-km cycling time trial | Placebo | 0 | 253.2 min | - | **Yes** |
| | | | | 7% CHO (5% maltodextrin + 2% FRU) | 37 | 241.0 min | 5.1% | |
| **O'Brien and Rowlands (2011)** | Endurance-trained males (58.4 ± 4.7 ml·kg·min$^{-1}$) (n = 10) | Subjects were provided with a pre-packaged pasta meal to be consumed the evening before each experimental trial (1.7 g·kg$^{-1}$ CHO). Began Trial after an overnight fast. | Cycling at 50% $W_{max}$ for 150 min immediately followed by an incremental test to exhaustion | Placebo | 0 | 549 s | - | **Yes (for 1.25 to 1 and 0.8 to 1)** |
| | | | | 2:1 GLU:FRU (2:1) | 108 | 545 s | -0.7% | |
| | | | | 1.25:1 GLU:FRU (1.25:1) | 108 | 594 s | 8.2% | |
| | | | | 0.8:1 GLU:FRU (0.8:1) | 108 | 578 s | 5.3% | |
| **O'Brien et al. (2013)** | Endurance-trained males (59.1 ± 5.2 ml·kg·min$^{-1}$) (n = 12) | Subjects were provided with a pre-packaged pasta meal to be consumed the evening before each experimental | Cycling at 57% $W_{max}$ for 2 h followed by 10 performance sprints (~2 min each sprint with ~5-min recovery) | Placebo | 0 | 275 W | - | **Yes (for all CHO vs. P)** |
| | | | | 2:1 GLU:FRU (2:1) | 108 | 294 W | 6.9% | |
| | | | | 1.25:1 GLU:FRU (1.25:1) | 108 | 303 W | 10.2% | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | trial (1.9 g·kg$^{-1}$ CHO). Began Trial after an overnight fast. | | 0.8:1 GLU:FRU (0.8:1) | 108 | 296 W | 7.6% | |
| **Triplett et al. (2010)** | Endurance-trained males (61.6 ± 2.6 ml·kg·min$^{-1}$) (n = 9) | Record diet for previous 2 days prior to trial and replicate and began trial after an overnight fast | 100-km cycling TT on ergometer | GLU | ~144 | 220 min | - | **Yes** |
| | | | | GLU:FRU (1:1 ratio) | ~144 | 204 min | 7.8% (vs. GLU) | |

**Note:** CHO, carbohydrate; FRU, fructose; GLU, glucose; HI, high intensity: LOW, low intensity; P, placebo; TT, time trial; SUC, sucrose. [a]No glucose-alone treatment

### 2.3.4.4 Dose-response effects of carbohydrate ingestion

The aforementioned sections have outlined the well-accepted ergogenic effects of CHO feeding upon endurance performance and/or capacity. However, for many years researchers have been trying to identify the optimal ingestion rates to elicit the greatest improvements in performance and the results from dose-response studies have found somewhat contradictory results, as can be seen in Table 2.5. Accordingly, an early study by Mitchel *et al.* (1988), reported that CHO intakes of 34, 39 and 50 $g \cdot h^{-1}$ during 2-h intermittent cycling resulted in similar times to complete an isokinetic TT lasting ~12-min at the end of the exercise protocol. In a further study by the same group which examined higher doses of CHO ingestion rates, a dose of 74 $g \cdot h^{-1}$ improved isokinetic TT performance following a 105-min constant intensity pre-load whilst 37 or 111 $g \cdot h^{-1}$ had equivocal results to the placebo trial (Mitchell *et al.*, 1989). Furthermore, there was no difference in muscle glycogen utilisation rates between the 0 or 74 $g \cdot h^{-1}$ despite the improvement in performance. In a study by Murray *et al.* (1991) the ingestion of glucose at a rate of 26 and 78 $g \cdot h^{-1}$ significantly increased 4.8-km time trial performance which was preceded by a 2-h intermittent cycling protocol at 65-75% $\dot{V}O_{2max}$ compared with a placebo, whilst the ingestion of 52 $g \cdot h^{-1}$ had no performance benefit.

The first study that measured exogenous CHO oxidation and exercise performance simultaneously found no difference in performance between ~20 (2% solution), ~60 (6% solution) and ~120 (12% solution) $g \cdot h^{-1}$ when subjects were required to cycle at 80% $\dot{V}O_{2max}$ to exhaustion in the cold (~10°C) (Galloway *et al.*, 2001). Interestingly, the 2% CHO solution resulted in reduced oxidation of exogenous glucose compared to the 6 and 12% solutions in the cold, but this had no subsequent effect upon cycling capacity (Galloway *et al.*, 2001). However, Smith *et al.* (2010) found in ambient conditions, that exogenous oxidation rates increased with dose when 15, 30 and 60 $g \cdot h^{-1}$ were consumed during 120-min of cycling at 77% $\dot{V}O_{2max}$. The authors also reported evidence for a dose-response effect upon power output during the 20-km time trial following the preload, as determined by meaningful change. Indeed, 30 $g \cdot h^{-1}$ was *very unlikely* to further improve performance over 15 $g \cdot h^{-1}$ while 60 $g \cdot h^{-1}$ was *likely* to improve performance over 30 $g \cdot h^{-1}$ with a mean percentage improvement of 2.3%. However, there was no statistical difference in power outputs between CHO ingestion rates. In addition, more recent work by Newell *et al.* (2015) reported no further benefit of CHO ingestion beyond 39 $g \cdot h^{-1}$ of glucose intake compared to a control solution, when 20, 39 and 64 $g \cdot h^{-1}$ were examined in trained cyclists completing 120-min of cycling at 95% lactate

threshold followed by a work matched simulate time trial lasting ~30-min. Additionally, there was no further performance benefit between CHO ingestion rates. The authors further concluded through dual glucose techniques, indirect calorimetry and blood analyses, that increasing rates of CHO ingestion, especially with 39 and 64 $g \cdot h^{-1}$ altered substrate utilisation by a greater preservation of endogenous glycogen stores, most likely hepatic stores and resulted in subsequent higher CHO oxidation rates with a blunting of plasma NEFA circulation to exercise. Moreover, there was a lack of any additional change in many metabolic parameters when consuming 64 $g \cdot h^{-1}$ compared to 39 $g \cdot h^{-1}$ whilst 20 $g \cdot h^{-1}$ was insufficient for these particular metabolic changes to occur.

In the most comprehensive investigation of CHO doses, Smith *et al.* (2013) used a large-scale multi-centre study examined 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110 and 120 $g \cdot h^{-1}$ with four doses being tested in each site. The CHO given was a 1:1:1 glucose, maltodextrin and fructose blend and endurance-trained cyclists or triathletes were required to undertake a 2-h constant load ride at ~71% $\dot{V}O_{2max}$ followed by a 20-km time trial. The findings of the study indicated an incremental performance improvement of 1.0, 2.0, 3.0, 4.0 and 4.7% improvement at 9, 19, 31, 48 and 78 $g \cdot h^{-1}$, with diminishing performance enhancement seen at CHO levels beyond 78 $g \cdot h^{-1}$. Following statistical modelling, the authors therefore identified 78 $g \cdot h^{-1}$ as the optimal dose but reported only a small 1.7% improvement in performance from 30 to 80 $g \cdot h^{-1}$ and a 0.7% improvement from 40 to 80 $g \cdot h^{-1}$. However, the linear regression model used to determine the optimal dose was not significant.

When specifically higher doses of CHO ingestion rates have been examined, King *et al.* (2018) observed no linear dose-response relationship effect as the ingestion of 90 $g \cdot h^{-1}$ of a glucose-fructose solution resulted in the highest mean power output during a 30-minute self-paced TT preceded by 120-min of cycling at 77% $\dot{V}O_{2max}$ compared to 60 and 75 $g \cdot h^{-1}$ of a glucose only solution and a 112.5 $g \cdot h^{-1}$ glucose-fructose solution. When the same group extended exercise duration beyond 3-h (King *et al.*, 2019) they observed similar results in that the ingestion of 90 $g \cdot h^{-1}$ of CHO in the form of 2:1 glucose and fructose increased power output during a 30-minute self-paced TT (preceded by 180-min at 60% $\dot{V}O_{2max}$) by 6.8% compared with 100 $g \cdot h^{-1}$ and 4.0% compared to 80 $g \cdot h^{-1}$, however this was not statistically significant. In both studies, they observed no significant difference in performance between CHO doses. The authors also suggested a potential "over-dose" effect of intestinal CHO transporters when 112.5 $g \cdot h^{-1}$ glucose-fructose solution was consumed, and there was an increased reliance on liver and

muscle glycogen with this dose, with no further oxidation of exogenous CHO (King *et al.*, 2018 and 2019). Such discrepancies in findings between the above-mentioned studies will most notably be due to the differences in exercise duration and intensity, the CHO dose ingested, the source of CHO, the format of CHO, pre-exercise nutritional status and the training status of subjects. Additionally, the performance measures used within these studies many not be sensitive enough to measure potentially small differences between varying doses.

**Table 2.5** Dose-response effects of carbohydrate ingestion during endurance exercise on exercise performance or capacity.

| Study | Training status of subjects ($\dot{V}O_{2max}$) | Pre-exercise nutritional status | Exercise and performance description/duration | CHO type: study-dependent variable | Mean CHO intake rate ($g \cdot h^{-1}$) | Performance effect vs. control trial | % Difference (compared with control unless otherwise stated) | Significant performance effect? | Dose-response effect? |
|---|---|---|---|---|---|---|---|---|---|
| Flynn *et al.* (1987) | Well trained cyclists (64 $ml \cdot kg \cdot min^{-1}$) (n = 8; male) | 500 $g \cdot day^{-1}$ CHO 48 hrs before trial. Began trial after overnight fast | Performed 120-min cycling with the aim of completing as much work as possible within that time ($x10^{-1}$ Nm) | Consumed 150 mL of water; MD & GLU (MG); MD & FRU (MF); MD & HFRU Corn Syrup (MHF) immediately prior and at 20-min intervals throughout exercise | Water: 0 | 1,340,241 Nm (avg W: 186) | - | No | No |
| | | | | | MG: 22.5 | 1,285,318 Nm (avg W: 178) | -4% vs. Water | | |
| | | | | | MF: 45 | 1,326,408 Nm (avg W: 184) | -1% vs. Water | | |
| | | | | | MHF: 45 | 1,345,593 Nm (avg W: 187) | ~0% vs. Water | | |
| Galloway *et al.* (2001) | Healthy male recreational subjects (~57 $ml \cdot kg \cdot min^{-1}$) (n = 6; male) | Began trial after overnight fast | Performed cycling at ~80% $\dot{V}O_{2max}$ to exhaustion (performed in the cold, 10°C) | Consumed bolus immediately before and every 10-min during exercise, 2% GLU; 6% GLU; 12% GLU; vs. P | P: - | 83.52-min | - | No difference in exercise capacity | No |
| | | | | | 2% GLU: ~20 | 103.19-min | ~23.6% vs. P | | |
| | | | | | 6% GLU: ~60 | 100.37-min | ~20.2% vs. P | | |
| | | | | | 12% GLU: ~120 | 94.76-min | ~13.5% vs. P | | |
| King *et al.* (2018) | Trained cyclists (61.6 $ml \cdot kg \cdot min^{-1}$) (n = 10; male) | Instructed to repeat the same diet in the 48-h period before each experimental trial. Began trials after 12-h overnight fast | Completed 120-min cycling at 77% $\dot{V}O_{2max}$ followed by a 30-min self-paced TT | Ingested 250 ml of one of five drinks solutions every 15-min (starting at minute 15) | P: 0 g/h | 187 w (avg power) | - | Yes (60, 90 and 112.5 g/h produced higher power output than 0 g/h) No difference between other comparisons | No |
| | | | | | LG: 60 | 206 w (avg power) | 11.2% vs. P | | |
| | | | | | HG: 75 | 196 w (avg power) | 5.1% vs. P | | |
| | | | | | LGF: 90 | 225 w (avg power) | 21.1% vs. P | | |
| | | | | | HGF: 112.5 | 213 w (avg power) | 14.9% vs. P | | |
| King *et al.* (2019) | Trained cyclists (60.0 $ml \cdot kg \cdot min^{-1}$) (n = 11; male) | Instructed to repeat the same diet in the 48-h period before each experimental trial. Began trials after 12-h overnight fast | Completed 180-min cycling at 60% $\dot{V}O_{2max}$ followed by a 30-min self-paced TT | Ingested 250 ml of one of four drinks solutions (2:1 GLU:FRU) every 15-min (starting at minute 15) | P: 0 | 186 w (avg power) | - | Yes (80, 90 and 100 g/h produced higher power output than 0 g/h) No difference between other comparisons | No |
| | | | | | 80 | 219 w (avg power) | ~17.7% vs. P | | |
| | | | | | 90 | 228 w (avg power) | ~22.6% vs. P | | |
| | | | | | 100 | 212 w (avg power) | ~14.0% vs. P | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Maughan *et al.* (1996) | Recreationally trained for endurance (n = 12; male) | Began trial after overnight fast | Four repeated trials of running at 70% $\dot{V}O_{2max}$ to exhaustion | Consumed 100 ml prior to exercise and every 20-min during exercise, 2.3% GLU (LGLU); 3.1% GLU (HGLU); vs. P | P: 0<br>LGLU: 16<br>HGLU: 22 | 93-min<br>110-min<br>107-min | -<br>18.3% vs. P<br>15.1% vs. P | Yes | No |
| Mitchell *et al.* (1988) | Endurance trained cyclists (~60 ml·kg·min$^{-1}$) (n = 8; male) | 400 g CHO 24 hrs before trial. 116 g CHO breakfast pre-exercise | Intermittent cycling protocol – 84-min (7 x 12 min bouts) of cycling at 70% $\dot{V}O_{2max}$ followed by 12-min TT | WP<br>5% CHO (CHO5)<br>6% CHO (CHO6)<br>7.5% CHO (CHO7.5) | WP: 0<br>33.5<br>39.4<br>50.1 | 1.83 Nm<br>1.98 Nm<br>1.96 Nm<br>2.05 Nm | -<br>7.1% vs. WP<br>6.6% vs. WP<br>7.1% vs. WP | Yes<br>(All CHO trials greater than WP) | No |
| Mitchell *et al.* (1989) | Endurance trained cyclists (~63 ml·kg·min$^{-1}$) (n = 10; male) | - | 105-min of cycling at 70% $\dot{V}O_{2max}$ followed by 15-min TT | Placebo (P)<br>6% CHO<br>12% CHO<br>18% CHO | 0<br>37<br>74<br>111 | 201 kJ<br>213 kJ<br>228kJ<br>217 kJ | -<br>6% vs. P<br>13.4% vs. P<br>8.0% vs. P | Yes (12% CHO vs. P); no for rest of comparisons | No |
| Murray *et al.* (1989b) | Moderately trained (~42.8 ml·kg·min$^{-1}$) (n= 7 males & 5 females) | Subjects had standard breakfast and lunch meal before experimental trials in the evening | 3 × 20-min cycling at 65% $\dot{V}O_{2max}$ with 5-min rest followed by a ~13-min TT sprint | Water Placebo<br>6% SUC<br>8% SUC<br>10% SUC | 0<br>31<br>41<br>52 | 13.62-min<br>13.03-min<br>13.30-min<br>13.57-min | -<br>4.5% vs. WP<br>2.4% vs. WP<br>0.4% vs. WP | Yes (31 g/h (6% SUC vs. 52 g/h (10% SUC) and WP) no for rest of comparisons. | No |
| Murray *et al.* (1991) | Healthy adults (~48 ml·kg·min$^{-1}$ (n= 8 males & 2 females) | ~1.8 g·kg$^{-1}$ CHO pre-exercise meal | 120-min of variable intensity cycling at 50, 65 and 75% $\dot{V}O_{2max}$ followed by 4.8-km TT (performed in the cold 10°C) | Placebo (P)<br>6% GLU<br>12% GLU<br>18% GLU | 0<br>26<br>52<br>78 | ~505 s<br>~475 s<br>~485 s<br>~475 s | -<br>~5.9% vs. P<br>~4.0% vs. P<br>~5.9% vs. P | Yes (26 (6% CHO) and 78 g/h (18% CHO) better than P) | No |
| Newell *et al.* (2015) | Trained cyclists (62 ml·kg·min$^{-1}$ (n = 20; male) | Commenced each trial after an overnight fast | 120-min of cycling at 95% LT followed by the time to complete a work matched simulated TT (~30-min) | 0%<br>2.0% GLU<br>3.9% GLU<br>6.4% GLU | 0<br>20<br>39<br>64 | 37:01.9-min<br>35:17.6-min<br>34:19.5-min<br>34:11.4-min | -<br>3.7% vs. P<br>6.1% vs. P<br>7.0% vs. P | Yes (39 and 64 g/h increased performance more than 0g/h) but no difference between any other conditions | No |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Smith *et al.* (2010) | Recreationally trained (~55 ml·kg·min$^{-1}$) (n = 12; male) | Commenced each trial after 10-h overnight fast | 120-min of cycling at 95% OBLA / 77% $\dot{V}O_{2max}$ followed by a 20-km TT | Placebo (P) vs. 3 CHO doses as 250-mL GLU solution every 15-min during exercise | Placebo (P)<br>15<br>30<br>60 | 36.4-min<br>35.2-min<br>35.0-min<br>34.7-min | -<br>3.4% vs. P<br>4.0% vs. P<br>4.8% vs. P | Yes (all trials vs. P) | Yes |
| Smith *et al.* (2013) | Endurance trained (~59.1 ml·kg·min$^{-1}$) (n = 51; male) | Commenced each trial after 10-h overnight fast | 120-min of cycling at 95% / ~71% $\dot{V}O_{2max}$ OBLA followed by a 20-km TT | 0%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11% and 12% (1:1:1 GLU-FRU-MD) CHO solution consumed during the 2-h constant load ride at a fluid intake rate of 1 L/H. | 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110 and 120 g/h | Raw data not provided | 9 g·h$^{-1}$ vs. P: 1.0% increase<br>19 g·h$^{-1}$ vs. P: 2.0% increase<br>31 g·h$^{-1}$ vs. P: 3.0% increase<br>48 g·h$^{-1}$ vs. P: 4.0% increase<br>78 g·h$^{-1}$ vs. P: 4.7% increase<br>108 g·h$^{-1}$ vs. P: 4.0% increase<br>120 g·h$^{-1}$ vs. P: 3.3% increase | Yes | No |
| Watson *et al.* (2012) | Recreationally trained (~54 ml·kg·min$^{-1}$) (n = 24; male) | Subjects asked to replicate same diet 24-h before each trial and undertook each trial after overnight fast | 2 groups of 12 subjects cycling at 70% $\dot{V}O_{2max}$ until exhaustion in cool (n=12; 10°C) or 60% $\dot{V}O_{2max}$ until exhaustion in cool (n=12; 30°C) | P vs. 2%, 4%, or 6% solutions (SUC, GLU, and FRU in a ratio of 50:25:25) at the start and every 10-min during exercise | *COOL*<br>Placebo<br>~15<br>~31<br>~46 | *COOL*<br>102.6-min<br>109.2-min<br>121.0-min<br>122.4-min | *COOL*<br>-<br>6.4% vs. P<br>17.9% vs. P<br>19.3% vs. P | *COOL*<br>Yes (~31 and ~46 g/h vs. P). | No |
| | | | | | *WARM*<br>Placebo<br>~16<br>~33<br>~50 | *WARM*<br>94.5-min<br>104.1-min<br>105.5-min<br>112.0-min | *WARM*<br>-<br>10.2% vs. P<br>11.6% vs. P<br>18.5% vs. P | *WARM*<br>Yes (~50 g/h vs. P). | No |

**Note:** TT, time trial; OBLA, onset blood lactate accumulation; CHO, carbohydrate; FRU, fructose; GLU, glucose; MD: maltodextrin; LG, low glucose; HG, high glucose; LGF, low glucose fructose; HGF, high glucose fructose; SUC, sucrose; WP, water placebo.

**Table 2.6** Effect of carbohydrate feeding during endurance-based exercise on whole muscle glycogen utilisation.

| Study Reference | Training status of subjects ($\dot{V}O_{2max}$) | Exercise modality | Pre-exercise feeding status | Duration of exercise | Intensity of exercise | Muscle sampled | Type and amount of CHO | Pre-exercise muscle glycogen (mmol·kg dm$^{-1}$) | Post-exercise muscle glycogen (mmol·kg dm$^{-1}$) | Δ Muscle glycogen utilisation (mmol·kg dm$^{-1}$) | Muscle glycogen utilisation between conditions |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Arkinstall *et al*. (2001) | Moderately-trained (53 ml·kg·min$^{-1}$) (n = 7) | Constant-intensity cycling | ~6 g·kg$^{-1}$ CHO 24 hrs before and undertook trial after an overnight fast | 60-min | Lactate threshold* 70% $\dot{V}O_{2max}$ | VL | G: 64 ± 3 g·h$^{-1}$<br>Placebo | 445 ±43<br>457 ± 46 | 223 ± 39<br>237 ± 27 | 227 ± 36<br>216 ± 39 | Similar |
| Arkinstall *et al*. (2001) | Moderately-trained (53 ml·kg·min$^{-1}$) (n = 7) | Constant-intensity running | ~6 g·kg$^{-1}$ CHO 24 hrs before and undertook trial after an overnight fast | 60-min | Lactate threshold* 70% $\dot{V}O_{2max}$ | VL | G: 64 ± 3 g·h$^{-1}$<br>Placebo | 480 ± 45<br>451 ± 38 | 368 ± 20<br>220 ± 38 | 112 ± 32<br>141 ± 34 | Similar |
| Bosch *et al.* (1994) | Endurance-trained cyclists (49 and 54 ml·kg·min$^{-1}$) (n = 14) | Constant-intensity cycling | ~600 g·day$^{-1}$ CHO 3 days prior and 70 g CHO pre-exercise meal | 180-min | 70% $\dot{V}O_{2max}$ | VL | GP: 50 g·h$^{-1}$<br>Placebo | 921 ± 65<br>843 ± 22 | 320 ± 54<br>256 ± 108 | ~600<br>~600 | Similar |
| Bosch *et al.* (1996) | Moderately-trained (49 and 47 ml·kg·min$^{-1}$) (n = 14) | Constant-intensity cycling | ~600 g·day$^{-1}$ CHO 3 days prior and 70 g CHO pre-exercise meal<br>No CHO load and 70 g CHO pre-exercise meal | 180-min | 70% $\dot{V}O_{2max}$ | VL | Placebo<br>GP: 50 g·h$^{-1}$ | 842 ± 17<br>535 ± 32 | 261 ± 31<br>261 ± 42 | ~580<br>~275 | *Intended different initial starting glycogen so difficult to ascertain differences* |
| Bosch *et al.* (1996) | Moderately-trained (47 and 53 ml·kg·min$^{-1}$) (n = 17) | Constant-intensity cycling | 70 g CHO pre-exercise meal | 180-min | 70% $\dot{V}O_{2max}$ | VL | GP: 50 g·h$^{-1}$<br>Placebo | 579 ± 19<br>535 ± 30 | 261 ± 40<br>122 ± 43 | ~320<br>~410 | Decreased |
| | | | | 120-min | | VL | MD + F: 60 g·h$^{-1}$ | ~425 ± 100 | ~200 ± 100 | ~225 ± 125 | Similar |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Clark *et al.* (2019) | Competitive athletes (52.5 ml·kg·min$^{-1}$) (n = 14) | Heavy-intensity cycling | Undertaken after overnight fast | | Power output at the GET plus 25% of the difference between the GET and C-EP | | Placebo | ~400 ± 120 | ~150 ± 75 | ~250 ± 125 | |
| Coyle *et al.* (1986) – Part 1 | Endurance-trained cyclists (~65 ml·kg·min$^{-1}$) (n = 5) | Constant-intensity cycling | Undertaken after 16-h fast | 105-min | 71% $\dot{V}O_{2max}$ | VL | GP: 100 g·h$^{-1}$<br>Placebo | 525 ± 37<br>507 ± 33 | 183 ± 36<br>183 ± 16 | 337 ± 31<br>324 ± 41 | Similar |
| Coyle *et al.* (1986) – Part 2 | Endurance-trained cyclists (~70 ml·kg·min$^{-1}$) (n = 7) | Constant-intensity cycling | Undertaken after 16-h fast | Time matched biopsy at: ~180-min | 71% $\dot{V}O_{2max}$ | VL | GP: ~114 g·h$^{-1}$<br>Placebo | 753 ± 48<br>709 ± 57 | 191 ± 52<br>161 ± 22 | ~560<br>~550 | Similar |
| De Bock *et al.* (2007) | Physically active (n = 8) | Constant-intensity cycling | Provided with ~6 g·kg·day$^{-1}$ CHO (3 days before)<br>2 g·kg$^{-1}$ CHO and pre-exercise meal<br>~6 g·kg·day$^{-1}$ CHO (3 days before) and fasted | 120-min | ~75% $\dot{V}O_{2max}$ | VL | MD: 75 g·h$^{-1}$<br>Placebo | 455 ± 40<br>466 ± 19 | 206 ± 31<br>111 ± 24 | 111 ± 24 (per hr)<br>149 ± 25 (per hr) | Similar |
| Hargreaves & Briggs (1988) | Competitive cyclists (~68 ml·kg·min$^{-1}$) (n = 5) | Constant-intensity cycling | Undertaken after overnight fast | 120-min | 70% $\dot{V}O_{2max}$ | VL | GP: 60 g·h$^{-1}$<br>Placebo | 774 ± 63<br>717 ± 44 | 501 ± 22<br>470 ± 11 | 273 ± 44<br>248 ± 44 | Similar |
| McConell *et al.* (2000) | Well-trained cyclists (66 ml·kg·min$^{-1}$) (n = 13) | Constant-intensity cycling | Provided with ~8 g g·kg$^{-1}$ kg CHO 24 hrs before and undertaken the trial after an overnight/prolonged fast | TTE: 68-min<br>TTE: 69-min | 83 ± 2% $\dot{V}O_{2peak}$ | VL | G: ~81 g·h$^{-1}$<br>Placebo | 567 ± 25<br>514 ± 46 | 201 ± 13<br>222 ± 33 | 366 ± 16<br>292 ± 42 | Similar |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Mitchell *et al*. (1989) | Highly trained cyclists (~63 ml·kg·min$^{-1}$) (n = 10) | Constant-intensity OR Intermittent cycling | - | 105-min (biopsy taken) followed by performance test | 70% $\dot{V}O_{2max}$ + all-out for 15-min | VL | GP + F: 74 g·h$^{-1}$<br>GP + F: 74 g·h$^{-1}$<br>Placebo | 572 ± 4<br>578 ± 10<br>594 ± 6 | 194 ± 25<br>242 ± 30<br>231 ± 21 | 377 ± 26<br>328 ± 34<br>363 ± 24 | Similar |
| Erickson *et al*. (1987) | Competitive cyclists (65.2 ml·kg·min$^{-1}$) (n = 5) | Constant-intensity cycling | Undertaken after overnight fast | 90-min | 65 - 70% $\dot{V}O_{2max}$ | VL | G: 45 g·h$^{-1}$<br>Placebo | 598 ± 59<br>657 ± 84 | 330 ± 61<br>262 ± 77 | 267 ± 23<br>395 ± 43 | Decreased |
| Yaspelkis *et al*. (1991) | Competitive cyclists (65.8 ml·kg·min$^{-1}$) (n = 12) | Constant-intensity cycling | Undertaken after overnight fast | 120-min in the heat | 49% $\dot{V}O_{2max}$ | VL | MD + F: 75 g·h$^{-1}$<br>Placebo | 608 ± 32<br>693 ± 53 | 402 ± 33<br>350 ± 26 | 207 ± 24<br>343 ± 42 | Decreased |
| Flynn *et al.* (1987) | Well trained cyclists (64 ml·kg·min$^{-1}$) (n = 8) | Constant-intensity cycling | 500 g g·day$^{-1}$ CHO 48 hrs before trial. Began trial after overnight fast | 120-min (biopsy at 90-min) | 55% $\dot{V}O_{2max}$ (total work: 0.9 x10$^{-1}$ Nm) | VL | MHF: 45 g·h$^{-1}$<br>MF: 45 g·h$^{-1}$<br>MG: 22.5 g·h$^{-1}$<br>Placebo | 835 ± 66<br>793 ± 31<br>824 ± 40<br>777 ± 47 | 386 ± 40<br>430 ± 59<br>409 ± 38<br>391 ± 28 | 454 ± 13<br>359 ± 14<br>415 ± 10<br>398 ± 12 | Similar |
| Yaspelkis *et al*. (1993) | Competitive cyclists (n = 7, male) | Intermittent cycling | Undertaken after overnight fast<br>Undertaken after overnight fast | 190-min | 45 & 75% $\dot{V}O_{2max}$ | VL | GP: ~75 g·h$^{-1}$<br>Placebo | ~540<br>~540 | ~346<br>~260 | ~195<br>~280 | Decreased |
| Hargreaves *et al*. (1984) | Trained (62 ml·kg·min$^{-1}$) (n = 10) | Intermittent cycling | Undertaken after overnight fast | 240-min | 50 & 100% $\dot{V}O_{2max}$ | VL | S: 43 g·h$^{-1}$<br>Placebo | 559 ± 54<br>623 ± 45 | 124 ± 37<br>77 ± 35 | 256 ± 30<br>404 ± 35 | Decreased |
| Fielding *et al*. (1985) | Trained (50 ml·kg·min$^{-1}$) (n = 9) | Intermittent cycling | Undertaken after overnight fast | 240-min | 50 & 100% $\dot{V}O_{2max}$ | VL | Dose; S: 21.5 g·h$^{-1}$<br>Frequency; S: 21.5 g·h$^{-1}$<br>Placebo | 479 ± 26<br>519 ± 26<br>462 ± 26 | 120 ± 19<br>199 ± 54<br>118 ± 31.14 | 359 ± 29<br>321 ± 53<br>350 ± 30 | Similar |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Foskett *et al.* (2008) | Recreational games players (60 ml·kg·min$^{-1}$) (n = 6) | Intermittent running | ~10 g·kg·day$^{-1}$ 48 hrs before trial. Began trial after overnight fast | CHO: 158 ± 28 min PLA: 131 ± 20 min *Time matched Biopsy: 90-min | 55 - 95% $\dot{V}O_{2max}$ + all out sprints | VL | MD: ~90 g·h$^{-1}$<br>Placebo | ~530<br>~510 | ~350<br>~370 | ~200<br>~150 | Similar |
| Tsintzas *et al*. (1995) | Recreational runners (54.5 ml·kg·min$^{-1}$) (n = 7) | Constant-intensity running | Undertaken after overnight fast | 60-min | 72% $\dot{V}O_{2max}$ | VL | GP+F+G: 50 g·h$^{-1}$<br>Placebo | 344 ± 27<br>342 ± 21 | 235 ± 33<br>191 ± 21 | 109 ± 16<br>151 ± 20 | Decreased |
| Tsintzas *et al*. (1996) | Recreational runners (61.8 ml·kg·min$^{-1}$) (n = 8) | Constant-intensity running | Undertaken after overnight fast | 100-min | 76% $\dot{V}O_{2max}$ | VL | GP+F+G: 45 g·h$^{-1}$<br>Placebo | 382 ± 20<br>396 ± 30 | 126 ± 22<br>60 ± 8 | ~256<br>~336 | Decreased |
| Nicholas *et al*. (1994) | Recreational games players (n = 6) | Intermittent running | Undertaken after overnight fast | 90-min | 55 - 95% $\dot{V}O_{2max}$ + all out sprints | VL | GP+G+F: ~47 g·h$^{-1}$<br>Placebo | 363 ± 27<br>405 ± 23 | 170 ± 23.6<br>160 ± 15 | ~193<br>~245 | Decreased |

**Note:** CHO, carbohydrate; FRU, fructose; GLU, glucose; MD: maltodextrin; GP, glucose polymer; VL, vastus lateralis.

## 2.4 Factors affecting the efficacy of carbohydrate supplementation upon performance and metabolism

Although the well-documented metabolic and performance effects of exogenous CHO provision during exercise have been detailed in preceding sections, the varying research designs applied within the literature are often limited in their translation potential to the elite sporting environment. Indeed, previous exercise protocols have typically been less than 180-min in duration for cycling-based exercise and less than 120-min for running based-exercise consisting of varying intensities and involving different performance tests. These exercise protocols are perhaps somewhat limited given typical endurance cycling events last >3 h in duration whilst certain endurance running events are >2-h in duration and ran at higher exercising intensities. In addition, many of studies examining CHO ingestion have also loosely controlled pre-exercise nutritional status and subjects have further commenced exercise after an overnight fast. Although this may be intended to better understand the metabolic association of CHO ingestion, this will in turn limit the 'true' magnitude of any associated performance effect. Furthermore, it is uncommon for athletes to typically begin competition after an over-night fast as they will indeed consume high-CHO both in the day(s) and hours before competition in order to promote the well-established performance effects. Moreover, the feeding strategies often employed within these exercise protocols are often comprised of CHO drinks *per se* as opposed to the feeding strategies typical of elite endurance athletes who will consume a mixture of drinks, solids and gels during both competition and certain training scenarios.

### 2.4.1 Initial feeding status

The initial feeding status of participants before and during exercise is well known to significantly alter metabolic responses to exercise (Horowitz *et al.*, 1997) in turn influencing the mechanistic underpinning of an experimental intervention. In this regard, exercise performance has also been found to be more pronounced with CHO feeding and mouth rinsing when exercise is commenced with low muscle glycogen availability and in the absence of a pre-exercise meal (Lane *et al.*, 2013; Widrick *et al.*, 1993). Additionally, there is an interaction between intake of CHO before and during prolonged exercise in terms of the effect of muscle CHO availability on performance as time to exhaustion was found to be 44%, 32% and 18% greater with CHO feeding before and during exercise, CHO feeding during exercise only and

pre-exercise CHO feeding only compared to no CHO intake before or during exercise (Wright *et al.*, 1991).

In addition, liver glycogen has been well documented to be influenced by diurnal variation and has been found to be significantly reduced following an overnight fast whilst muscle glycogen seems relatively unaffected (Iwayama *et al.*, 2020). This is an important consideration during exercise trials as liver glycogen provides an important energy substrate during prolonged endurance exercise (Gonzalez *et al.*, 2015) and subjects may produce more prominent sparing effects of liver glycogen during exercise after an overnight fast. Therefore, consuming CHO in the hours before a performance trial restores liver glycogen after an overnight fast.

Moreover, Table 2.6 also highlights the varying muscle glycogen concentrations between studies prior to experimental trials. Although, many of these studies have perhaps not intended to maximise muscle glycogen stores prior to exercise, this should be an important consideration from both a mechanistic and performance outcome. Indeed, greater muscle glycogen availability has been demonstrated to influence fuel metabolism over and above that of exercise intensity (Arkinstall *et al.*, 2004) and muscle glycogen use during exercise is also directly related to the pre-exercise muscle glycogen level (Hargreaves *et al.*, 1995). In relation to exercise performance, the linear relationship between pre-exercise muscle glycogen availability and endurance exercise capacity (Bergström *et al.*, 1967) could underpin the importance of appropriate muscle glycogen levels prior to prolonged endurance exercise protocols. Limited studies have achieved "supercompensated" or maximal muscle glycogen levels prior to prolonged cycling protocols utilising exogenous CHO provision (see Table 2.6) which can reduce the true magnitude and translation potential of any findings as it is uncommon for elite endurance athletes to undertake competition without an appropriate prior competition CHO load.

Collectively, strict dietary controls through pre-exercise feeding status and muscle glycogen availability prior to examining exogenous CHO provision during endurance-based exercise is an important consideration to minimise the difference in nutritional status between and within subjects and to further isolate the true effects of CHO ingestion during exercise. Such controls can promote the direct relevance of any findings to the elite sporting environment through the incorporation of dietary protocols that are a true reflection of the dietary habits of elite

endurance athletes prior to competition or training whilst ensuring the outcome of interest is isolated from potentially confounding variables.

### 2.4.2 Carbohydrate intake rate

A varying range of CHO ingestion rates have been examined amongst the literature (Stellingwerff & Cox, 2014) with Smith *et al.* (2013) demonstrating in their dose-response investigation that as little as 10 $g \cdot h^{-1}$ of CHO induced a 1% improvement in 20-km time trial performance (preceded by a 2-h pre-load) compared to a placebo whilst the optimal dose was suggested to be around 70 – 80 $g \cdot h^{-1}$ with no further benefits up to 120 $g \cdot h^{-1}$ . The dose-response relationship of CHO ingestion and performance has been detailed in Section 2.3.4.4 and Table 2.5. Accordingly, current sport nutrition guidelines from the American College of Sports Medicine (ACSM) recommend CHO ingestion at a rate of small amounts or CHO mouth rinse during exercise <60 mins in duration, 30-60 $g \cdot h^{-1}$ during 1-2.5-h of exercise and 90 $g \cdot h^{-1}$ during >2.5-h of exercise (Jeukendrup, 2014; Thomas *et al.*, 2016).

### 2.4.3 Carbohydrate type and blend

Both the type and blend of CHO has been found to be an important consideration when examining the metabolic and performance efficacy of exogenous CHO provision, given that different types of CHO in isolation and combination are oxidised at varying rates during prolonged exercise (Jeukendrup, 2010). Indeed, the rate of gastric emptying, intestinal digestion, intestinal absorption and hepatic metabolism of different carbohydrates may modulate their availability to skeletal muscle to be oxidised during exercise. In this regard, glucose and glucose polymers have been found to be oxidised at maximal rates of up to 1.0 – 1.1 $g \cdot min^{-1}$ during exercise, even when higher amounts of glucose have been ingested and both fructose and galactose have been found to be oxidised at a rate almost 50% lower than glucose at 0.6 $g \cdot min^{-1}$ (Jeuekndrup & Jentjens, 2000). The rate of exogenous glucose oxidation is limited due to the maximal saturation of sodium dependent glucose transporters (SGLT1) which is how glucose is absorbed from the intestine via SGLT1 (Daniel & Zietek, 2015). Fructose is absorbed independently from glucose by a distinct class of CHO transporters, glucose transporter 5 (GLUT5) (Shi *et al.*, 1995). The combination of both glucose and fructose has been confirmed to further increase absorption and higher subsequent oxidation rates up to 1.8 $g \cdot min^{-1}$ compared to an equivalent amount of glucose alone through the saturation of both transporters (Jentjens *et al.*, 2004; Jeukendrup, 2014; Shi *et al.*, 1995; Wallis *et al.*, 2005).

Moreover, sucrose combines glucose and fructose, and has also been found to report higher exogenous CHO oxidation rates when combined with glucose compared to glucose alone, with similar oxidation rates to a glucose and fructose blend also being identified (Trommelen *et al.*, 2017). However, interesting findings by Hulston *et al.* (2009) demonstrated that higher exogenous CHO oxidation rates of glucose and fructose mixtures was only apparent when maximal glucose absorption rates are reached therefore demonstrating that CHO doses >60 $g \cdot h^{-1}$ may only benefit from glucose and fructose blends.

This apparent increase in exogenous CHO oxidation rates has also equated to an increase in prolonged endurance performance with 108 $g \cdot h^{-1}$ of a 2:1 glucose and fructose blend compared to an isocaloric glucose only blend improving ~1-h time trial performance after 2-h of cycling at 55% $W_{max}$ (Currell & Jeukendrup, 2008) and over 100-km of cycling with 37 $g \cdot h^{-1}$ of a 5% maltodextrin and 2% fructose blend (Triplett *et al.*, 2010). Similarly, both O'Brien and Rowlands (2011) and O'Brien *et al.* (2013) further reported improvements in performance with 108 $g \cdot h^{-1}$ of different glucose and fructose ratios over multiple sprints after an endurance pre-load. These findings further suggest that intake rates of greater than 60 – 70 $g \cdot h^{-1}$ of CHO are required to show the added benefit of using blends of CHO containing both glucose and fructose (see Table 2.4).

Moreover, the ratio of the CHO blend has also been found to vary amongst studies and it has also been previously demonstrated to affect exogenous CHO oxidation rates (O'Brien *et al.*, 2011). O'Brien and Rowlands (2011) examined the effects of different ratios of glucose and fructose on an incremental cycling test to exhaustion following 150-min at 50% $W_{max}$. The authors reported that a 1.25 to 1 and 0.8 to 1 ratio of glucose and fructose improved time to exhaustion compared to a placebo whilst a 2 to 1 ratio had no effect over the placebo. Further work by the same group determined that an ingested fructose:glucose ratio of 0.8-1.0:1 may provide the most practical benefit for endurance athletes (O'Brien *et al.*, 2013) (see Table 2.4).

Collectively, the current sport nutrition recommendations for exogenous CHO provision during exercise outlined in the preceding section have further identified that the 30-60 $g \cdot h^{-1}$ recommended intake during exercise lasting approximately 1-2-h should come from single source glucose or glucose polymers whilst recommended CHO ingestion rates of 90 $g \cdot h^{-1}$ during >2.5-h of exercise should come from multiple transportable carbohydrate sources like glucose and fructose (Thomas *et al.*, 2016). Therefore, the efficacy of higher amounts of CHO

ingestion during exercise protocols should be considered based of the CHO type and blend, especially during those exercise protocols employing exercise duration beyond 2.5-h.

### 2.4.4 Carbohydrate sources

Elite endurance athletes will typically consume CHO in the form of solids, gels and liquids during competition and specific training sessions (Pfeiffer *et al.,* 2012). However, the bulk of studies investigating exogenous CHO ingestion during exercise have used CHO drinks *per se* compared to a mixture of CHO sources which seems a more convenient method for athletes to ingest CHO in large amounts. Accordingly, a series of independent studies by Pfeiffer *et al.* assessed the exogenous CHO oxidation rates of a CHO gel and CHO solid bar compared to a CHO drink and reported that both the gel and solid induced similar high peak oxidation rates and oxidation efficiencies (Pfeiffer *et al.,* 2010a; Pfeiffer *et al.,* 2010b) compared to the drink during 180-min of cycling at ~59% $\dot{V}O_{2max}$. However, interestingly, oxidation rates were found to be 15% lower in the solid bar compared with the drink at 120, 135 and 150-min of exercise within that study. The CHO drink, solid and gel all consisted of glucose and fructose at a ratio of 2:1 and was ingested at a rate of 93 $g \cdot h^{-1}$ in the solid versus drink study and at 108 $g \cdot h^{-1}$ in the gel versus drink study.

In regards to performance differences, limited studies have assessed the performance effects of the ingestion of solid CHO but those which did collectively all found improvements in performance compared to a placebo (Campbell *et al.,* 2008; Flynn *et al.,* 1987; Hargreaves & Briggs, 1988) and similar improvements in performance, when the same amounts of CHO was ingested in the form of solids compared with liquids (Campbell *et al.,* 2008; Flynn *et al.,* 1987; Hargreaves & Briggs, 1988; Murdoch *et al.,* 1993). Moreover, studies investigating the effects of CHO gels upon performance are also scarce and Lee *et al.* (2014) found no difference in half-marathon performance between a glucose gel, glucose and fructose gel and a glucose drink delivering the same amount of CHO. However, more recent work by Guillochon and Rowlands (2017) compared the effects of a CHO drink, gel, bar or a mixture of all three formats during a time to exhaustion ramp cycling test preceded by 140-min of a race simulation. CHO was comprised of fructose and maltodextrin and was ingested at a rate of 80 $g \cdot h^{-1}$ during the 140-min race simulation. The main finding of this study was that mean peak power during the ramp to exhaustion was highest in the gel trial and least in the bar trial, with the ingestion of the bar likely to lower peak power by 4%. Such performance decrements were concluded to be likely

related to increases in GI discomfort and perceived exertion, relative to the drink, gel and mixture of formats whilst the mixture of formats likely increased nausea and stomach fullness relative to the gel but did not translate to a reduction in performance. Figure 2.10 therefore provides an overview of key recommendations for carbohydrate intake rate, carbohydrate type, and form over varying exercise durations and intensities (Stellingwerff & Cox, 2014).

### 2.4.5 Exercise protocol – intensity, duration and type

The laboratory exercise protocols which have been devised to study the ergogenic and mechanistic effects of CHO ingestion during exercise have largely employed exercising durations, intensities and performance assessments that are unreflective of typical endurance events. Firstly, the bulk of studies have used cycling modality exercise protocols that have required subjects to cycle for less than 3-h in duration (Stellingwerff & Cox, 2014) which is uncharacteristic of professional road cycling multi-day stage races by which stages typically last 3-5 h in duration (Saunders & Heijboer, 2019; Vogt *et al.*, 2007). Similarly, running studies have used exercise durations of less than ~120-min in duration, whereby prolonged endurance running like the marathon are often well beyond 2-h in duration. Additionally, research designs have involved a variety of exercise protocols, which most notably have incorporated either a constant or variable-intensity prolonged pre-load followed by a time to exhaustion test or a time trial comprised of a set distance or work completed. Other protocols have included a prolonged time to exhaustion test lasting up to 4-h at a set intensity whilst others have used a prolonged time trial up to 100-km involving variable intensity throughout in order to create a race simulation (Madsen *et al.*,1996).

Such protocols have also included varying intensities between studies during pre-loads and time to exhaustion tests along with different distances and set workloads to complete in time trials, with intensities often based of a percentage of $\dot{V}O_{2max}$ or $W_{max}$. More constant load protocols have involved intensities ranging from ~60 – 75% $\dot{V}O_{2max}$ and have enabled a controlled environment for comparisons of metabolic variables whilst variable-intensity protocols have included intermittent higher intensities throughout. Such characteristics may not be applicable to all professional cycling stages as multi-day stage races are often devised of flat, mountain and time trial stages. These previous characteristics seem to be more in line with flat stages which are often characterised by a sprint finish whilst mountain stages have been observed to mostly require submaximal, constant power outputs for longer periods than

flat stages alongside undertaking higher maximal mean power for longer durations than flat stages (Vogt *et al.*, 2007; Sanders *et al.*, 2018). Thus, specific laboratory-based exercise protocols should be devised line with the specific categorisation of a cycling stage.

With this in mind, many of the performance tests employed to assess the performance effect of exogenous CHO have used both time trials and time to exhaustion as previously stated. It is important to consider the efficacy of exogenous CHO ingestion based upon the specific research question being addressed (Hopkins, 2000). As such, both a time trial and time to exhaustion test could be considered as a valid measure of performance within road cycling given that both situations present themselves in the form of designated time-trial stages and the ability to respond to 'attacks' on mountain climbs, respectively. Furthermore, the prescription of the relative exercise intensity within these studies can also produce a confounding factor when attempting to standardise the physiological responses from the same relative intensity. Indeed, Baldwin *et al.* (2000) concluded that markers of exercise stress were greater in untrained subjects compared with trained subjects when the exercise was performed at 70% $\dot{V}O_{2peak}$. In contrast, the metabolic and cardiac stress in trained and untrained subjects were similar during exercise at 95% of lactate threshold. Therefore, selected work rate of between 60 and 80% $\dot{V}O_{2peak}$ may be inappropriate as a relative exercise intensity because it may be below the lactate threshold in some participants whilst above the lactate threshold in others. As such, lactate threshold may be a more appropriate method for prescribing exercise intensity.

Collectively, more studies are therefore required to promote the efficacy of the mechanistic and performance effects of CHO feeding as comprised from a mixture of CHO sources during exercise protocols which closely replicate the demands of endurance events. Additionally, there is a definitive requirement to ensure that subjects are commencing trials under conditions of best nutritional practice, whereby they are appropriately CHO loaded before the trial in combination with a high CHO pre-exercise meal.

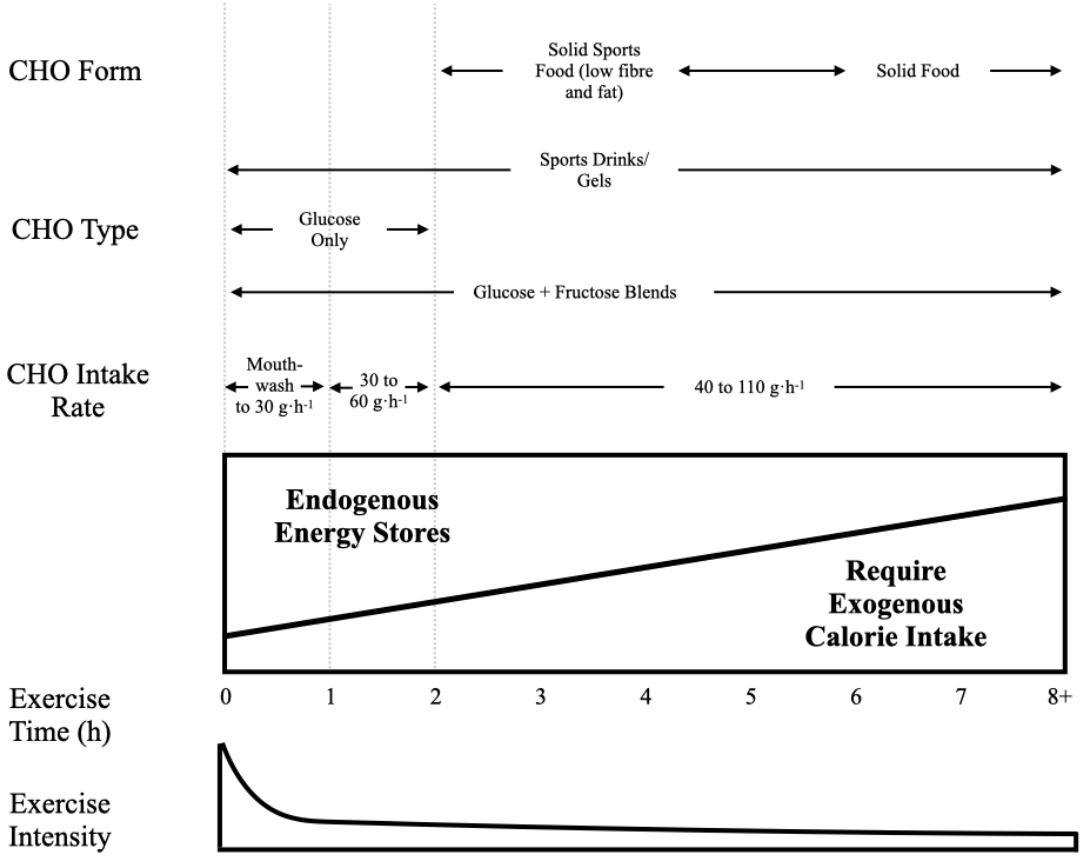


**Figure 2.10** Schematic overview of key recommendations for carbohydrate intake rate, carbohydrate type, and form over varying exercise durations and intensities (taken from Stellingwerff & Cox, 2014). CHO: carbohydrate.

### 2.4.6 Carbohydrate feeding in real world setting – athlete competition intake

Much of the recommendations for CHO intake during competition, as detailed in previous sections are largely based on laboratory research. Such recommendations have subsequently been used to inform the ACSM position statement on nutrition and athletic performance (Thomas *et al.*, 2016). However, the nutritional intake of elite endurance athletes during competition is scarce within the literature, and it is not clear whether athletes are able to match recommendations. Within professional road cycling, nutritional intake has been examined during both mutli-day stage races (Ebert *et al.*, 2007; García-Rovés *et al.*, 1998; Muros *et al.*, 2019; Pfeiffer *et al.*, 2012; Rehrer *et al.*, 2010; Ross *et al.*, 2014;Sànchez-Muñoz *et al.*, 2016; Saris *et al.*, 1989) and single-day "Spring Classic" races (Heikura *et al.*, 2019). The most recent observations by Muros *et al.* (2019) during the Vuelta a Esapaña Grand Tour reported the dietary intakes of the whole team (9 riders) throughout the 3-wk race. The average daily overall CHO intake was found to be 12.5 $g \cdot kg^{-1}$ with the distribution of absolute CHO intake between

meals consisting of 198.5 ± 43.0, 146.7 ± 33.0 and 146.2 ± 21.5 g at breakfast, lunch and dinner with an average of 90.8 $g \cdot h^{-1}$ being consumed during the stage. García-Rovés *et al.* (1998) and Sànchez-Muñoz *et al.* (2016) reported similar daily CHO intakes of 12.6 and 12.5 $g \cdot kg^{-1}$ during the Vuelta a España and Tour of Andalusia. Furthermore, earlier observations by Saris *et al.* (1989) during the Tour de France reported CHO intake of ~61% of total daily energy intake equating to ~900 $g \cdot day^{-1}$. Moreover, García-Rovés *et al.* (1998) reported the average CHO intake during the stages examined to be 25 $g \cdot h^{-1}$. In contrast, Saris *et al.* (1989) reported the average CHO intakes of five cyclists during the Tour de France to be as high as 94 $g \cdot h^{-1}$, similar to that of Muros *et al.*, (2019) and in line with ACSM guidelines of ~90 $g \cdot h^{-1}$ during exercise > 3-h. However, these studies highlight an important limitation of the current literature in that nutritional intakes have been described as an average across 3 weeks of racing, thereby ignoring the likely significant day-by-day variation or periodisation in nutrition across stages alongside the relative CHO intake distribution across meals. Nevertheless, interesting insights from Heikura *et al.* (2019) documented the CHO intake of six professional cyclists during a "Spring Classics" campaign which consist of 1-day races compared to the traditional multi-day cycling stage races. Such observations highlighted the slightly lower average daily total CHO intakes of 10.7 ± 1.3 $g \cdot kg^{-1}$ by these riders to those documented earlier. Furthermore, the average CHO intakes 3-h pre-race (3.4 ± 0.7 $g \cdot kg^{-1}$), during the race (51 ± 9 $g \cdot h^{-1}$) and 3-h post-race (1.6 ± 0.5 $g \cdot kg^{-1}$) were also described.

Pfeiffer *et al.* (2012) also aimed to quantify the habitual CHO intake of competitors in difference endurance-based events and found that the lowest mean CHO intake rates were reported during the measured marathon with 35 ± 26 $g \cdot h^{-1}$ being ingested. This was found to be significantly lower than intake rates during cycling events (53 ± 22 $g \cdot h^{-1}$) and triathlons (62 ± 26, 71 ± 25 and 65 ± 25 $g \cdot h^{-1}$ during the three separate events. Additionally, individual CHO intakes among athletes, regardless of the event, varied greatly from 6 – 136 $g \cdot h^{-1}$. The authors also reported that CHO was ingested in solid, liquid and gel form during all events and during both Ironman events and a marathon, faster times were correlated with high CHO intake.

**Table 2.7** Nutritional intake of male professional road cyclists during professional stage racing and one-day races (updated from Heikura *et al.*, 2019).

| Reference | Participants | Race period | Dietary assessment | Daily nutrient intake | In race nutrition |
|---|---|---|---|---|---|
| Muros *et al.* (2018) | Male professional (UCI World Tour Team) cyclists (n = 9):<br>31.3 ± 3.0 y<br>1.79 ± 0.07 m<br>69.1 ± 7.3 kg | Tour of Spain 2015: a 3-wk stage race; total distance of 3356.1 km; 6 flat, 8 mid-mountain, 5 high mountain, 1 team TT, 1 individual TT; 2 rest days | Daily for the whole tour | Energy: 5415 ± 567 kcal·d$^{-1}$<br>CHO: 12.5 ± 1.8 g·kg$^{-1}$·d$^{-1}$<br>PRO: 3.3 ± 0.3 g·kg$^{-1}$·d$^{-1}$<br>Fat: 1.5 ± 0.5 g·kg$^{-1}$·d$^{-1}$ | During the race:<br>CHO: 91 ± 15 g·h$^{-1}$<br>After the race (between race finish and dinner):<br>CHO: 147 ± 33 g<br>PRO: 55 ± 17 g<br>Fat: 55 ± 17 g |
| Saris *et al.* (1989) | Male professional cyclists (n = 4):<br>1.78 m<br>69.2 kg<br>$\dot{V}O_{2max}$: 79.4 ml·kg$^{-1}$·min$^{-1}$ | Tour de France; a 3-wk stage race; total distance of ~4000 km; 30 mountain passages; 1 rest day | Daily for the whole tour | Energy Intake:<br>Overall mean: 24.7 ± 2.4 MJ·d$^{-1}$<br>Highest (mountain stage): 32.4 ± 4.4 MJ·d$^{-1}$<br>Lowest (rest day): 16.1 ± 3.9 MJ·d$^{-1}$<br>CHO: 61% total energy intake (~900 g·d$^{-1}$)<br>PRO: 217 ± 47 g·d$^{-1}$<br>FAT: 147 ± 39 g·d$^{-1}$ | During the race:<br>CHO: 94 g·h$^{-1}$ |
| García-Rovés *et al.* (1998) | Male professional cyclists (n = 10):<br>27.6 ± 2.0 y<br>1.7 ± 0.04 m<br>66.9 ± 3.2 kg<br>$\dot{V}O_{2max}$: 71.0 ± 6.2 ml·kg$^{-1}$·min$^{-1}$ | Tour of Spain: a 3-wk stage race; total distance of 3600 km; average distance of 170 km per stage; no rest days | Weighed food records (by RD) for 2 separate 24-h periods: 1 flat stage (day 2, 178 km) and 2 mountain stages (day 14, 174 km; day 16, 148 km) | Energy: 23.5 ± 1.8 MJ·d$^{-1}$<br>CHO: 12.6 ± 1.1 g·kg$^{-1}$·d$^{-1}$<br>PRO: 3.0 ± 0.3 g·kg$^{-1}$·d$^{-1}$<br>Fat: 2.4 ± 0.3 g·kg$^{-1}$·d$^{-1}$ | During the race:<br>CHO: 94 g·h$^{-1}$<br>After the race (between race finish and dinner):<br>CHO: 2.0 ± 0.5 g·kg$^{-1}$<br>PRO: 0.3 ± 0.1 g·kg$^{-1}$<br>Fat: 0.2 ± 0.1 g·kg$^{-1}$ |

| | | | | | |
|---|---|---|---|---|---|
| Ebert *et al.*(2007) | Male professional cyclists (n = 8):<br>25 ± 5 y<br>1.77 ± 0.05 m<br>71.4 ± 7.4 kg<br>$\dot{V}O_{2max}$: 71.0 ± 6.2 ml·kg$^{-1}$·min$^{-1}$ | Tour Down Under: a 6-d stage race; total distance of 719 km (stages between 50 and 152 km) | Recall immediately after each stage. | NR | During the race:<br>CHO: 48 g·h$^{-1}$ |
| Ross *et al.* (2014) | Male international-level cyclists (n = 10):<br>19.7 ± 0.8 y<br>1.80 ± 0.05 m<br>72.0 ± 6.1 kg | Tour of Gippsland (n = 5): 9 stages over 5 d<br>Tour of Geelong (n = 5); 6 stages over 5 d | Recall immediately after each stage. | NR | Gippsland:<br>CHO: 40.5 ± 24.2 g·h$^{-1}$<br>Geelong:<br>CHO: 64.2 ± 23.7 g·h$^{-1}$ |
| Sanches-Munoz *et al.* (2016) | Male professional cyclists (n = 6):<br>25.5 ± 1.5 y<br>1.76 ± 0.06 m<br>67.7 ± 3.6 kg | Tour of Andalucia 2009; a 4-d stage race; total distance of 647.6 km | Weighed food records collected by investigators | Energy: 5644 ± 593 kcal·d$^{-1}$<br>CHO: 12.8 ± 1.7 g·kg$^{-1}$·d$^{-1}$<br>PRO: 3.0 ± 0.3 g·kg$^{-1}$·d$^{-1}$<br>Fat: 2.1 ± 0.2 g·kg$^{-1}$·d$^{-1}$ | During the race:<br>CHO: 278 ± 91 g<br>After the race (between race finish and dinner):<br>CHO: 74 ± 20 g<br>PRO: 42 ± 9 g<br>Fat: 14 ± 2 G |
| Rehrer *et al.* (2010) | Male elite cyclists (n = 4):<br>20 ± 3 y<br>1.91 ± 0.06 m<br>84.1 ± 8.2 kg<br>$VO_{2peak}$: 57.6 ± 3.9 ml·kg$^{-1}$·min$^{-1}$<br>PPO: 415 ± 35 W | Tour of Southland 2005; a 6-d stage race with 10 stages; total distance of 883 km | Weighed food records collected for the 6-d period | Energy: 27.3 ± 3.8 MJ·d$^{-1}$<br>CHO: 12.9 ± 1.4 g·kg$^{-1}$·d$^{-1}$<br>PRO: 2.9 ± 0.3 g·kg$^{-1}$·d$^{-1}$<br>Fat: 128 ± 61 g·d$^{-1}$ | NR |

| | | | | | |
|---|---|---|---|---|---|
| Pfeiffer *et al.* (2012) | Male professional cyclist teams at Dauphine Libere (n = 7):<br>31 ± 5 y<br>1.81 ± 0.05 m<br>70 ± 5 kg<br>and Tour of Spain:<br>29 ± 3 y<br>1.81 ± 0.05 m<br>71 ± 7 kg | Dauphine Libere 2009: An 8-day stage race; this study focused on two flat stages (228 km and 182 km)<br>Tour of Spain 2009; a 3-wk stage race; this study focused on 2 mountain stages (204.7 and 188.8 km) and 1 flat stage (171.2 km) | Self-report retrospective questionaire | NR | During the race:<br>CHO: 64 ± 20 g·h$^{-1}$<br>Caffeine: 21 ± 29 mg·h$^{-1}$<br>Sodium: 208 ± 183 mg·h$^{-1}$ |
| Heikura *et al.* (2019) | Male professional (UCI World Tour Team) cyclists (n = 6):<br>30.0 ± 5.7 y<br>1.87 ± 0.04 m<br>77.4 ± 2.7 kg<br>37.2 ± 4.0 mm Sum of 7 skinfolds<br>20’ MMP, W: 399 ± 5.2 W<br>20’ MMP, W· kg$^{-1}$: 5.2 ± 0.4 W | This study was built around an 8-day window of the 2018 Spring Classics (4 single-day races interspersed with 1-2 recovery days) | Weighed food records collected for the 8-d period | Race-day intakes:<br>Energy: 6216 ± 789 kcal<br>EA: 14.4 ± 8.5 kcal·kg$^{-1}$ FFM<br>CHO: 10.7 ± 1.3 g·kg$^{-1}$<br>PRO: 2.8 ± 0.3 g·kg$^{-1}$<br>Fat: 2.7 ± 0.5 g·kg$^{-1}$<br>Rest-day intakes:<br>Energy: 5050 ± 519 kcal<br>EA: 56.9 ± 9.8 kcal·kg$^{-1}$ FFM<br>CHO: 6.4 ± 0.8 g·kg$^{-1}$<br>PRO: 3.3 ± 0.5 g·kg$^{-1}$<br>Fat: 2.7 ± 0.5 g·kg$^{-1}$ | 3-h pre-race:<br>CHO: 3.4 ± 0.7 g·kg$^{-1}$<br>During the race:<br>CHO: 51 ± 9.0 g·h$^{-1}$<br>After the race (between race finish and dinner):<br>CHO: 1.6 ± 0.5 g·kg$^{-1}$<br>24- post-race CHO intake:<br>CHO: 7.4 ± 1.0 g·kg$^{-1}$ |

**Note:** BM: Body mass, CHO: Carbohydrate; PRO: Protein; EA: Energy availability; NR: not reported; MMP: Maximal mean power; RD: Registrered Dietitian; UCI, Union Cycliste Internationale; $\dot{V}O_{2max}$: maximal oxygen uptake.

## 2.5 Carbohydrate post-exercise

For athletes who compete in multi-day sporting events (i.e., cycling tours), undertake a congested competition schedule (i.e., soccer competitions) and/or undertake a prodigious volume of training with multiple sessions in a 24-h period (i.e., distance runners, rowers, swimmers), the replenishment of endogenous glycogen stores after such events or between specific training sessions is of upmost importance to promote performance in the subsequent bout of exercise. As expected, the main substrate for stimulating muscle glycogen resynthesis is dietary CHO intake. In order to maximise glycogen resynthesis, CHO intake in the first hours following exercise is important. Indeed, the translocation of glucose transporter 4 (GLUT4) to the plasma membrane as a consequence of exercise alongside the increased activity of muscle glycogen synthase (Zachwieja *et al.*, 1991) which has been found to increase linearly with the degree of glycogen depletion, provide metabolic mechanisms by which plasma glucose uptake into skeletal muscle is increased at the early stages of recovery. However, this exercise-induced increase in muscle glucose uptake reverses within 2 – 4-h and as such glycogen resynthesis could remain low unless further dietary CHO is consumed. Moreover, the insulin sensitivity of the muscles engaged in exercise is increased for many hours following exercise, so when CHO is consumed, this leads to a greater ability of insulin to increase microvascular perfusion (Sjøberg *et al.*, 2017) and, hence, deliver glucose and insulin to the muscles. Increased insulin sensitivity is also a result of further GLUT4 translocation to the plasma membrane (Hansen *et al.*, 1998) and activation of glycogen synthase (Wojtaszewski *et al.*, 2001).

With this in mind, many studies have focused on the early 0 – 4-h period of immediate post-exercise recovery. Ivy *et al.* (1988a) reported that muscle glycogen repletion rates are approximately 45% lower when CHO intake of 2 $g \cdot kg^{-1}$ is delayed by 2-h compared to when consumed immediately post-exercise. However, a 2-h delay in intake of CHO-rich meal had no effect when glycogen content was evaluated at 8-h post-exercise (Parkin *et al.*, 1997). Initially, it was also suggested that the provision of 0.7 $g \cdot kg^{-1} \cdot h^{-1}$ given immediately after exercise as a single bolus eliciting ~20 $mmol \cdot kg^{-1}$ $dw \cdot h^{-1}$ rate of resynthesis was the maximal rate attainable over 4-h (Ivy *et al.*, 1988b). Importantly, later work using smaller but more frequent (15-30-min interval) provision of CHO feeds following exercise providing 1.2 $g \cdot kg^{-1} \cdot h^{-1}$ elicited a much greater synthesis rate of 44.8 $mmol \cdot kg^{-1}$ $dw \cdot h^{-1}$ (Howarth *et al.*, 2009; Van Loon *et al.*, 2000). Several other studies have observed similar rates of synthesis when providing 1.0 $g \cdot kg^{-1} \cdot h^{-1}$ or greater of CHO at 15 – 60-min intervals in the first 0 – 5-h post

exercise, reported synthesis rates of 40 – 50 mmol·kg$^{-1}$ dw·h$^{-1}$ (Blom, 1989; Doyle *et al.*, 1993; Hickner *et al.*, 1997; Jentjens *et al.*, 2001; Piehl, 1974; Tarnopolsky *et al.*, 1997; Van Hall *et al.*, 2000). Moreover, no obvious differences in synthesis rates have been observed between liquid or CHO sources (Reed *et al.,* 1989). Interestingly, training status also seems to be a determinant of muscle glycogen resynthesis (Fritzen *et al.*, 2019) as the same CHO intake resulted in elevated synthesis rates in trained individuals (~48 – 52 mmol·kg$^{-1}$ dw·h$^{-1}$) compared with recreationally active participants ($\dot{V}O_{2max}$: < 55 ml·kg$^{-1}$·min$^{-1}$; ~22 – 26 mmol·kg$^{-1}$ dw·h$^{-1}$).

The co-ingestion of other macronutrients with CHO may directly influence muscle glycogen restoration. As such, previously it was speculated that the addition of protein may have an augmenting effect upon resynthesis rates given the insulemic effects of protein. However, several studies have tested multiple amino acids and protein combinations with CHO between 0.8 – 1.5 g·kg$^{-1}$·h$^{-1}$ and showed no further benefit to the provision of 1.2 g·kg$^{-1}$·h$^{-1}$ of CHO alone (Jentjens *et al.*, 2001; Van Hall *et al.*, 2000; Van Loon *et al.*, 2001; Zawadzki *et al.*,1992).

Other important factors for ensuring maximal glycogen resynthesis rates with CHO provision post-exercise have been considered, for example the type of CHO ingested. CHO of high glycaemic index (GI) has been shown to produce 61% greater rate of resynthesis of glycogen than provision of CHO with low-GI (Burke, 1993). This effect is likely mediated via increased gastric emptying and thus elevated concentration in the blood available for absorption by skeletal muscle. Several studies have also examined the effect of multi-transportable CHO upon post-exercise muscle glycogen repletion rates and lower resynthesis rates have been observed with the ingestion of fructose compared to glucose (Blom *et al.,* 1987; Conlee *et al.,* 1987; Van Den Bergh *et al.*, 1996). Such augmented rates of resynthesis with fructose are most likely related to a slower absorption rate in the gut compared with glucose and/or the need for fructose to be converted into glucose in the liver before it can be utilized as substrate for muscle glycogen repletion. Nonetheless, combining glucose-fructose in recovery period may provide a strategy to further augment glycogen resynthesis in line with the findings of such strategies during exercise. Wallis *et al.* (2008) found no additional benefit of the addition of fructose to glucose (2:1 glucose:fructose mix) for glycogen resynthesis following exercise, however glucose in the glucose-fructose trial was provided at 0.8 g·kg$^{-1}$·h$^{-1}$, well below the 1.2 g·kg$^{-1}$·h$^{-1}$ recommended. Further work by Trommelen *et al.* (2016) examined glucose, glucose-fructose and glucose-sucrose combinations feeding at 1.5 g·kg$^{-1}$·h$^{-1}$ at 30-min intervals for 5-h and also

noted no additive benefit of fructose or sucrose over glucose alone at facilitating glycogen synthesis. In spite of this, utilising multi-transportable CHO in the immediate recovery may be a more potent strategy for accelerating liver glycogen repletion. For example, glucose-fructose co-ingestion has been shown to increase liver glycogen repletion rates when compared to an isolcaloric amount of glucose only (Décombaz *et al.*, 2011). Moreover, Fuchs *et al.* (2016) reported that 1.5 $g \cdot kg^{-1} \cdot h^{-1}$ of sucrose ingestion for 5-h following glycogen-depleting exercise accelerated post-exercise liver, but not muscle glycogen repletion compared with glucose ingestion alone as measured by $^{13}C$ magnetic resonance spectroscopy and magnetic resonance imaging (MRI). This combination of post-exercise glucose-fructose co-ingestion at a rate of 1.5 and 1.2 $g \cdot kg^{-1} \cdot h^{-1}$ has been found to enhance cycling capacity following short-term (4-h) and overnight (15-h) recovery durations compared with isocaloric glucose alone (Gray *et al.*, 2020). Similarly, Maunder *et al.* (2018) found similar improvements in endurance capacity. In contrast, a similar rate of glucose and fructose ingestion during a 4-h recovery period after exhaustive exercise, had no further performance benefit upon a subsequent ~40 min time trial (preceded by 1-h steady state cycling at 50% $W_{max}$) compared to the ingestion of glucose only (Podlogar & Wallis, 2020).

Taken together, provision of a mix of fluid and solid CHO is recommended to facilitate repletion of endogenous glycogen stores following exercise. The CHO provided should be high-GI and contain multi-transportable CHO so as to induce muscle and liver glycogen repletion. 1.2-1.5 $g \cdot kg^{-1} \cdot h^{-1}$ at 30-min intervals for up to 5-h seems optimal for maximising resynthesis rates, depending on duration and intensity of exercise in addition to consideration of the demands of the next training session or proximity of competition.

## 2.6 Summary

The effects of CHO feeding during prolonged endurance exercise on muscle metabolism and exercise performance has been well studied. It is apparent, however, that the exercise protocols, nutritional status and training status may not always be representative to the practical situations in which elite endurance athletes often compete. Accordingly, further work is required to identify the effects of "real world feeding protocols" on muscle fuel selection and exercise performance so as to increase the translational potential of experimental findings.

# Chapter Three:

# General methodology

*This chapter presents the methodologies used within this thesis. Where required, more specific details are also presented in the subsequent chapters.*

## 3.1 General Methodology

The General Methods Chapter serves to outline the methods that are common across the experimental chapters, as well as describing in detail the staining procedures for the immunofluorescence microscopy work and biochemical analysis. Many of the methods undertaken in this thesis are replicated throughout Studies 1-5. For brevity, these methods have therefore been detailed in the present Chapter with reference to this section made in each of the experimental Chapters, where appropriate.

### 3.1.1 Location of testing and ethical approval

All of the experimental protocols and related biochemical procedures undertaken were conducted in the Exercise Physiology and Biochemical Laboratories of the Research Institute for Sport and Exercise Science, Liverpool John Moores University. Ethical approval was granted from the local ethics committee at Liverpool John Moores University.

### 3.1.2 Subject Characteristics

All of the subjects who volunteered to participate in Study 1-4 were young healthy trained male cyclists whilst Study 5 included an elite professional World Tour cyclist. Subjects were classified as trained according to their $\dot{V}O_{2max}$, lactate threshold and number of hours involved in active cycling training and annual race days, according to the criteria of subject characterisation as defined by Jeukendrup, Craig & Hawley (2000). A comparison of subjects' physical and physiological characteristics is shown in Table 3.1. The physiological profile of the elite World Tour professional cyclist was conducted 1 week before competing in the 2015 Vuelta a España and has been published previously (Bell *et al*., 2017). All participants gave written informed consent to participate after details of the study had been fully explained. None of the participants had a history of neurological disease or skeletal muscle abnormality and none were under pharmacological intervention during any study. Participants were asked to maintain consistent cycling training volumes throughout the entirety of the study and refrain from any additional exercise, they were also asked to maintain habitual caffeine intake and refrain from alcohol for at least 24 hours prior to any testing session. Participation was entirely voluntary, and subjects were free to withdraw from the study at any time.

**Table 3.1** Physical and physiological characteristics of the subjects who participated in the experimental studies (mean ± SD).

| | Study 1 | Study 2-4 | Study 5 |
|---|---|---|---|
| Age (years) | 26 ± 7 | 31 ± 7 | 31 |
| Height (m) | 1.78 ± 0.1 | 1.77 ± 0.1 | 185.7 |
| Body mass (kg) | 74.6 ± 7.2 | 73.6 ± 6.1 | 67.0[#] |
| Total body fat (kg) | - | - | 6.7 |
| Total body fat (% of mass) | - | - | 9.8 |
| Total lean mass (kg) | - | - | 61.5 |
| $\dot{V}O_{2max}$ ($mL \cdot kg^{-1} \cdot min^{-1}$) | 60.0 ± 4.5 | 60.5 ± 5.4 | 88.2 |
| $\dot{V}O_{2max}$ ($L \cdot min^{-1}$) | 4.6 ± 0.4 | 4.6 ± 0.4 | 5.91 |
| PPO (W) | 392 ± 40 | 379 ± 24 | 525 |
| PPO ($W \cdot kg^{-1}$) | 5.3 ± 0.4 | 5.2 ± 0.4 | 7.5 |
| Lactate Threshold (W) | 198 ± 29 | 208 ± 11 | 350 |
| Lactate Threshold ($W \cdot kg^{-1}$) | 2.7 ± 0.4 | 2.9 ± 0.3 | 5.2 |
| Lactate Threshold (%$\dot{V}O_{2max}$) | 63 ± 6 | 64 ± 3 | - |
| Lactate Threshold (% PPO) | 51 ± 5 | 55 ± 3 | 67 |
| *Weekly Training Duration (hh:min) | 05:51 ± 02:50 | 05:12 ± 01:03 | 28:06 ± 04:36 |
| *Weekly Training Distance (km) | 133 ± 93 | 139 ± 33 | 805 ± 174 |
| Race Days per Season | 7 ± 10 | 10 ± 2 | 67 |

*Denotes for the 6-month period before beginning the study. #Denotes known racing weight, all performance/physiological data (Study 5) are expressed relative to the athlete's known race weight. Missing gaps denote no available data.

### 3.1.3 Anthropometry

Subject's height was measured whilst standing in the Frankfurt plane using a stadiometer (SECA, Birmingham, UK). Subjects' body mass was recorded, whilst nude, using precision calibrated weighing scales (SECA, Birmingham, UK) on their preliminary visit to the laboratories that coincided with their assessment of physiological fitness. Subjects' body mass was subsequently recorded at every additional visit to the laboratory for experimental trials.

### 3.1.4 Exercise Protocols

Each exercise related study involved cycling as the exercise modality. All tests were conducted on an electrically braked cycle ergometer (Lode Excaliber Sport, Groningen, Netherlands) as can be seen in Figure 3.1. The electrically braked cycle ergometer was calibrated to intensities

of 100-1000 W and each participant's setup (saddle height, reach, handle-bar height, clip pedals and set crank lengths) was registered during their first visit to the laboratory for preliminary testing to ensure similar settings in all subsequent trials.


**Figure 3.1** Illustration of a subject on the electrically braked cycle ergometer (Lode Excaliber Sport, Groningen, Netherlands) that was used in all exercise-related studies.

## 3.2 Physiological measures

### 3.2.1 Heart rate

Subjects were fitted with a short-range radio telemetry system for the measurement of heart rate (Polar H7 bluetooth smart, Kempele, Finland) in all exercise related experiments.

### 3.2.2 Assessment of respiratory gases during exercise

Subjects were fitted with a mouthpiece supported by a secured head piece (AEI Technologies, Pittsburgh, PA, USA) during longer sampling periods (for assessment of $\dot{V}O_{2max}$ and during submaximal test) or a mouthpiece alone (for durations of sampling of 5 mins) (Figure 3.2).

Expired fractions of oxygen and carbon dioxide were averaged over each 10-second period and were analysed via an on-line gas analysis system, Moxus Modular Metabolic System (AEI Technologies, Pittsburgh, PA, USA) (see Figure 3.3), after calibration with known reference gases according to the manufacturer's guidelines (Beltrami *et al.*, 2014).

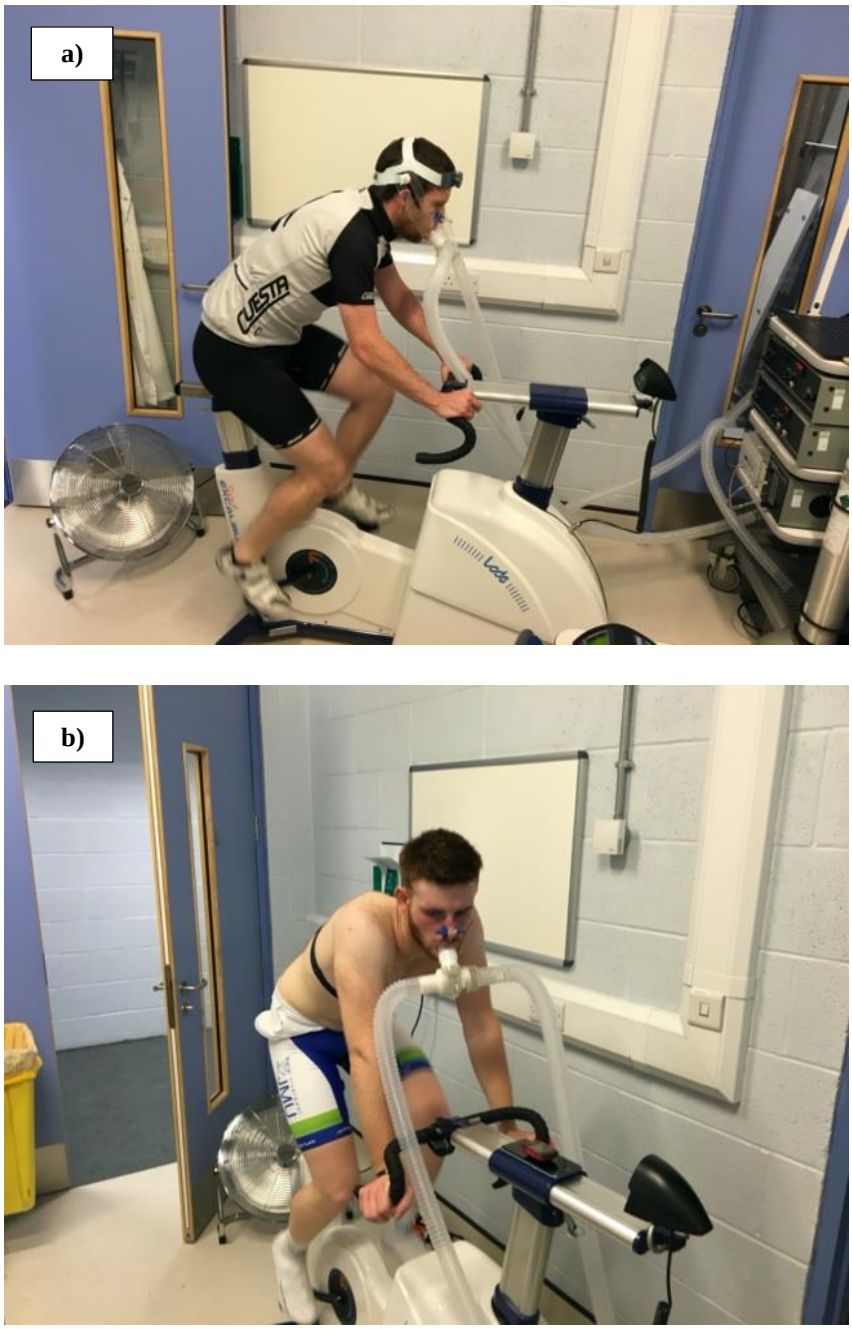


**Figure 3.2** (a) Example of mouthpiece supported by head piece used during assessment of submaximal and $\dot{V}O_{2max}$ tests and (b) mouthpiece used for the collection of respiratory gases during exercise trials.

### 3.2.3 Moxus Modular Metabolic System

A Hans-Rudolph two-way breathing T-shaped valve (Hans Rudolph Inc., Shawneee, Kansas, USA) was mounted on a mouthpiece and connected to a hose leading expired air to the Moxus instrument. The automated gas analyser consists of a mask incorporating a turbine for determination of ventilation volume. The turbine is attached on the inspiratory side of the valve, thus avoiding problems related to condensation of water vapour and accumulation of saliva (Bassett *et al.*, 2001). The use of a nose clip and mask covered the mouthpiece and prevented subjects from inhaling the room air. All the expired gas travelled through a tube from the two-way breathing T-shaped valve into a 4.2 L mixing chamber. Samples were constantly drawn from the mixing chamber (flow regulated to 250 mL $min^{-1}$), through a dual-stage nafion dryer and into the $O_2$ and $CO_2$ content analysers (S-3A/I with an N-22M $O_2$ sensor and CD-3A with a P-61B $CO_2$ sensor, respectively, AEI Technologies, IL, USA). The analysers use the zirconia and non-dispersive infrared methods for $O_2$ and $CO_2$ analysis. Data from the Moxus system were averaged for each 10-second period. Prior to start of each testing session, the automated gas analyser system was calibrated for gas volume and composition according to the manufacturers' instructions. Volume calibration was performed using a high-precision 3 L calibration syringe (Hans-Rudolph, Kansas City, MO, USA) and gas calibration was made using two-point calibration curve, consisting of room air and a gas of known concentration (15.00 % $O_2$, 6.00 % $CO_2$, AGA Norgas, Norway). The coefficient of variation for the Moxus modular metabolic system used in all studies was 2.4 ± 0.4% as calculated from the measurements of gas exchange (whole body $O_2$ consumption and $CO_2$ production). This was determined by eight subjects completing three repeated exercise trials under the same dietary conditions.

**Figure 3.3** Illustration of the Moxus Modular Metabolic System (AEI Technologies, Pittsburgh, PA, USA) that was used in all exercise-related studies.

### 3.2.4 Assessment of lactate threshold, lactate turn point, peak oxygen uptake ($\dot{V}O_{2max}$) and peak aerobic power

A two-part incremental cycling test at a self-selected cadence on an electrically braked cycle ergometer (Lode Excaliber Sport, Groningen, Netherlands) was used to determine lactate threshold (LT), lactate turn point (LTP), maximal oxygen uptake ($\dot{V}O_{2max}$) and peak aerobic power (PPO). The appropriate seat position, handlebar height and orientation that was used during this test was recorded and replicated in each subsequent visit. Part 1 commenced at 125 W and each stage increased by 25 W every 4 minutes. The wattage continued to increase until the blood lactate concentration increased to 4 $mmol \cdot L^{-1}$ or above. The LT and LTP was defined as the first and second sustained increase in blood lactate above baseline values (Carter *et al.*, 1999) (see Figure 3.4). Expired gas was recorded continuously throughout via an automated gas analyser machine (Moxus Modular Metabolic System, AEI Technologies, Pittsburgh, PA, USA) and heart rate (Polar H7, Kempele, Finland), ratings of perceived exertion (RPE) (Borg, 1970) and a capillary blood sample (fingertip) obtained for blood lactate concentration analysis

by micro-assay (Lactate Pro 2, ArkRay Inc., Kyoto, Japan) being taken and recorded in the last 30 seconds of each stage. Part 1 was followed by a 10-minute recovery period consisting of continuous cycling at 100 W. The coefficient of variation for the Lactate Pro used in all studies was 3.5% within a blood lactate range of 2.0 – 4.9 mmol·$L^{-1}$ (Bonaventura *et al*., 2015).

Participants commenced Part 2, starting at an intensity of the penultimate stage of Part 1, with each stage lasting 1-minute and increasing by 25 W until volitional exhaustion or (1) heart rate within 10 beats·$min^{-1}$ of age predicted maximum (2) respiratory exchange ration > 1.1 (3) plateau of oxygen consumption despite increase in workload. The end time and power output of the stage was used to calculate PPO using the following equation (Kuipers, Verstappen, Keizer, Geurten & Van Kranenburg, 1985):

$$PPO = Wfinal + ([t/60] \cdot PI)$$

Where, Wfinal = the power output of the final completed stage in watts, t = time spent in the final uncompleted stage (seconds), 60 = duration of each stage (seconds) and PI = the increase in power output between each stage (W). Expired gas was recorded continuously throughout via an automated gas analyser machine (Moxus Modular Metabolic System, AEI Technologies, Pittsburgh, PA, USA) and heart rate (Polar H7, Kempele, Finland) and RPE (Borg, 1970) was collected at the end of each stage. $\dot{V}O_{2max}$ was determined as the highest average $\dot{V}O_2$ captured in a 30-second period within the last stage.

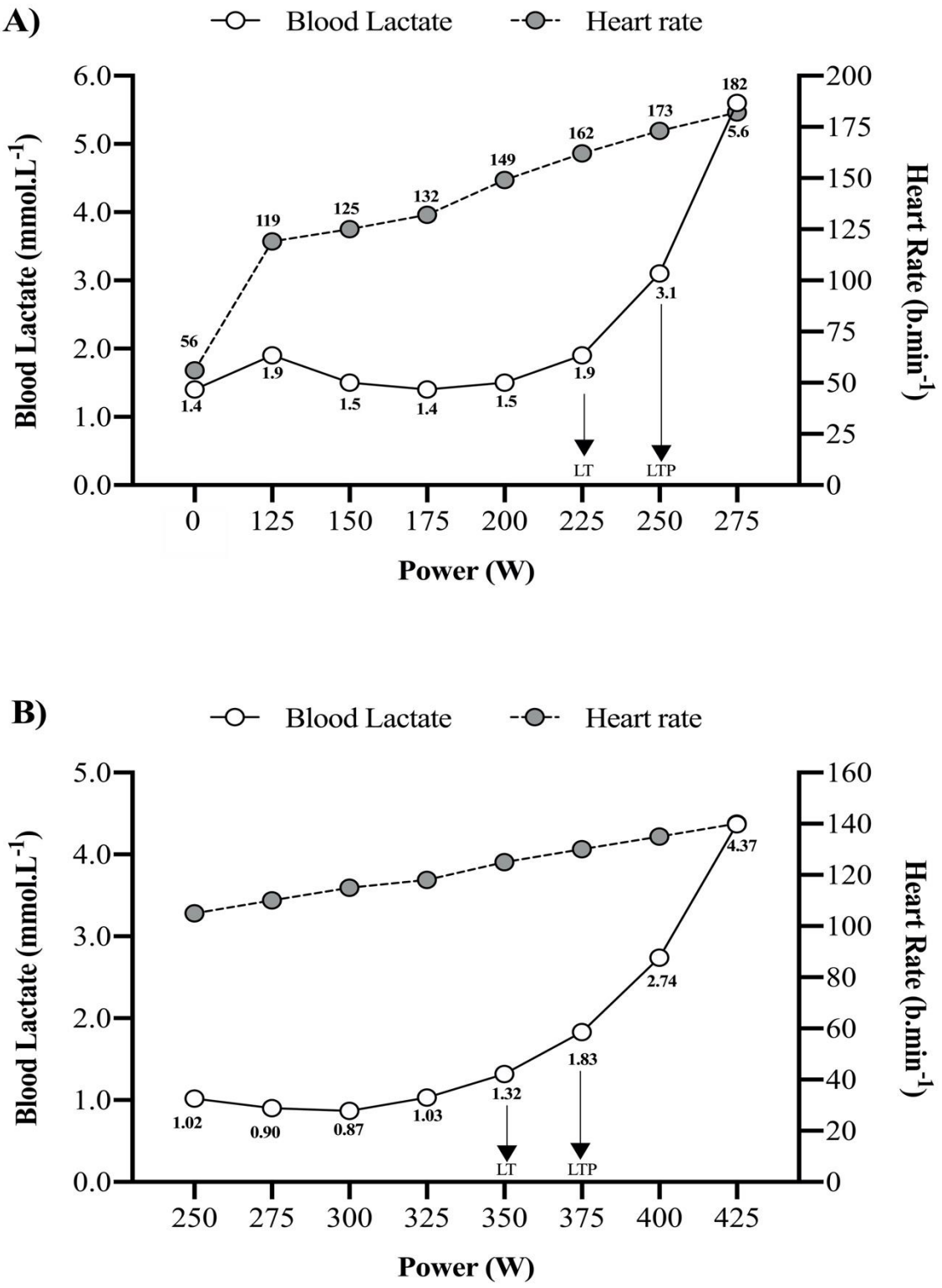


**Figure 3.4** Illustration of plots of blood lactate concentration and heart rate against power output from A) a subject who participated within Study 2 and B) multiple Tour de France winner (https://www.gskhpl.com/dyn/_assets/_pdfs/chris-froome-bodycompaerophys.pdf.), with lactate threshold and lactate turn point determined as the first and second sustained increase in blood lactate above baseline values. LT: lactate threshold; LTP: lactate turn point.

### 3.2.5. Assessment of substrate utilisation and energy expenditure

Rates of whole-body carbohydrate and fat oxidation ($g \cdot min^{-1}$) were estimated via indirect calorimetry (Moxus Modular Metabolic System, AEI Technologies, Pittsburgh, PA, USA) and calculated during exercise in all studies using the equations of Jeukendrup and Wallis (2005). This equation estimates substrate utilisation from $\dot{V}O_2$ and $\dot{V}CO_2$ measurements using non-protein RER equations which are based on the assumption that $\dot{V}O_2$ and $\dot{V}CO_2$ accurately reflect tissue $O_2$ consumption and $CO_2$ production, where $\dot{V}CO_2$ and $\dot{V}O_2$ are measured in litres per minute.

$$\textbf{CHO oxidation rate } (g \cdot min^{-1}) = (4.210 \times \dot{V}CO_2) - (2.962 \times \dot{V}O_2)$$

$$\textbf{Fat oxidation rate } (g \cdot min^{-1}) = (1.695 \times \dot{V}O_2) - (1.701 \times \dot{V}CO_2)$$

Oxidation of 1 gram of carbohydrate was assumed to be energetically equivalent to 17.57 kJ whilst oxidation of 1 gram of fat was assumed equivalent to 39.33 kJ. Volumes of oxygen and carbon dioxide are expressed in litres throughout the calculation.

### 3.2.6 Assessment of cycling gross efficiency

The calculation of gross efficiency divides the work accomplished by the total energy cost required to do the work (Hopker *et al.*, 2009):

$$\textbf{Gross efficiency \%} = (\text{Work done} \div \text{Energy Expenditure}) \times 100$$

(Gaesser & Brooks, 1975)

In order to establish 'Work Done', the average $\dot{V}O_2$ and Respiratory Exchange Ratio (RER) from the given sampling time point was required. The calorific equivalent of $O_2$ was then determined from the corresponding RER according to the table of Zuntz (1901).

$$\textbf{Work Done } (kcal \cdot min^{-1}) = \dot{V}O_2\,(L \cdot min^{-1}) \times kcal \cdot L^{-1} \text{ of } O_2$$

In order to establish the 'Energy Expenditure', the mean power output for the given time point was determined and converted into $kcal \cdot min^{-1}$ via the following equation:

**Energy Expenditure (kcal·min$^{-1}$)** = Power (W) × 0.01433

(Astrand & Rodahl, 1988)

### 3.2.7 Indirect calorimetry principle

The method of indirect calorimetry uses measurements of gas exchange at the lungs (whole body $O_2$ consumption and $CO_2$ production) as an approximate measure of substrate oxidation during exercise given that CHO, fats and proteins differ in their chemical composition and thus, the amounts of $O_2$ needed and $CO_2$ produced when oxidised. Therefore, rates of substrate utilisation for energy production can be estimated through the measurement of $\dot{V}O_2$ consumed and $CO_2$ produced during exercise.

**Table 3.2** Energy and volumes of $O_2$ needed and $CO_2$ produced in the oxidation of carbohydrate, fat and amino acids. Adapted from Jeukendrup & Wallis (2005).

| | **Energy (kcal.g$^{-1}$)** | **$O_2$ required (L.g$^{-1}$)** | **$CO_2$ produced (L.g$^{-1}$)** | **RQ** | **Energy equivalent of $O_2$ (kcal.L)** |
|---|---|---|---|---|---|
| Glucose | 3.74 | 0.7455 | 0.7426 | 0.996 | 5.02 |
| Glycogen | 4.15 | 0.8283 | 0.8251 | 0.996 | 5.02 |
| Fatty acid | 9.75 | 2.0092 | 1.4136 | 0.704 | 4.85 |
| Amino acid | 4.09 | 0.9842 | 0.7931 | 0.807 | 4.16 |

## 3.3 Psycho-physiological measures

### 3.3.1 Ratings of perceived exertion

Subjects' reported ratings of perceived exertion during exercise were recorded using a 15-point Likert scale devised by Borg (1970) (see Figure 3.5). Subjects were familiarised to the scale during preliminary laboratory visits to ensure comprehension of the subjective nature of this measure.

| Rating | Description |
|---|---|
| 6 | No Exertion at All |
| 7 | Extremely Light |
| 8 | |
| 9 | Very Light |
| 10 | |
| 11 | |
| 12 | |
| 13 | Somewhat Hard |
| 14 | |
| 15 | Hard |
| 16 | |
| 17 | Very Hard |
| 18 | |
| 19 | Extremely Hard |
| 20 | Maximal |

**Figure 3.5** Borg scale used for the assessment of ratings of perceived exertion (RPE)

### 3.3.2 Rating of global gastrointestinal symptoms

Global gastrointestinal (GI) symptoms were recorded during each experimental protocol using a GI discomfort scale adapted from Pfeiffer *et al.* (2009) (see Figure 3.6). Participants rated their symptoms on a 10-point scale, ranging from 0 ('no problem at all') to 10 ('the worst it has ever been'), with a score > 4 being regarded as 'moderate'. Participants were instructed to consider symptoms of bloating, the urge to defecate, burping, nausea, flatulence, and/or urge to vomit.

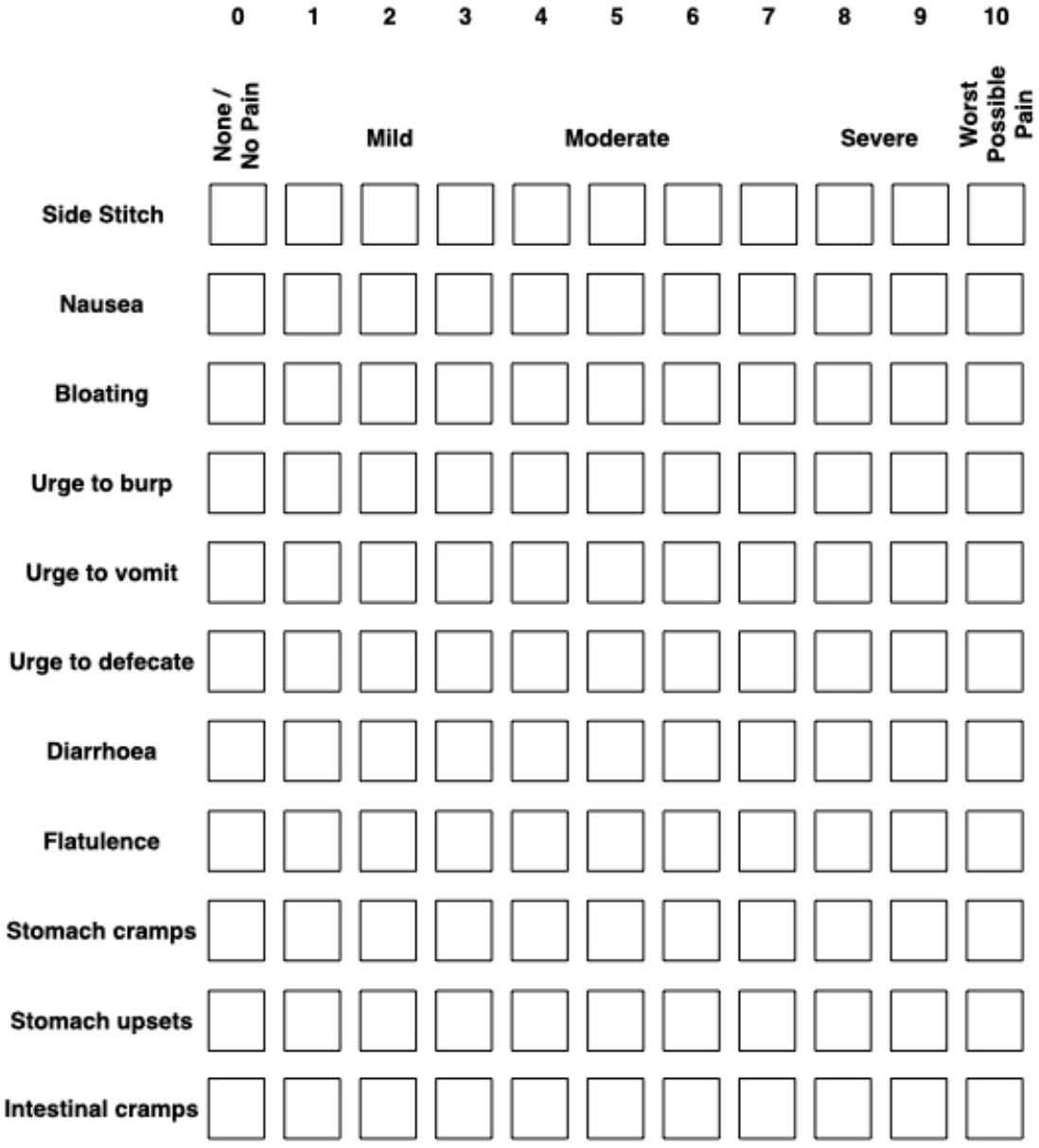


**Figure 3.6** Gastrointestinal discomfort scale adapted from Pfeiffer *et al.* (2009).

### 3.4 Procurement, storage and analysis of blood samples

Blood samples were drawn from a superficial vein in the antecubital crease of the forearm using an indwelling cannula (Safety Lock 22G, BD Biosciences, West Sussex UK). To keep the cannula patent and sterile, this procedure was repeated after each subsequent blood draw. Samples were collected into serum separation tubes (SST), $K_2$ EDTA or lithium heparin (LH) (BD Biosciences, UK). $K_2$ EDTA and LH tubes were stored on ice while SST vacutainers were stored at room temperature, both for 1 h before centrifugation at 1500 g for 15 mins at 4˚C. Following centrifugation, plasma and serum was separated into 4 aliquots and stored at -80˚C for later analysis.

#### 3.4.1 Circulating metabolite analysis

Samples were later analysed for plasma glucose, lactate, non-esterified fatty acids (NEFA), glycerol and β-hydroxybutyrate (β-OHB). Plasma glucose, lactate, NEFA and glycerol were analysed using the Randox Daytona spectrophotometer with commercially available kits (Randox, Ireland), as per the manufacturer's instructions. The coefficient of variation for the measured metabolites was as follows

| | | |
|---|---|---|
| Plasma glucose | (Glucose-Hexokinase, Randox Laboratories, Antrim, UK) | 1.3 ± 1.1% |
| Plasma Lactate | (L-Lactate, Randox Laboratories, Antrim, UK) | 1.2 ± 1.0% |
| Plasma NEFA | (NEFA, Randox Laboratories, Antrim, UK) | 3.5 ± 2.8% |
| Plasma Glycerol | (Glycerol, Randox Laboratories, Antrim, UK) | 0.9 ± 1.3% |
| Plasma β-hydroxybutyrate | (Ranbut, Randox Laboratories, Antrim, UK) | 5.1 ± 3.4% |

#### 3.4.2 Spectrophotometry principle

The spectrophotometry method works by measuring the amount of light that each sample absorbs which is then used for the determination of plasma metabolite concentrations by plotting the absorbance value against the absorbance of standards of known concentrations.

Within the spectrometer, the lens transmits a straight beam of light (photons) that passes through the prism in order to be split into several component wavelengths. The desired wavelength is then selected by the silt and is transmitted through the sample in the cuvette. The amount of photons that is absorbed by the sample is then detected by the photometer (see Figure 3.7).

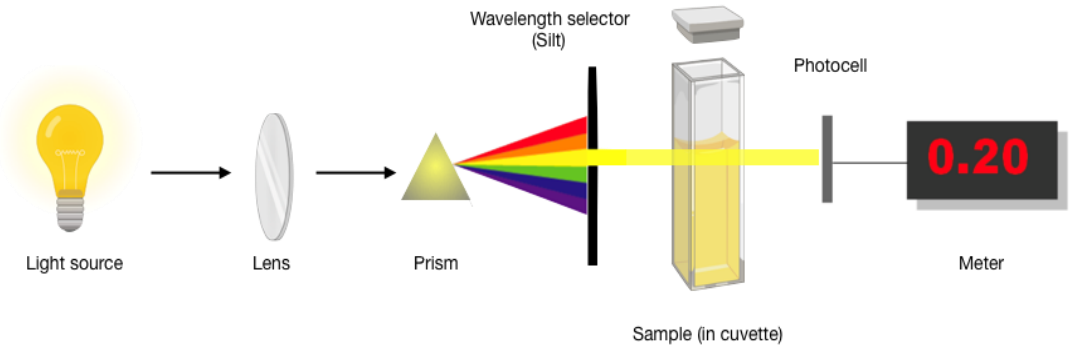


**Figure 3.7** An overview of the general principle of spectrophotometry.

### 3.5 Dietary controls

During Study 1 (Table 3.3), 2, 3 and 4 (Table 3.4) subjects were provided with pre-packaged high CHO meals and foods for 40 h following the glycogen depletion exercise in order to maximise endogenous CHO stores in line with current sport nutrition recommendations (Thomas *et al.*, 2016). Following participant feedback in Study 1 whereby high levels of fullness were reported in relation to the 10 $g \cdot kg^{-1}$ of CHO provided to participants on Day 2 of each experimental trial alongside the pre-exercise meal consisting of 3 $g \cdot kg^{-1}$ CHO (see Table 3.3 and Figure 4.), we felt it necessary to reduce these amounts in Study 2, 3 and 4 to 8 $g \cdot kg^{-1}$ of CHO being provided to participants on Day 2 of each experimental trial alongside 2 $g \cdot kg^{-1}$ CHO in the pre-exercise meal, which would still be considered sufficient to highly elevate endogenous muscle and liver glycogen concentrations.

**Table 3.3** Outline of the high carbohydrate diet provided to subjects' in preparation for experimental trial during Study 1. The below outline is based on a 75 kg individual.

| **Post Depletion** | | | | | |
|---|---|---|---|---|---|
| **Meal** | **Description** | **CHO (g)** | **Protein (g)** | **Fat (g)** | **Kcal** |
| **Immediately Post** | SiS GO Energy (50 g) | 47 | 0 | 0 | |
| | SiS GO Energy Bar (40 g) | 28 | 5 | 0 | |
| | SiS REGO Protein (50 g) | 22 | 23 | 2 | |
| **Total (g)** | | **97** | **28** | **2** | **518** |
| **Total (g·kg$^{-1}$)** | | **1.2** | **0.3** | **0** | |
| **(Post +1 h)** | SiS GO Energy (50 g) | 47 | 0 | 0 | |
| | 2 x SiS GO Energy Bar (40 g) | 56 | 10 | 0 | |
| **Total (g)** | | **103** | **10** | **0** | **452** |
| **Total (g·kg$^{-1}$)** | | **1.3** | **0.1** | **0** | |
| **(Post +2 h)** | SiS GO Energy (50 g) | 47 | 0 | 0 | |
| | 2 x SiS GO Energy Bar (40 g) | 56 | 10 | 0 | |
| **Total (g)** | | **103** | **10** | **0** | **452** |
| **Total (g·kg$^{-1}$)** | | **1.3** | **0.1** | **0** | |
| **Total (g)** | | **303** | **48** | **2** | **1422** |
| **Total (g·kg$^{-1}$)** | | **4.0** | **0.6** | **0.0** | |
| **The following day** | | | | | |
| **Breakfast** | Coco pops (100 g) | 84 | 6 | 3 | |
| | Whole Milk (300 mL) | 14 | 9 | 11 | |
| | Orange Juice (200 mL) | 21 | 1 | 0 | |
| | White Toast x 2 | 38 | 6 | 2 | |
| | Strawberry Jam (15 g) | 9 | 0 | 0 | |
| **Snack** | Muller Rice Original (180 g) | 29 | 7 | 5 | |
| | 2 x SiS GO Energy Bars (40 g) | 56 | 9 | 1 | |
| | SiS GO Hydro Tab | 1 | 0 | 0 | |
| **Lunch** | Cooked Penne Pasta (220 g) | 75 | 11 | 2 | |
| | Tesco Tomato & Basil Pasta Sauce (175 g) | 12 | 2 | 3 | |
| | Chicken Breast (125 g) | 0 | 29 | 8 | |
| | Bread Roll | 30 | 5 | 3 | |
| | SiS GO Energy (50 g) | 47 | 0 | 0 | |
| **Snack** | SiS REGO Protein (50 g) | 22 | 23 | 2 | |
| | Banana | 23 | 1 | 0 | |
| | SiS GO Energy (50 g) | 47 | 0 | 0 | |
| **Dinner** | Tilda flavoured white rice (250 g) | 76 | 7 | 2 | |

| | | | | | |
|---|---|---|---|---|---|
| | Salmon fillet (120 g) | 0 | 25 | 19 | |
| | Sweet chilli sauce (50 g) | 25 | 0 | 0 | |
| | Roast Veg (1/2 Pepper and onion) | 5 | 1 | 0 | |
| | Muller Rice Original (180 g) | 29 | 7 | 5 | |
| **Snack** | Coco pops (100 g) | 85 | 4 | 2 | |
| | Whole Milk (200 mL) | 9 | 7 | 7 | |
| **Total (g)** | | **737** | **160** | **75** | **4263** |
| **Total (g·kg$^{-1}$)** | | **9.8** | **2.1** | **1.0** | |
| **Pre-trial Meal** | | | | | |
| | Porridge oats (100 g uncooked) | 60 | 10 | 7 | |
| | Semi-skimmed milk (500 mL) | 25 | 20 | 10 | |
| | Honey (50 g) | 40 | 0 | 0 | |
| | White Toast (40 g) | 19 | 3 | 1 | |
| | Strawberry jam (15 g) | 9 | 0 | 0 | |
| | Plain Omelette (3 x eggs) | 0 | 18 | 25 | |
| | Orange juice (400 mL) | 40 | 1 | 0 | |
| | Banana | 23 | 1 | 0 | |
| **Total (g)** | | **216** | **53** | **43** | **1463** |
| **Total (g·kg$^{-1}$)** | | **2.9** | **0.7** | **0.6** | |
| **Total (g)** | | **1256** | **261** | **120** | **7148** |
| **Total (g·kg$^{-1}$)** | | **16.7** | **3.5** | **1.6** | |

**Note.** CHO: carbohydrate.

**Table 3.4** Outline of the high carbohydrate diet provided to subjects' in preparation for experimental trial during Study 2-4. The below outline is based on a 75 kg individual.

| **Post Depletion** | | | | | |
|---|---|---|---|---|---|
| **Meal** | **Description** | **CHO (g)** | **Protein (g)** | **Fat (g)** | **Kcal** |
| **Immediately Post** | SiS GO Energy (50 g) | 47 | 0 | 0 | |
| | SiS GO Energy Bar (40 g) | 28 | 5 | 0 | |
| | SiS REGO Protein (50 g) | 22 | 23 | 2 | |
| **Total (g)** | | **97** | **28** | **2** | **518** |
| **Total (g·kg$^{-1}$)** | | **1.2** | **0.3** | **0** | |
| **(Post +1 h)** | SiS GO Energy (50 g) | 47 | 0 | 0 | |
| | 2 x SiS GO Energy Bar (40 g) | 56 | 10 | 0 | |
| **Total (g)** | | **103** | **10** | **0** | **452** |
| **Total (g·kg$^{-1}$)** | | **1.3** | **0.1** | **0** | |
| **(Post +2 h)** | SiS GO Energy (50 g) | 47 | 0 | 0 | |
| | 2 x SiS GO Energy Bar (40 g) | 56 | 10 | 0 | |
| **Total (g)** | | **103** | **10** | **0** | **452** |
| **Total (g·kg$^{-1}$)** | | **1.3** | **0.1** | **0** | |
| **Total (g)** | | **303** | **48** | **2** | **1422** |
| **Total (g·kg$^{-1}$)** | | **4.0** | **0.6** | **0.0** | |
| **The following day** | | | | | |
| **Breakfast** | Coco pops (100 g) | 84 | 6 | 3 | |
| | Whole Milk (300 mL) | 14 | 9 | 11 | |
| | Orange Juice (200 mL) | 21 | 1 | 0 | |
| | White Toast x 2 | 38 | 6 | 2 | |
| | Strawberry Jam (15 g) | 9 | 0 | 0 | |
| **Snack** | Muller Rice Original (180 g) | 29 | 7 | 5 | |
| | 2 x SiS GO Energy Bars (40 g) | 56 | 9 | 1 | |
| | SiS GO Hydro Tab | 1 | 0 | 0 | |
| **Lunch** | Cooked Penne Pasta (220 g) | 75 | 11 | 2 | |
| | Tesco Tomato & Basil Pasta Sauce (175 g) | 12 | 2 | 3 | |
| | Chicken Breast (125 g) | 0 | 29 | 8 | |
| | SiS GO Energy (50 g) | 47 | 0 | 0 | |
| **Snack** | SiS REGO Protein (50 g) | 22 | 23 | 2 | |
| | Banana | 23 | 1 | 0 | |
| **Dinner** | Tilda flavoured white rice (250 g) | 76 | 7 | 2 | |
| | Salmon fillet (120 g) | 0 | 25 | 19 | |
| | Sweet chilli sauce (50 g) | 25 | 0 | 0 | |

| | | | | | |
|---|---|---|---|---|---|
| | Roast Veg (1/2 Pepper and onion) | 5 | 1 | 0 | |
| | SiS GO Hydro | 1 | 0 | 0 | |
| **Snack** | Coco pops (80 g) | 68 | 4 | 2 | |
| | Whole Milk (200 mL) | 9 | 7 | 7 | |
| **Total (g)** | | **615** | **148** | **67** | **3655** |
| **Total (g·kg$^{-1}$)** | | **8.2** | **2.0** | **0.9** | |
| **Pre-trial Meal** | | | | | |
| | Porridge oats (80 g uncooked) | 50 | 5 | 4 | |
| | Semi-skimmed milk (300 mL) | 20 | 13 | 6 | |
| | Honey (25 g) | 20 | 0 | 0 | |
| | White Toast (40 g) | 19 | 3 | 1 | |
| | Strawberry jam (15 g) | 9 | 0 | 0 | |
| | Plain Omelette (3 x eggs) | 0 | 18 | 25 | |
| | Orange juice (200 mL) | 20 | 1 | 0 | |
| | Banana | 23 | 1 | 0 | |
| **Total (g)** | | **161** | **42** | **36** | **1136** |
| **Total (g·kg$^{-1}$)** | | **2.0** | **0.6** | **0.5** | |
| **Total (g)** | | **1079** | **238** | **105** | **6213** |
| **Total (g·kg$^{-1}$)** | | **14.4** | **3.2** | **1.4** | |

**Note.** CHO: carbohydrate.

### 3.5.1 CHO feeding during exercise

The experimental feeding strategies employed within Study 2-4 were typical of elite cyclists whereby they consume a mixture of drinks, solids and gels during competition (Morton, unpublished). Table 5.1 and 5.2 provide the hourly nutritional composition provided during the 45 and 90 g·h$^{-1}$ feeding strategy that were provided at 20 (90 g·h$^{-1}$ trial) and 30-min (45 g·h$^{-1}$ trial) intervals during exercise. Table 5.3 also provides the nutritional breakdown between the different CHO forms of solid (rice cakes, see Figure 3.8), gel and drink utilised in Study 2-4.

**Figure 3.8** Batch of rice cakes that are typically consumed by road cyclists during competition and were used in Study 2-4 as part of the experimental feeding strategies.

### 3.6 Muscle biopsies

Skeletal muscle biopsies (100-150 mg) were obtained from the lateral portion of the vastus lateralis (VL) muscle immediately before and immediately after 180-min of submaximal cycling during Study 2-4. Muscle biopsies were obtained from separate incision sites 2-3 cm apart with the Weil-Blakesley conchotome technique. The Weil-Blakesley conchotome has a sharp biting tip with a 4-6 mm wide hollow. It is inserted through a 5 – 10 mm skin incision can be maneuvered for controlled tissue penetration. This tip is opened and closed within the tissue and then rotated through 90-180˚ to cut the muscle. This technique enables larger amounts of muscle tissue to be obtained which is needed for histology studies (Baczynska *et al.*, 2016). Subjects were asked to relax on a pre-sterilised bed, whist the biopsy area of the VL was prepared. Avoiding immediate areas of previous incision, the site was shaved, washed with an alcohol swab and washed again with Hydrex surgical scrub (ECOLAB Ltd. Leeds, UK), before a sterile sheet was placed around the site of interest and participants were informed to

avoid direct contact with the surgical area. A local anaesthetic (0.5% Marcaine) (bupivacaine hydrochloride; Kays Medical Supplies, Liverpool, UK) was administered to the incision site, at a concentration of 5 $mg \cdot ml^{-1}$ (~1.5 – 2 mL) to anaesthetise the area. A sterile, one-use, disposable scalpel (needle size 10; Swann-Morton, Nu-Care Ltd., Bedfordshire, UK) was used to make an incision, penetrating the skin and muscle fascia. An autoclaved sterilised conchotome biopsy tool was used to retrieve a muscle sample from the VL muscle of subjects (see Figure 3.9 and 3.10).

Skeletal muscle samples were first blotted to remove excess blood, and surgically sterile tweezers and a scalpel were used to dissect the obtained muscle on an irradiated sterile petri-dish for preparation of down-stream analysis (see below). In the unlikely event of the biopsy containing any fibrous/fat tissue this was removed using a scalpel, leaving only lean muscle tissue. A portion of the muscle tissue was then prepared for immunohistochemical analysis (~30-50 mg) by embedding in Tissue-Tek OCT compound (Sakura Finetek Europe, The Netherlands) on cork and freezing in liquid nitrogen-cooled isopentane (Sigma-Aldrich, Dorset, UK) Embedded tissue was stored in an aluminium cryotubes (LuBio Science, Switzerland) which were subsequently submerged in liquid nitrogen and stored at -80˚C for later analysis (see Figure 3.11). ~5-10 mg was then prepared for transmission electron microscopy analysis through fixation in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) for 24-48 h. Samples submerged in 2.5% glutaraldehyde were stored at room temperature for 15-30 minutes before being stored at 4˚C throughout the remaining fixation period. The remaining muscle tissue was sliced into 3 separate sections (~20 mg each section) and snap frozen in liquid nitrogen and stored at -80˚C for later analysis.

Post-incision treatment and follow-up aftercare was given to all participants in alignment with our laboratories operating procedures.

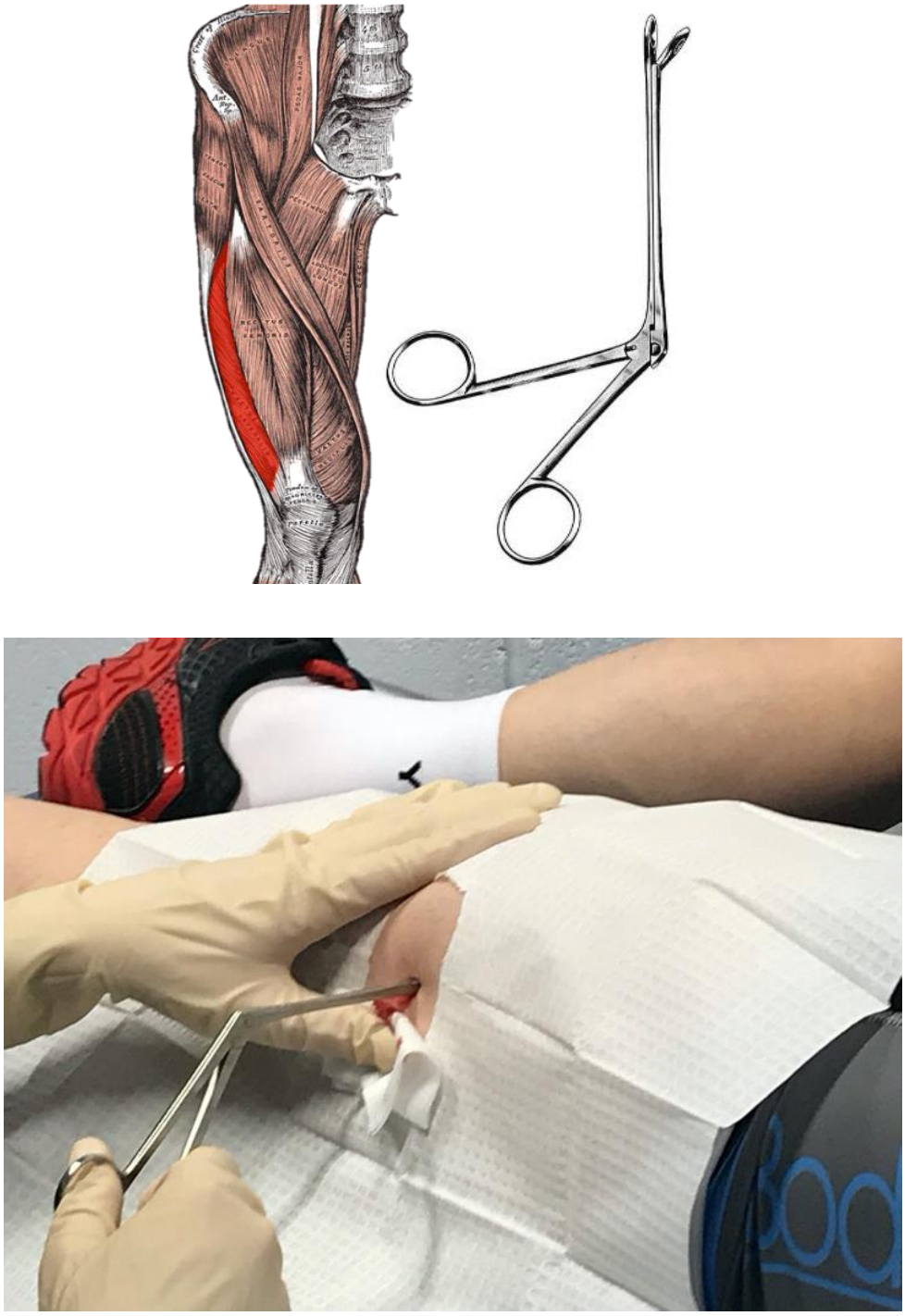

**Figure 3.9** Anatomical location of the muscle biopsy site (*Vastus Lateralis*), the Weil-Blakesley conchotome instrument used and a muscle biopsy procedure from an investigation within this thesis.

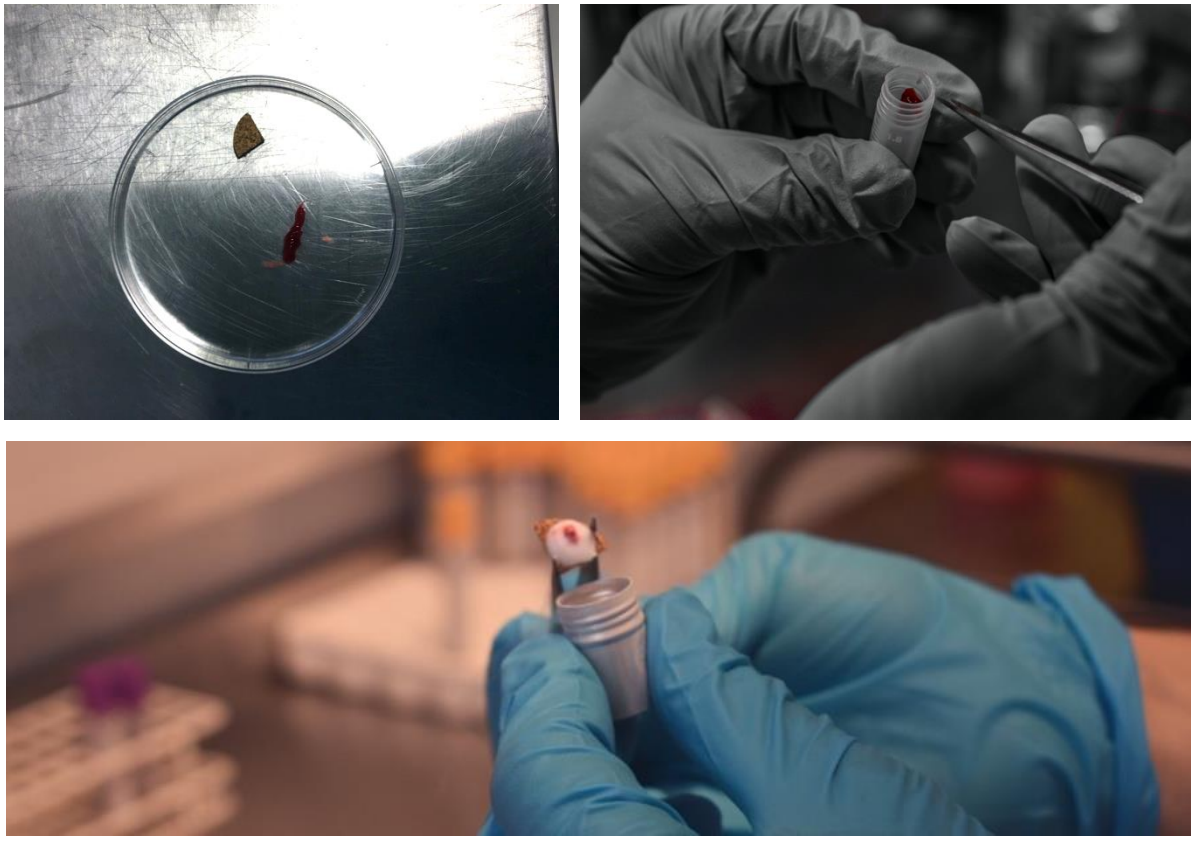

**Figure 3.10** Muscle biopsy preparation for subsequent analysis.

### 3.7 Muscle glycogen concentration

Muscle glycogen concentration was determined according to the methods described by Van Loon *et al.* (2000). Approximately 3-5 mg of freezer dried muscle was powdered, and all visible blood and connective tissue were removed. The freeze-dried sample was then hydrolysed by incubation of in 500 µl of 1 M HCl for 3 h at 100˚C. After cooling to room temperature for ~20 min, samples were neutralised by the addition of 250 µL 0.12 mol.$L^{-1}$ Tris/2.1 mol.$L^{-1}$ KOH saturated with KCl. After centrifugation of 1500 RCF for 10 min at 4˚C, 200 µl of the supernatant was analysed in duplicate for glucose concentration according to hexokinase method using a commercially available kit (GLUC-HK; Randox Laboratories, Antrim, UK) as described in Section 3.4.1 and 3.4.2. Glycogen concentration is expressed as mmol $kg^{-1}$ dry weight (dw) and was calculated, and intra-assay coefficients of variation were 4.4 ± 5.7%. Muscle glycogen concentrations were determined from the average glycogen concentrations from two muscle portions from the same muscle biopsy sample with each portion being run in duplicate.

### 3.7.1 Acid hydrolysis principle

The acid hydrolysis method allows for the cleavage of glyosidic bonds which are necessary for the attachment of glucose molecules to one another, allowing for the breakdown of di- and polysaccharide chains into single sugar monosaccharides. Once boiled, an acid-base neutralisation reaction is performed with the addition of KOH, saturated with KCl in order to neutralise the pH of the sample.

## 3.8 Quantitative immunofluorescence

Details of the specific quantification techniques used can be found in Table 3.5 and all techniques have been previously validated (Bradley *et al.,* 2014; Cocks *et al.,* 2016; Shepherd *et al.,* 2013) with an illustration of the process detailed in Figure 3.12. The muscle mounted in Tissue-Tek was cut into 5 μm thick cryosections with a cryostat at -30˚C (Bright OTF 5000, Bright instruments, Luton, UK) and transferred onto ethanol-cleaned glass slides so that transverse orientated samples could be used for analysis. Cryosections were initially examined on a light microscope to ensure sufficient cell count and determine if cells were cross-sectionally or longitudinal orientated. If cells were orientated longitudinal, the angle of the mounted muscle being sliced within the cryostat was adjusted in an attempt to provide cross-sectional muscle cells. Cryosections of both pre- and post-exercise samples from one participant were placed on a single slide but samples between trials were placed on separate slides to account for any variation in staining intensity between sections (e.g. slide 1: 0 $g \cdot h^{-1}$ pre and post; slide 2: 45 $g \cdot h^{-1}$, pre and post; slide 3: 90 $g \cdot h^{-1}$, pre and post), one slide of each was then used for IMTG staining and analysis (Prats *et al.*, 2013).

Slides were immediately fixed in 3.7% formaldehyde in doubly distilled water ($ddH_2O$) for 1 h followed by three rinses (each for 30s) in $ddH_2O$ before permeabilization in TritonX-100 (0.5%) in Phosphate Buffered Saline (PBS, 137 $mmol.L^{-1}$ sodium chloride, 3 $mmol.L^{-1}$ potassium chloride, 8 $mmol.L^{-1}$ sodium phosphate dibasic and 3 $mmol.L^{-1}$ potassium phosphate monobasic, pH of 7.4) for 5-min. Following three 5-min washes in PBS, slides were incubated for 1 h with appropriate primary antibodies targeting myosin heavy chain type I and myosin heavy chain type IIa (see *antibodies*). Following this incubation period, a further three 5-min PBS washes were completed before the slides were incubated with appropriate Alexa Fluor secondary fluorescence-conjugated antibodies for 30-min. Three more 5-min washes in PBS preceded a 20-min incubation with BODIPY 493/503 (Invitrogen, Paisley, UK, D3922,

dilution 1:100 with PBS) in the dark in order to visualise IMTG. Following a final single 5-min wash in PBS solution, coverslips were mounted with Vectashield (H-1000, Vector Laboratories, Burlingame, CA, USA) and sealed with nail varnish. All staining was completed in batches of three slides (one participant) and each stained cross-section was imaged within three days following.

### 3.8.1 Antibodies and staining combinations

For the lipid analysis the primary antibodies applied targeted myosin heavy chain type I (MHC1 - A4.840c) and myosin heavy chain type IIa (MCHIIa - N2.261c; both Developmental Studies Hybridoma Bank (DSHB), University of Iowa, Iowa, USA developed by Dr. Blau, dilution 1:100 with PBS) and visualised using the secondary antibodies goat anti-mouse (GAM) IgM 546 and goat anti-mouse IgG blue 405 both diluted 1:200 in PBS. Wheat germ agglutinin (WGA) Alexa Fluor 633 conjugate (Invitrogen, Paisley, UK) diluted 1:100 in PBS was used to visualize the cell border.

**Table 3.5** Staining protocol for intramuscular triglycerides (IMTG).

| Treatment | |
|---|---|
| Fixation | Formaldehyde 3.7% in PBS for 1 hour |
| Washes | 3 × 30 second dd$H_2O$ washes |
| Permeabilization | 0.5% Trition X-100 for 5 mins |
| Washes | 3 × 5 min PBS washes |
| Incubation | Primary antibodies for 1 hour<br>MHCI A4.840 (1:100)<br>MHCIIa N2.261 (1:100) |
| Washes | 3 × 5 min PBS washes |
| Incubation | Secondary antibodies for 30 mins<br>GAM IgM 546 (1:200); GAM IgG 405 (1:200);<br>WGA (1:100) |
| Washes | 3 × 5 min PBS washes<br>Move to dark room |
| Incubation | BODIPY 493/503 for 20 mins (1:200) |
| Mounting | Vectashield |

**Note.** MHCI = myosin heavy chain I; MHCIIa = myosin heavy chain IIa; PBS = phosphate-buffered saline; GAM = goat anti-mouse

### 3.8.2 Image capture

Images of cross-sectionally orientated sections, used to investigate fibre type-specific IMTG content and LD morphology, were captured using an inverted confocal microscope (Zeiss LSM710; Carl Zeiss AG, Oberkochen, Germany) with a 63 × 1.4 NA oil immersion objective. A diode laser was used to excite the Alexa Flour 405 fluorophore, an argon laser for the Alex Flour 488 fluorophone and BODIPY 493/503 and a helium-neon laser for the Alexa Fluor 546 and 633 fluorophones.

To assess fibre specific IMTG content, cross-sectional images were obtained at 1.1x digital zoom. Type I and type IIa fibres were identified through positive staining, and any fibres without positive staining for either MHCI or MHCIIa were assumed to be type IIx fibres.

Although, some images were captured for type IIx fibres, there was an insufficient number to include within the data. Approximately 20 images were captured per time point, aiming for an even split across type I and IIa fibres. The images were scanned in projection of 4 lines and acquired at a resolution of 1,024 × 1,024 pixels and stored in 24-bit tagged image file format. No image processing was carried out prior to intensity analysis and identical settings were used for all image capture for each variable within each participant. Overall, 10 ± 1 fibres were analysed per time point and condition for type I fibres and 9 ± 1 fibres for type IIa fibres, equating to approximately 111 ± 30 fibres per participant.

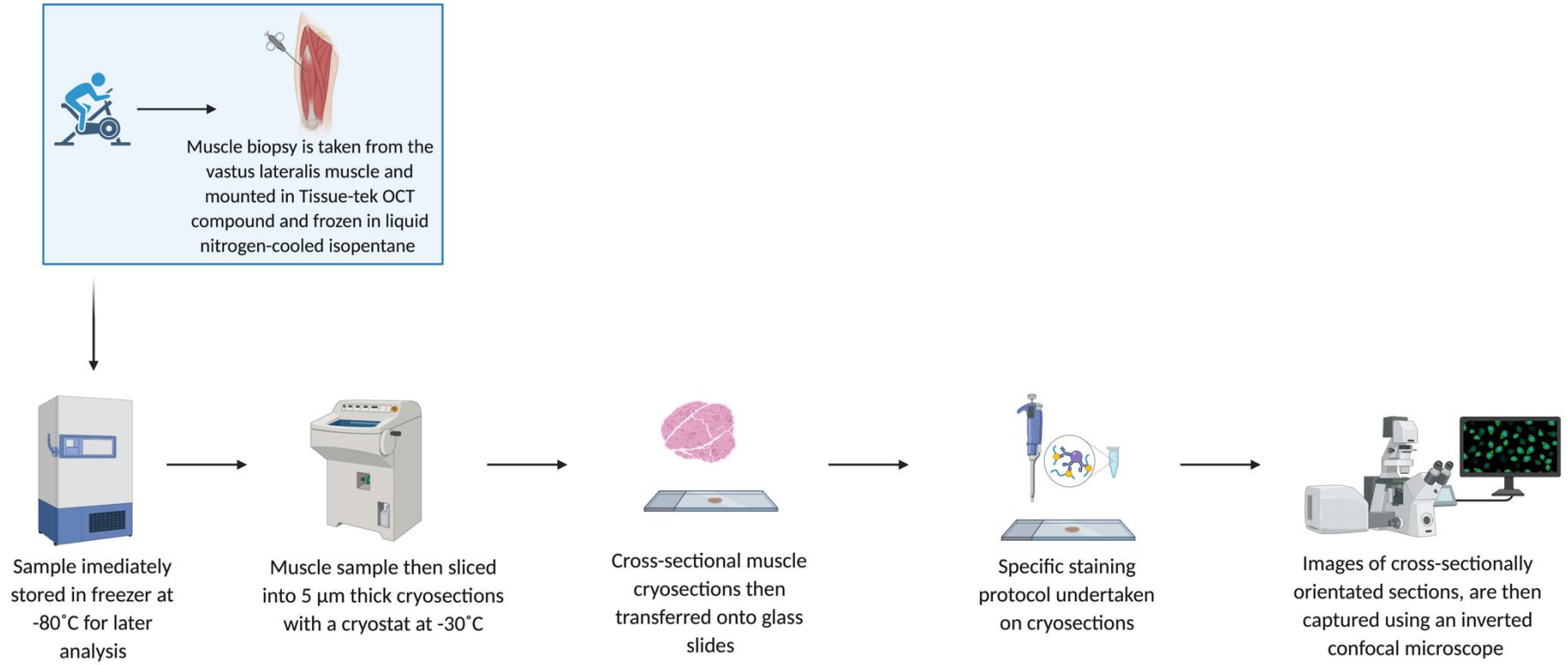


**Figure 3.11** Illustration outlining the process for quantitative immunofluorescence microscopy analysis using an inverted confocal microscope.

### 3.8.3 Image analysis

All image analysis was performed using ImagePro Plus 5.1 (Media Cybernetics Inc, Bethesda, MD, USA).

### 3.8.4 Intramuscular triglyceride analysis

To assess IMTG content and LD morphology on a fibre type-specific basis, the fibre was first separated into a peripheral region to measure subsarcolemmal LD (first 2 μm from the cell border) and the central region to measure intermyofibrillar LD (remainder of the cell). This approach of using a fixed 2 μm distance from the membrane to represent the subsarcolemmal region has been utilized previously to examine IMTG content in differing populations (Jevons *et al.*, 2020; Van Loon, 2004) (see Figure 3.12). An intensity threshold was uniformly selected to represent a positive signal for IMTG. The content of IMTG was expressed as the positively stained area relative to the total area of the peripheral or central region of each muscle fibre. IMTG density was calculated as the number of IMTG objects relative to area. The mean area of individual IMTG (lipid droplets) objects was used as a measure of lipid droplet size. Before conducting analysis, numerous controls were performed to check for bleed through and nonspecific secondary antibody binding. No positive staining was observed in the opposite channel when single staining (IMTG) was performed, and omission of the primary or secondary antibody.

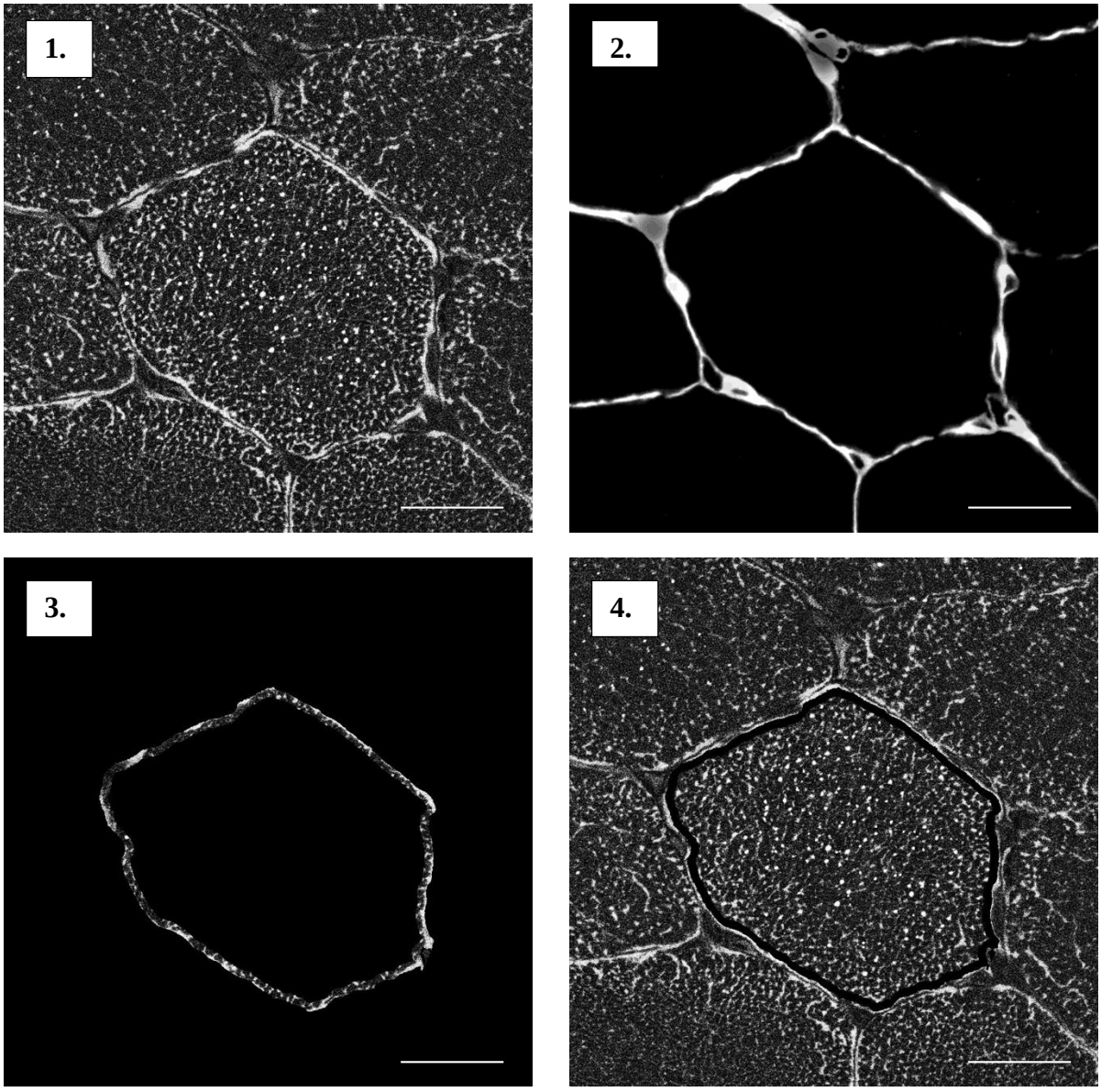


**Figure 3.12** Illustration of the process of quantifying IMTG content and LD morphology within the peripheral and central region of the cell of interest using ImagePro Plus 5.1 (Media Cybernetics Inc, Bethesda, MD, USA). Image 1) represents the initial processing of the image; 2) represented the extracted cell membrane which was manually drawn around for each image to create a 2 µm band defined as the peripheral region; 3) shows the extracted peripheral region and 4) represents the remaining central region of the cell of interest. IMTG: Intramuscular triglycerides; LD: Lipid droplet. Scale bars represent 30 µm.

### 3.9 Semi-quantitative fibre type specific muscle glycogen analysis

Details of the specific quantification technique used can be found in Table 3.6 Fibre type-specific glycogen was quantified by combining a brightfield periodic acid-Schiff stain (PAS) with an immunofluorescence myosin heavy chain (MHC) stain. As such, 5 µm thick muscle cryosections which had been previously prepared on to ethanol-cleaned glass slides (see

Section 3.8) were fixed in 3.7% formaldehyde in PBS (137 mmol.$L^{-1}$ sodium chloride, 3 mmol.$L^{-1}$ potassium chloride, 8 mmol.$L^{-1}$ sodium phosphate dibasic and 3 mmol.$L^{-1}$ potassium phosphate monobasic, pH of 7.4) for 60 min at 4˚C. Sections were then washed for 5 min in PBS and treated for 5 min with 0.1% Triton X-100 (Sigma-Aldrich, Dorset, UK) in PBS and again washed for 5 min in PBS. After this fixation and permeability step the slides were pre-treated for 5 min with 1% periodic acid (Sigma-Aldrich, Dorset, UK) in mQ-water followed by a washing step for 1 min in tap water and a wash dip for 5 s in mQ-water. The slides were then applied with 25% diluted Schiff's reagent in PBS (Sigma-Aldrich, Dorset, UK) (diluted in PBS) was then applied to the muscle sections and incubated for 15 min at room temperature. The Schiff's reagent was diluted to 25% as a result of prior optimisation, given that when we applied the reagent without any dilution the dye intensity saturated the signal in both the pre- and post-exercise samples. After Schiff's reagent, the sections were washed for 5 s in mQ-water followed by a 10 min rinse with slow running tap water. Thereafter, sections were washed for 5 min in PBS prior to fluorescence staining in which slides were incubated for 45-min at room temperature with an appropriate primary antibody targeting myosin heavy chain type I (MHCI – A4.840c; DSHB, University of Iowa). Following this incubation period, a further three 5-min PBS washes were completed before the slides were incubated with an appropriate Alexa Fluor secondary fluorescence-conjugated antibody (Goat anti-mouse IgM 488) for 30-min. Three more 5-min PBS washes followed and finally coverslips were mounted with a glycerol and mowiol 4-88 solution in 0.2 M Tris buffer (pH 8.5; including 0.1% DABCO anti-fade medium) and sealed with nail varnish. All staining was completed in batches of three slides (one participant) and each stained cross-section was imaged the following day.

**Table 3.6** Summarised staining protocol for fibre-specific semi-quantitative muscle glycogen content through for periodic acid-Schiff (PAS) staining with immunofluorescence (IF).

| Treatment | PAS with IF |
|---|---|
| Fixation | Formaldehyde 3.7% in PBS for 1 hour |
| Rinsing | PBS for 5 min |
| Permeabilization | 0.1% Trition X-100 in PBS for 5 mins |
| Rinsing | PBS for 5 min |
| Rinsing | mQ-water for 30 s |
| Pre-treatment | 1% Periodic acid in mQ-water for 5 min |
| Rinsing | Tap water for 1 min and mQ-water for 5 s |
| Staining | 25% Schiff's reagent for 15 min |
| Rinsing | mQ-water for 5 s and tap water for 10 min |
| Rinsing | PBS for 5 min |
| Incubation | Primary antibody for 45 min<br>MHCI A4.840 (1:100) |
| Rinsing | PBS for 3 × 5min |
| Incubation | Secondary antibody for 30 mins<br>GAM IgM 488 (1:200) |
| Rinsing | PBS for 3 × 5 min |
| Mounting | Mowiol |

**Note.** MHCI = myosin heavy chain I; PBS = phosphate-buffered saline; GAM = goat anti-mouse

### 3.9.1 Immunofluorescence in combination with Periodic acid-Schiff optimisation

The above section outlining the specific PAS quantification methodology used within this thesis was based on a previous method described elsewhere (Schaart *et al.*, 2004). However, as previously stated we were unable to combine PAS, fibre-specific and muscle cell border stains on single muscle cross-section when the relevant antibodies and methods replicated those used within Schaart *et al.* (2004) (see Table 3.7), as there seemed to be a significant interference effect between PAS and the immunofluorescent antibodies specifically targeting the plasma membrane. Indeed, substantial time was dedicated in attempting to optimise the intended combination of the PAS and immunofluorescent antibodies on single cross-sections. Previous investigations which employed such techniques had also stained PAS and immunofluorescent antibodies on separate muscle sections and merged the neighbouring images in attempts to match the corresponding muscle fibres. However, this may have been deemed a potential limitation given the near impossible task of exactly merging muscle fibres. The following Table 3.8 provides an outline of the combination of different primary and secondary antibodies used in combination with/without PAS in attempts to optimise the relevant staining. Each aligned primary and secondary antibody was also tested in isolation together to ensure it was functional.

**Table 3.7** Summarised staining protocol for periodic acid-Schiff (PAS) staining with and without immunofluorescence (IF). (PBS: Phosphate-buffer saline) (Shaart *et al.*, 2004).

| Treatment | PAS | PAS with IF |
|---|---|---|
| Fixation | Formaldehyde 3.7% in 90% ethanol for 1 hour | Formaldehyde 3.7% in PBS for 1 hour |
| Rinsing | - | PBS for 5 min |
| Permeabilization | - | 0.1% Trition X-100 in PBS for 5 mins |
| Rinsing | - | PBS for 5 min |
| Rinsing | - | mQ-water for 30 s |
| Pre-treatment | 1% Periodic acid in mQ-water for 5 min | 1% Periodic acid in mQ-water for 5 min |
| Rinsing | Tap water for 1 min and mQ-water for 5 s | Tap water for 1 min and mQ-water for 5 s |
| Staining | Schiff's reagent for 15 min | Schiff's reagent for 15 min |
| Rinsing | mQ-water for 5 s and tap water for 10 min | mQ-water for 5 s and tap water for 10 min |
| Nuclear stain | Haematoxylin for 2 min | (Optional: Haematoxylin for 2 min) |
| Rinsing | Tap water for 10 min | (Optional: Tap water for 10 min) |
| Rinsing | - | PBS for 5 min |
| Incubation | - | Primary antibody in 0.05% Tween 20/PBS for 45 min |
| Rinsing | - | PBS for 3 × 5min |
| Incubation | - | Secondary antibody in 0.05% Tween 20/PBS for 45 min |
| Rinsing | PBS for 3 × 5 min | PBS for 3 × 5 min |
| Mounting | Mowiol | Mowiol |

**Note.** PBS = phosphate-buffered saline.

**Table 3.8** Outline of the unsuccessful combination of different primary and secondary antibodies attempted in combination with/without PAS in attempts to optimise the relevant staining.

| Attempt | PAS Used | Primary antibodies | Secondary antibodies |
|---|---|---|---|
| 1 | Yes | MHC I (A4.840) 1:100<br>Dystrophin | GAM IgM 350 - 1:200<br>GAM $IgG_{2a}$ - 1:200 |
| 1 | No | MHC I (A4.840) 1:100<br>Dystrophin | GAM IgM 350 - 1:200<br>GAM $IgG_{2a}$ - 1:200 |
| 2 | Yes | MHC I (A4.840) -1 :100 | GAM IGM 488 - 1:200<br>WGA 350 - 1:200 |
| 3 | No | MHC I (A4.840) – 1:100 | GAM IGM 488 - 1:200<br>WGA 350 - 1:200 |
| 4 | Yes | 2E8-C (laminin) – 1:100 | GAM $IgG_1$ – 1:200 |
| 5 | No | 2E8-C (laminin) – 1:100 | GAM $IgG_1$ – 1:200 |
| 6 | Yes | MHC I (A4.840) – 1:100<br>MHC IIa (N2.261) – 1:100 | GAM IgM 488 - 1:200<br>GAM IgG 350 - 1:200 |

**Note.** GAM = goat anti-mouse; MHCI = myosin heavy chain I; MHCIIa = myosin heavy chain IIa;

### 3.9.2 Image Capture

Images of sections used to investigate fibre type-specific muscle glycogen, were captured using a Leica DM6 B upright microscope coupled to a Leica DFC7000 T camera and LAS X software (Leica, Wetzlar, Germany), with images captured at ×40,000 magnification. Periodic acid-Schiff (PAS) stained sections were captured in bright-field with the same exposure time, gain, brightness, contrast and offset settings brightness across all samples whilst corresponding fluorescence images for determining fibre type were captured by a simple shift in filters with images captured using the excitation filter (465 – 495 nm) in the green channel. To assess fibre-specific muscle glycogen content, fibres that were positively stained for myosin heavy chain type I were classified as type I fibres and all remaining fibres negatively stained were classified as type II fibres. Unfortunately, I could not acquire a positive stain for type IIa/x fibres due to

interaction effects between the current dye and antibodies used. The entire available section per biopsy sample was imaged in order to capture as many muscle fibres as possible.

### 3.9.3 Image Analysis

Image processing was completed using Image-Pro Plus 5.1 software (Media Cybernetics, Rockville, MD). To assess muscle glycogen content, the bright-field image of the PAS stain was converted *post hoc* to 8-bit greyscale values (0 - 255) (see Figure 3.13). The mean optical density of the PAS-stained muscle fibres was determined by averaging the optical density measured in every pixel in the cell, corrected for the mean optical density of the background stain, containing no muscle fibres. The relative staining intensity of each muscle fibre was classified into 4 separate sections based of optical density increments of 25% in order to visualise the relative scale of semi-quantitative glycogen content (empty, partially empty, partially full and full). The 25% increment sections were calculated from the lowest to the highest recorded optical density value within the current data set. For PAS analysis, on average per subject, a total of 154 ± 101 muscle fibres was analysed for each muscle cross-section (85 ± 54 type I, 76 + 51 type II).

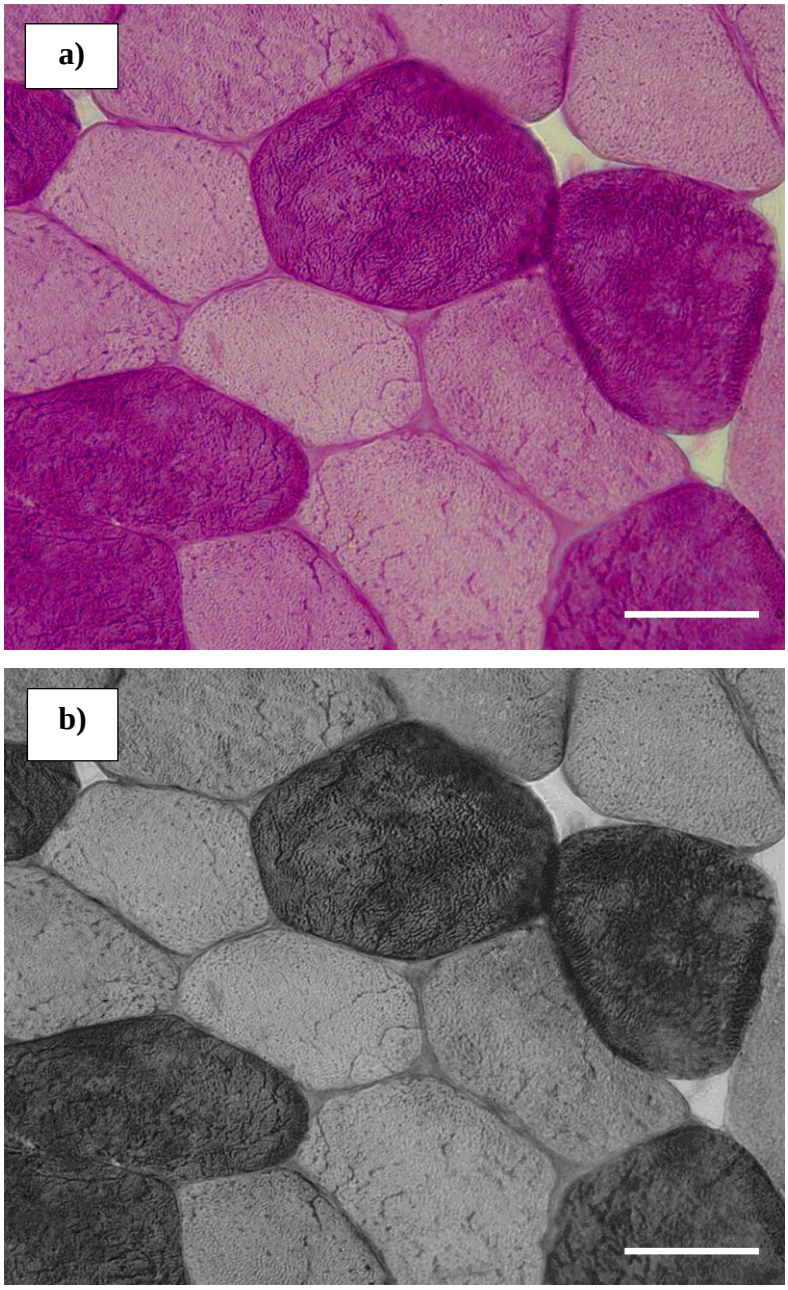


**Figure 3.13** Illustration of the process of quantifying PAS optical density content using ImagePro Plus 5.1 (Media Cybernetics Inc, Bethesda, MD, USA). Image a) represents the initial bright-field PAS image; b) represents the PAS image converted to *post hoc* to 8-bit greyscale for optical density quantification. PAS: Periodic acid-Shiff. Scale bars represent 50 µm and image taken at ×40,000 magnification.

### 3.10 Subcellular muscle glycogen analysis

Transmission electron microscopy was used in order to quantify subcellular muscle glycogen as outlined in the following sections.

### 3.10.1 Transmission electron microscopy sample preparation

Fresh muscle specimens were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) for 24-48 h with fixation starting at room temperature and continued at 4˚C after 15 – 30 min. Specimens were then rinsed four times in 0.1 M sodium cacodylate buffer. Following rinsing, muscle specimens were post fixed with 1% osmium tetroxide ($OsO_4$) and 1.5% potassium ferrocyanide ($K_4Fe(CN)_6$) in 0.1 M sodium cacodylate buffer for 90 min at 4˚C. The use of reduced osmium tetroxide containing potassium ferrocyanide is favouring a high electron density of glycogen particles (De Bruijn, 1973). After post-fixation, the muscle specimens were rinsed two times in 0.1 M sodium cacodylate buffer at 4˚C for 10 min, dehydrated through a graded series of acetone (30, 50, 70, 90 and 100%) at 4-20˚C for 10 min, infiltrated with graded mixtures of propylene oxide and epoxy resin (Sigma-Aldrich, Dorset, UK) at 20˚C for 1 h for each mix and embedded in 100% epoxy resin (Sigma-Aldrich, Dorset, UK) at 45˚C for 12 h, followed by 24 hr at 60˚C. Blocks were then left to air-cure for a few days after polymerisation prior to sectioning. Longitudinal orientated ultra-thin (~60 nm) sections were obtained using a ultramicrotome (Reichert Jung Ultracut, Vienna, Austria) fitted with a diamond blade and collected on to formvar coated grids (200 copper mesh size). To obtain as many fibres as possible, sections were collected at three different depths separated by ~150 nm.

### 3.10.2 Post-staining procedures

Post-staining is advised on samples for TEM to increase the contrast of subcellular structures. Prior to use, stock solutions of both the Reynold's lead citrate and uranyl acetate (UA) (3% by weight in $ddH_2O$) were aliquoted and micro centrifuged for 5 min. Samples were then incubated in UA for 14 min, after which excess UA was removed by manual washing of the sample in $ddH_2O$ (only moving the sample in an up and down motion). Subsequently, the sample was incubated in Reynold's lead citrate for 5 min in a dark room at room temperature, followed by washing in $ddH_2O$. Samples were air dried at room temperature for at least 30 min prior to imaging.

### 3.10.3 Imaging

For each muscle sample, all longitudinal orientated fibres were imaged using a transmission electron microscope (FEI Morgagni, Field Electron and Ion Company, Oregon, USA), coupled

with an Olympus Megaview III camera, which provided an average of 10 fibres per biopsy (range: 6-12 fibres). Each fibre was initially viewed at ×1,000 magnification in order to locate the plasma membranes and visually assess the available myofibrillar area. For mitochondria and glycogen analysis, images were collected at ×36,000 magnification, where a total of 24 images were obtained per fibre in a randomized systematic order (see Figure 3.14). Of these 24 images collected per fibre, 12 images were obtained of the subsarcolemmal region (SS), 6 images of the superficial region of the myofibrillar space (superficial myofibrillar, see Figure 3.14) and 6 images of the central region of the myofibrillar space (central myofibrillar, see Figure 3.14).

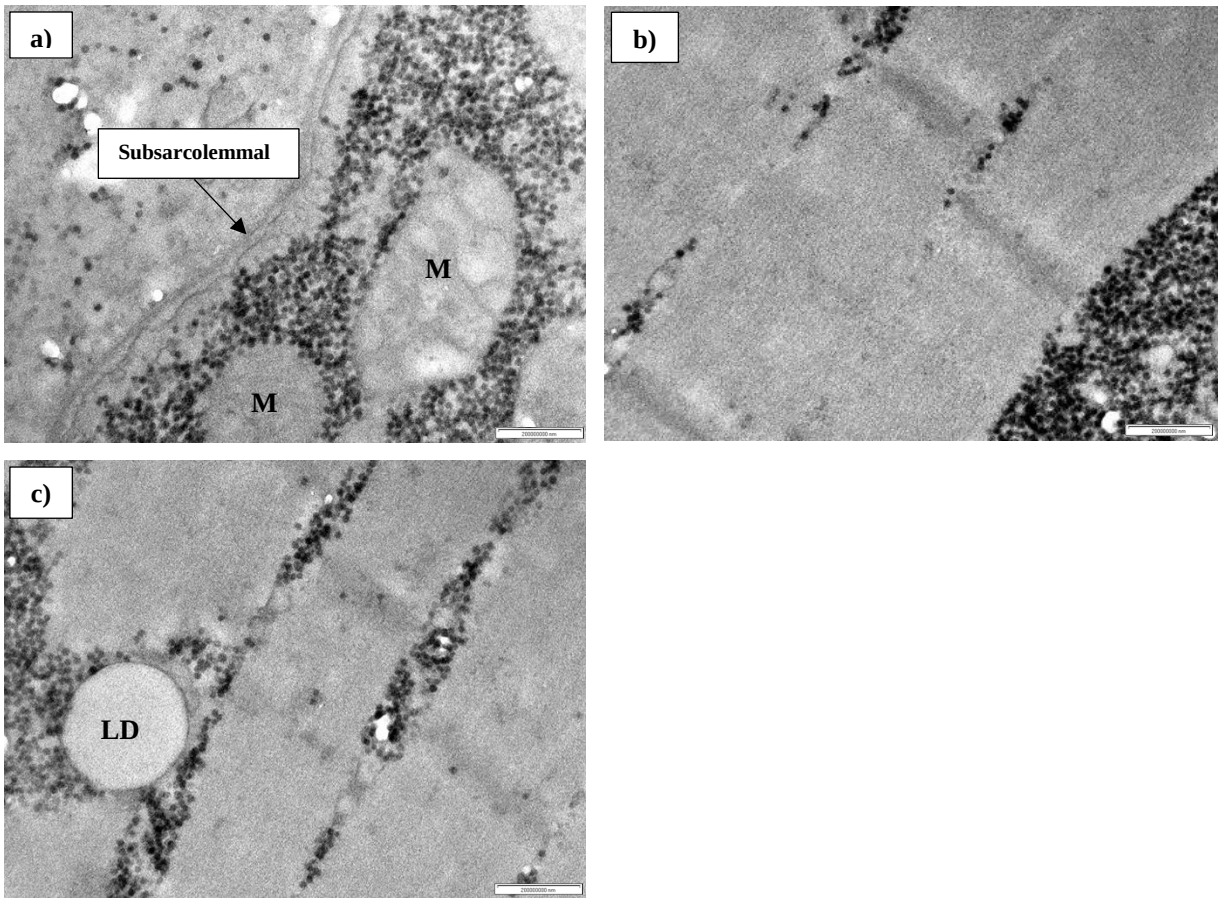


**Figure 3.14** Images illustrate the a) subsarcolemmal region, b) superficial myofibrillar and c) central myofibrillar. Images taken at Liverpool John Moores University, microscopy laboratory by J.M. Fell. M: mitochondria, LD: lipid droplet.

### 3.10.4 Image analysis

Image analysis was undertaken using ImageJ software (LOCI, University of Wisconsin, USA) and for each type I and type II fibres, three spatial localizations of glycogen were defined in

the following: 1) the subsarcolemmal (SS) space, 2) the intermyofibrillar (IMF) space, and 3) the intramyofibrillar (Intra) space, defined as being inside the myofibrils, between the contractile elements of the fibre. Subsarcolemmal images were used to determine SS glycogen, whereas the combination of superficial and central myofibrillar images were used to determine IMF and Intra glycogen pools.

### 3.10.5 Fibre type determination

Fibre type differences were assessed through classification of the fibres as either type I or type II by combining measures of IMF mitochondria volume and Z-line width (Sjostrom *et al*, 1982). First, a 135 nm grid was applied to the image (see Figure 3.15) before all points of the grid with mitochondria underneath were counted and recorded. The Z-line width of each image was then measured twice and recorded. The IMF mitochondria volume fraction was subsequently plotted against the Z-line width for each fibre obtained from each biopsy, and the fibres with the greatest IMF mitochondria volume fraction and the thickest Z-line width were classified as type I fibres, with the opposite being true for type II fibres (i.e., lowest IMF mitochondria volume fraction and narrowest Z-line width). Only the 2 – 3 most distinct type I or type II fibres were included in the analysis.

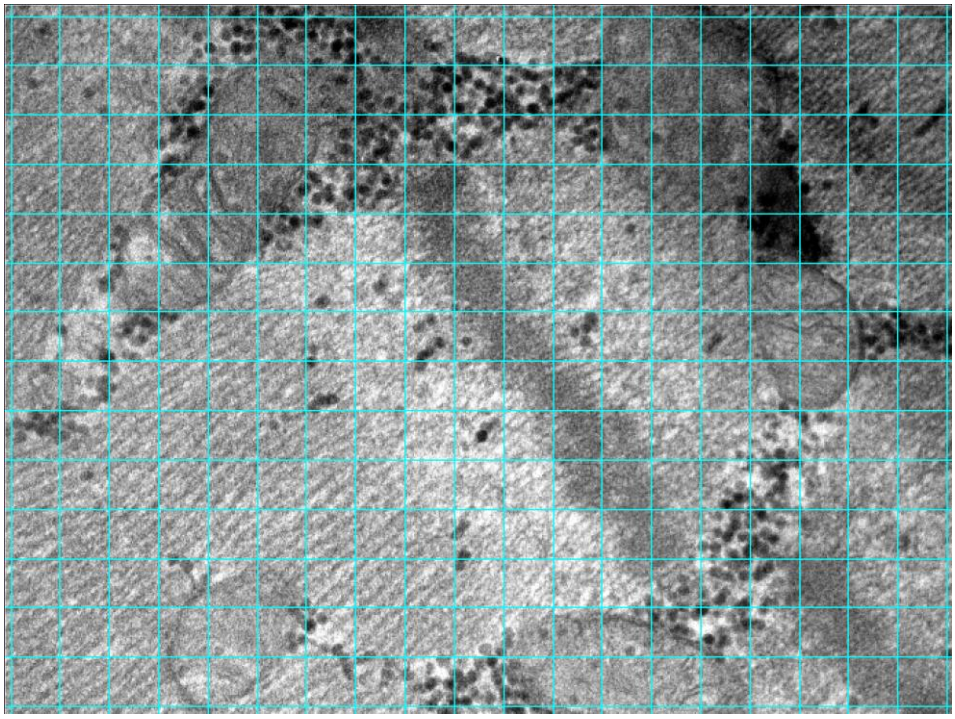

**Figure 3.15** Image illustrating the 135 nm grid applied to the image in order to count mitochondria in the intermyofibrillar region.

### 3.10.6 Glycogen counting

Following fibre type determination, glycogen quantification was undertaken in the three distinct localisations within the myofiber: 1) the subsarcolemmal space, 2) the intermyofibrillar space and 3) the intramyofibrillar space. In relation to the subsarcolemmal space, the available length of the subsarcolemmal region is firstly measured before a 180 nm grid (see Figure 3.16) is applied to the image and glycogen is counted by counting every glycogen particle that appears directly under the cross of each grid. Approximately 8 glycogen particles were then measured to gather particle diameter data. To calculate the myofibrillar glycogen, first the interspace points were counted and recorded using a 300 nm grid, this measure refers to any part of the image that is muscle fibre. Glycogen points in the IMF region were then counted using a 180 nm grid (see Figure 3.17), whilst the intra glycogen was counted under a 60 nm grid (see Figure 3.18). The glycogen particle diameter was measured for both of these regions individually.

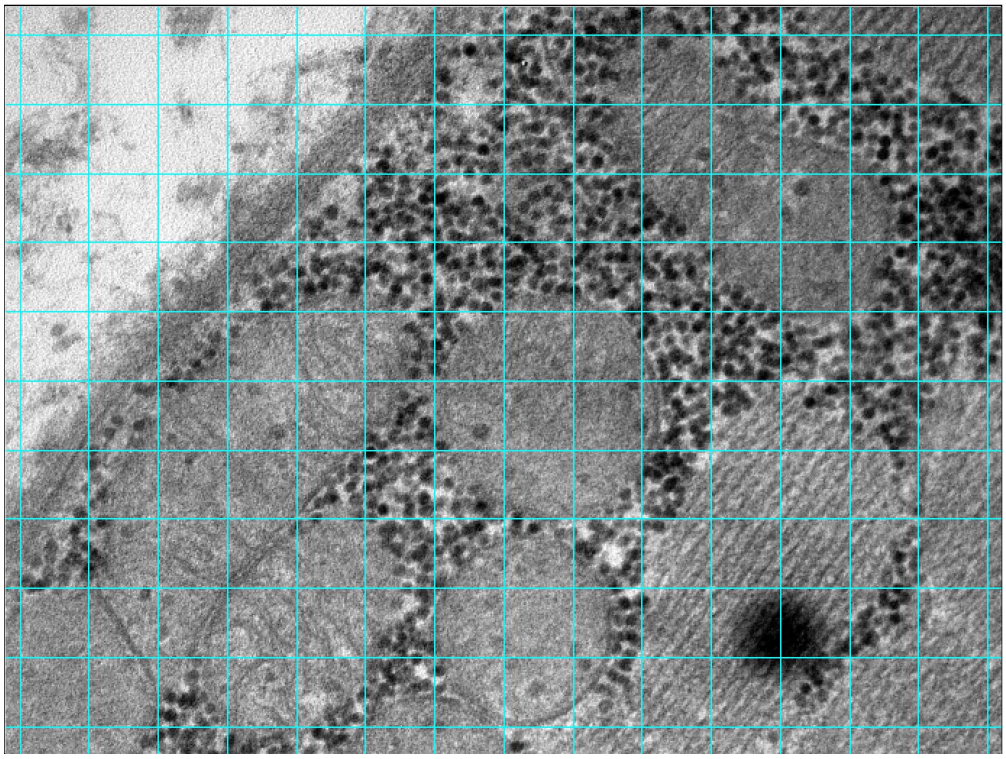

**Figure 3.16** Image illustrating the 180 nm grid applied to the image in order to count glycogen and mitochondria in the subsarcolemmal region.

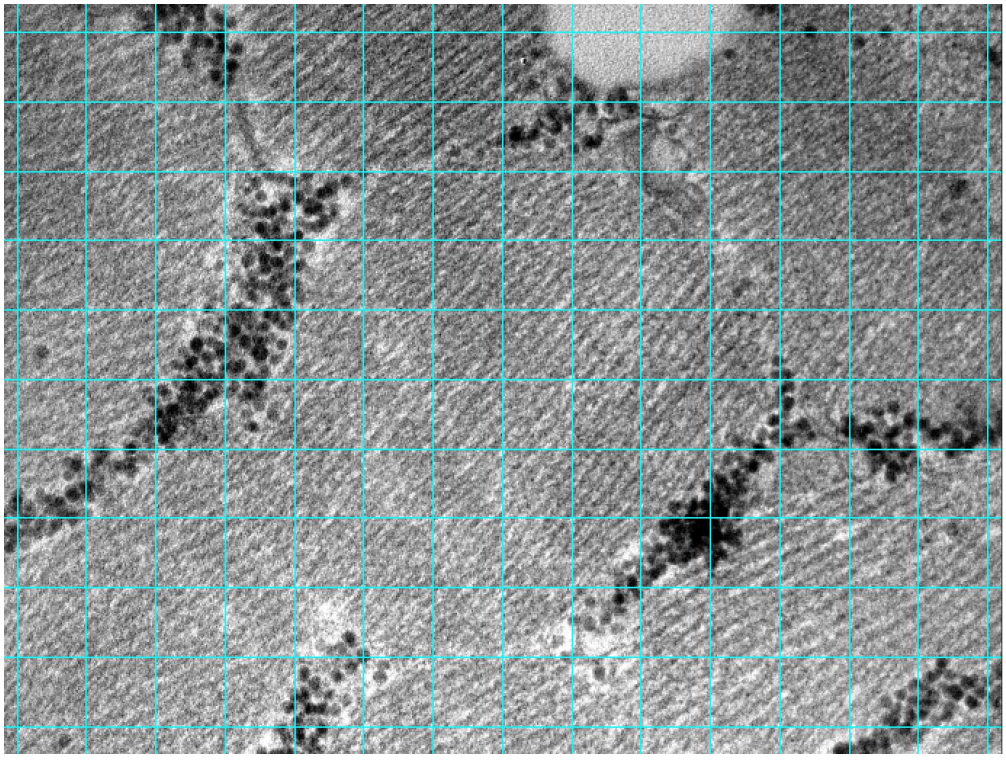

**Figure 3.17** Image illustrating the 180 nm grid applied to the image in order to count intermyofibrillar glycogen.

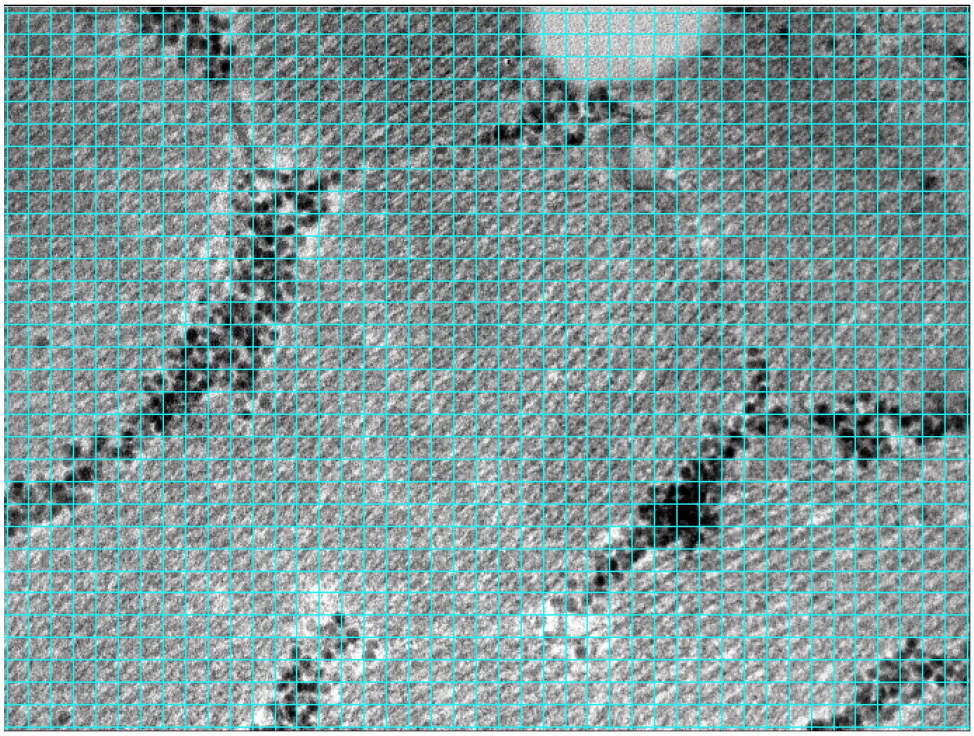

**Figure 3.18** Image illustrating the 60 nm grid applied to the image in order to count intramyofibrillar glycogen.

### 3.10.7 Glycogen quantification

In relation to Study 4 (Chapter 7), the following calculations explain how the absolute and relative distributions of mitochondria and glycogen in the IMF, intramyofibrillar (intra) and SS locations were calculated. For each pool, the glycogen volume fraction ($V_v$) was estimated as proposed by Weibel (Weibel, 1980), under the assumption that glycogen particles are spherical (Melendez-Hevia *et al.*, 1993) and where the effect of section thickness has also been taken into account:

$$V_v = A_A\text{-}t\{(1|\pi)\}B_A - N_A[t + H)]\}$$

Where $A_A$ is glycogen area fraction, $t$ is section thickness, $B_A$ is the glycogen boundary length density, $N_A$ is the number of particles per area and ǂ is the average profile diameter.

The glycogen area fraction was estimated by point counting (as previously described in section 3.11.4) relative to myofibrillar space (400 nm, including space occupied by myofibrils, mitochondria, sarcoplasmic reticulum, *t*-system, and lipids), intramyofibrillar space (400 nm, space occupied by myofibrils), and muscle fibre surface area (estimated from direct length measurement) for intermyofibrillar (180 nm), intramyofibrillar (60 nm), and subsarcolemmal glycogen (180 nm), respectively. The subsarcolemmal glycogen was expressed relative to the muscle fibre surface (estimated from direct length measurement) providing assurance that the results were unbiased, from differences in other subsarcolemmal organelles (e.g. mitochondria, nuclei and lipids). Different grid sizes were chosen based on expected area fractions in each pool to balance the coefficient of error (CE) and workload of analyses. The estimated CE as proposed by Howard and Reed (2005) was 0.15, 0.18 and 0.21 for intermyofribillar, intramyofribillar and subsarcolemmal glycogen, respectively.

The relative contribution of glycogen in each subcellular location to total glycogen per fibre is then calculated using the following equations:

IMF: IMF glycogen $V_v$ / Total glycogen

Intra: (Intra glycogen * Interspace) / total glycogen

SS: (SS glycogen / 20) / total glycogen) / 20 in order to correct for unit difference of absolute data.

To validate the stereological quantification of glycogen, inter-investigator variability between the present investigator and an experienced investigator in quantifying glycogen from TEM images was evaluated. The inter-investigator agreement was 96% and the intra-investigator agreement was 98%.

### 3.10.8 Mitochondria quantification

IMF mitochondrial volume fraction was calculated by using the reference point for mitochondria grid space divided by the number of fibres. The sum of IMF mitochondria is then divided by this to give the area fraction of IMF mitochondria in the Superficial region. SS mitochondria is not a volume fraction but is expressed as volume per surface area. The volume of the image is multiplied by the volume fraction to obtain an absolute volume. The absolute volume per length of fibre is divided by the thickness of the section to obtain an estimate per surface area of the fibre.

## 3.11 Ethical approval

All participants of each study gave written informed consent prior to participation after all experimental procedures and potential risks had been fully explained. Study 1-4 was approved by the Ethics Committee of Liverpool John Moores University (Ethics No: 16 SPS 030; see Appendix 1) and conformed to the standards set by the latest revision of the *Declaration of Helsinki* (except for registration in a database). Study 5 data collection was undertaken in line with routine data often collected for this athlete as part of his contractual obligation (Harris *et al.*, 2020).

## 3.12 Sample size estimation

In relation to Study 2, Smith *et al.* (2010) had previously calculated that a sample size of 15 (post hoc power calculations) was required to confidently conclude if there was a dose-response relationship between CHO ingestion during prolonged submaximal cycling exercise (>2 h) and subsequent performance. Although our aim was to try and recruit this number of participants, our sample size was considerably less based on pragmatic reasons, balancing logistical, financial and recruitment-related considerations for such an invasive and arduous experimental protocol. However, in regard to estimates of whole-body metabolism, respiratory

exchange ratio (RER) would also be assessed in Study 2. As such, a previous study (Coyle *et al.*, 1996) similar to that of Study 2, where 7 endurance-trained cyclists exercised at 71% $\dot{V}O_{2max}$ to fatigue, while ingesting either a CHO solution or a placebo, reported a higher RER in the CHO condition compared to the placebo with a difference of 0.06 ± 0.02. The exercise intensity and duration in that previous study was similar to the exercise protocol proposed in Study 2. Therefore, based on this estimate of RER, a sample size of 5 male subjects completing the study will have 90% power to detect to a change in RER of 0.06 with a standard deviation of 0.02 (Coyle *et al.*, 1986) as estimated using MiniTab Statistical Software (v. 17.2.1; MiniTab Inc, Pennsylvania, USA). Therefore, to account for dropouts, up to 8 subjects will be randomised in Study 2.

In relation to Study 3, in a previous study (Stellingwerff *et al.*, 2007a) similar to that of Study 3, where 10 male endurance-trained cyclists were studied twice during 3 h of cycling exercise at 50% $W_{max}$ with either CHO ingestion or without, the net decline in muscle glycogen content after exercise was 38 ± 19 and 57 ± 22% greater in type I and II muscle fibres without CHO ingestion during exercise compared to CHO ingestion during exercise. As such, based on these findings a sample size of 7 male subjects completing the study will have 90% power to detect a change in muscle glycogen content in type I and II fibres as estimated using MiniTab Statistical Software (v. 17.2.1; MiniTab Inc, Pennsylvania, USA). Therefore, to account for dropouts, up to 8 subjects will be randomised in Study 3.

### 3.13 Statistical analysis

All statistical analyses were performed using the statistical package for social sciences (SPSS, Version 26, IBM, USA) with the specific statistical tests presented in each subsequent chapter. All data in text, figures and tables are presented as means ± standard deviation (SD) with $P$ values ≤ 0.05 indicating statistical significance. All figures were designed in Prism (Version 8, Graphpad, USA).

# Chapter Four:

# The development of a prolonged submaximal endurance cycling protocol and exercise capacity test in trained male cyclists

The aim of this chapter was to develop a suitable exercise protocol that could be used in subsequent experimental chapters to examine the dose-response of CHO feeding during exercise on muscle fuel selection and exercise capacity. Importantly, the exercise protocol was to be completed after prior CHO loading and consumption of a pre-exercise CHO rich meal.

## 4.1 Abstract

**Purpose:** To develop a prolonged steady state submaximal endurance cycling protocol and exercise capacity test that is deemed suitable to study the dose-response effects of CHO feeding on muscle fuel selection and exercise capacity. **Methods:** In a repeated measures design, eight endurance trained male cyclists completed 180-min submaximal cycling at lactate threshold (198 ± 29 W) followed immediately by a cycling capacity test to exhaustion at 150% lactate threshold (299 ± 42 W) on three separate occasions. Subjects commenced each exercise session after a 40-h exercise and dietary standardisation protocol consisting of prior glycogen depleting exercise, a CHO loading regimen (13.6 g·kg$^{-1}$ body mass (BM)) and a pre-exercise CHO rich meal (3 g·kg$^{-1}$ BM) at 3 hours prior to commencing each trial. **Results:** Mean CHO (2.32 ± 0.51, 2.35 ± 0.45, 2.37 ± 0.49 g·min$^{-1}$; $P$ = 0.759) and fat oxidation rates (0.52 ± 0.20, 0.53 ± 0.20, 0.51 ± 0.20 g·min$^{-1}$; $P$ = 0.699) during exercise did not differ between Trial 1, 2 and 3, respectively, although rates of CHO oxidation decreased ($P$ < 0.001) and fat oxidation increased throughout exercise ($P$ < 0.001). Capillary lactate was found to progressively decrease throughout exercise ($P$ = 0.004), with lower mean lactate concentrations during Trial 3 compared with Trial 1 ($P$ = 0.039) whereas no differences were apparent between Trial 1 and 2 ($P$ = 0.691) or Trial 2 and Trial 3 ($P$ = 0.384). In addition, both RPE and heart rate (HR) decreased between Trial 1 and 3 (RPE: $P$ = 0.009; HR: $P$ = 0.003) with no apparent differences between Trial 1 and 2 (RPE: $P$ = 0.065; HR: $P$ = 0.219) and Trial 2 and 3 (RPE: $P$ = 0.229; HR: $P$ = 0.071, respectively). After completion of the 180-min submaximal exercise protocol, exercise capacity was significantly different ($P$ = 0.042) between Trial 3 and 1 (386 ± 262 vs. 260 ± 247 s: 95% CI = 5 to 246 s) whereas no differences were observed between Trial 1 and 2 (260 ± 247 vs. 324 ± 262 s: 95% CI for differences = -37 to 164 s; $P$ = 0.268) or Trial 2 and 3 (324 ± 262 vs. 386 ± 262 s: 95% CI for differences = -65 to 189 s; P = 0.515). **Conclusion:** In conditions of high pre-exercise CHO availability, data demonstrate that trained male cyclists are capable of completing the 180-min submaximal exercise protocol and that fatigue during the subsequent exercise capacity test typically occurs within <10 minutes. However, the significant reductions in capillary lactate, RPE and HR during the submaximal protocol between Trial 1 and 3 in combination with the significant extension in exercise capacity between Trial 1 and Trial 3 suggest that at least one full familiarisation session should be completed prior to studying the effects of a specific CHO feeding strategy on metabolism and performance in trained male cyclists.

## 4.2 Introduction

In order to investigate the effects of nutritional interventions on metabolism and exercise performance, it is imperative to consider the use of an appropriately valid and reliable exercise protocol and performance test. Such protocols, within an appropriate experimental design, will allow researchers to ascertain the true effects of any performance and/or metabolic effects whilst promoting the translational potential of any findings to the chosen sporting arena (Close *et al.*, 2019). In this regard, time trial (TT) and time to exhaustion (TTE) exercise capacity tests are two of the most common approaches used in the literature. Traditionally, TTE capacity tests require subjects to exercise at a fixed intensity (most commonly represented as a percentage of maximal oxygen uptake ($\dot{V}O_{2max}$; maximal power output, $W_{max}$; or lactate threshold, LT) until volitional exhaustion. In contrast, a simulated TT requires subjects to complete a set distance or set amount of work as fast as possible. This type of protocol has been reported to have a higher reliability compared to TTE protocols with TTs typically reporting a coefficient of variation (CV) less than 5% whilst TTE reporting a CV of more than 10% (Currell & Jeukendrup, 2008; Jeukendrup, *et al*., 1996; Laursen *et al.*, 2007; McLellan *et al*., 1995; Palmer *et al.*, 1996; Schabort *et al.*, 1998). In spite of this, other studies have demonstrated similar sensitivity between TT and TTE endurance tests (Alghannam *et al.*, 2016; Amann *et al.*, 2008). In addition, TTs have been considered to be more ecologically valid than TTE tests given athletes are generally required to complete a set distance as quickly as possible during real world competition (Currell *et al.*, 2006). However, it must be noted that TT protocols are likely to produce variability in exercise intensity between trials, whereas TTE protocols enable a controlled intensity to be set, thus providing a controlled environment for comparisons of certain metabolic variables between trials (Currell & Jeukendrup, 2008).

Ultimately, the selection of an exercise protocol should also be considered in the context of the specific research question being addressed and in accordance with the specific demands and performance characteristics of the sport of interest. As such, both a TT and TTE capacity test could both be considered as a valid measure of performance within professional road cycling given that both situations present themselves in the form of designated time-trial stages and the ability to respond to ‘attacks’ on mountain climbs, respectively (Close *et al.*, 2019; Padilla *et al.*, 2007). Importantly, both of these competitive situations are known to have a major impact upon the final overall standings of a multi-day cycling stage race (Padilla *et al.*, 2007). Mountain stages within professional road cycling have typically been observed to require long

periods of submaximal, constant power outputs, when riding conservatively within the 'peloton' in attempts to save energy, followed by higher maximal mean power outputs during later parts of the stage, for example during final mountain climbs and summit finishes (Padilla *et al.*, 2007; Sanders *et al.*, 2018; Van Erp *et al.*, 2020; Vogt *et al.*, 2007). In addition, during periods of climbing and summit finishes, team leaders will often attempt to gain time over their competitors through accelerations in power output otherwise known as 'attacks'. In turn, rivals will endeavour to respond by attempting to 'hold the wheel' of that attacking rider in order to minimise or nullify these attacks. In addition, in other specific race scenarios, key domestique riders will aim to maintain a prolonged high tempo on a mountain climb in attempts to reduce the size of the main General Classification (GC) group of riders and put extra strain on rival teams. Such scenarios involve key domestiques to hold a given power output for as long as possible and once they can no longer hold the required power output they will "swing off" and allow another key domestique to take up the pace or alternatively set up an "attack" for their team's GC rider. When considered this way, TTE tests may be more applicable in such scenarios given the performance requirement to maintain the given power output of the attacking rider for as long as possible. Therefore, specific laboratory-based exercise protocols should be devised in line with the specific categorisation of a cycling stage and resemble the performance which is being simulated as closely as possible (Hopkins, 2000).

In addition to the described considerations in devising an ecologically valid and reliable endurance cycling protocol, many previous studies examining the reliability of endurance exercise protocols have not necessarily adhered to best nutritional practice and have commenced trials after an overnight fast (Alghannam *et al.*, 2015) or reported limited information regarding pre-trial nutritional information (Amann *et al.*, 2008; Jeukendrup *et al.*, 1996; McLellan *et al*., 1995). Such practices can reduce the ecological validity of any findings given these conditions are rarely practiced by elite endurance athletes during competition, whereby they will typically consume high-CHO in the day(s) and hours before competition. Moreover, the importance of standardising pre-exercise nutritional feeding and energy status prior to reliability trials is apparent given that feeding before and/or during exercise can profoundly alter metabolic responses to exercise and influence performance (Braun & Brooks, 2008; Jeacocke & Burke, 2010; Hopkins *et al.*, 1999; Horowitz *et al.*, 1997). This suggests, that the reliability of specific exercise protocols should be examined in ecologically valid nutritional situations in accordance with the competition nutritional habits of elite endurance athletes (Burke and Hawley, 2018a; Burke *et al.*, 2018b; Ebert *et al.*, 2007; García-Rovés *et*

*al.,* 1998; Heikura *et al.,* 2019; Muros *et al.,* 2019; Pfeiffer *et al.,* 2012; Rehrer *et al.,* 2010; Ross *et al.,* 2014; Sànchez-Muñoz *et al.,* 2016; Saris *et al.,* 1989; Stellingwerff, 2012).

Accordingly, the aim of the present study is therefore to develop a prolonged steady state submaximal endurance cycling protocol and exercise capacity test that is deemed suitable to study the dose-response effects of CHO feeding on muscle fuel selection and exercise capacity in subsequent chapters. In this way, our experimental design utilised an exercise protocol aimed at simulating real-world endurance road cycling undertaken in conditions of best pre-exercise nutritional practice, i.e after prior CHO loading and consumption of a pre-exercise CHO rich meal.

## 4.3 Methods

### 4.3.1 Participants

Eight endurance trained male cyclists (mean (SD): age 26 ± 7 years, body mass 74.6 ± 7.2 kg) volunteered to take part in this study. Mean (SD) $\dot{V}O_{2max}$ and peak power output (PPO) for this cohort were 60.0 ± 4.5 $mL.kg^{-1}.min^{-1}$ and 392 ± 40 W (5.3 ± 0.4 $W \cdot kg^{-1}$), respectively. All participants provided written informed consent before commencement of the study, which was approved by the local Ethics Committee of Liverpool John Moores University. Subjects were defined as trained in accordance with the criteria of Jeukendrup, Craig & Hawley (2000) whereby the participants were competitive club level road cyclists as detailed by the subject characteristics and the training and racing data described in Table 3.1. None of the subjects had any history of musculoskeletal or neurological disease nor were they under any pharmacological treatment during the course of the testing period. All participants provided written informed consent before commencement of the study, which was approved by the local Ethics Committee of Liverpool John Moores University.

### 4.3.2 Experimental Design

In a repeated-measures design, separated by a minimum of 7 days, subjects undertook 180 minutes of steady state submaximal exercise (undertaken at LT, 198 ± 29 W) followed by a cycling capacity test to exhaustion (undertaken at 150% of LT, 299 ± 42 W) on three separate occasions. This protocol was designed in an attempt to replicate a real-world endurance road cycling race whereby riders will typically ride in the “peloton” at a submaximal intensity for

the first few hours before “attacking” on mountain climbs / finishes in order to gain time over their opponents (Padilla *et al.*, 2007). To promote high CHO availability prior to the main experimental trials and in accordance with real world practice and current sport nutrition competition recommendations (Thomas *et al.*, 2016), subjects commenced the main experimental trials after completion of a CHO loading protocol (13.6 $g \cdot kg^{-1}$ consumed over 36 h) and consumption of a pre-exercise meal (3 $g \cdot kg^{-1}$ of CHO consumed at 3 h prior to exercise). A schematic of the experimental procedure is shown in Figure 4.1.

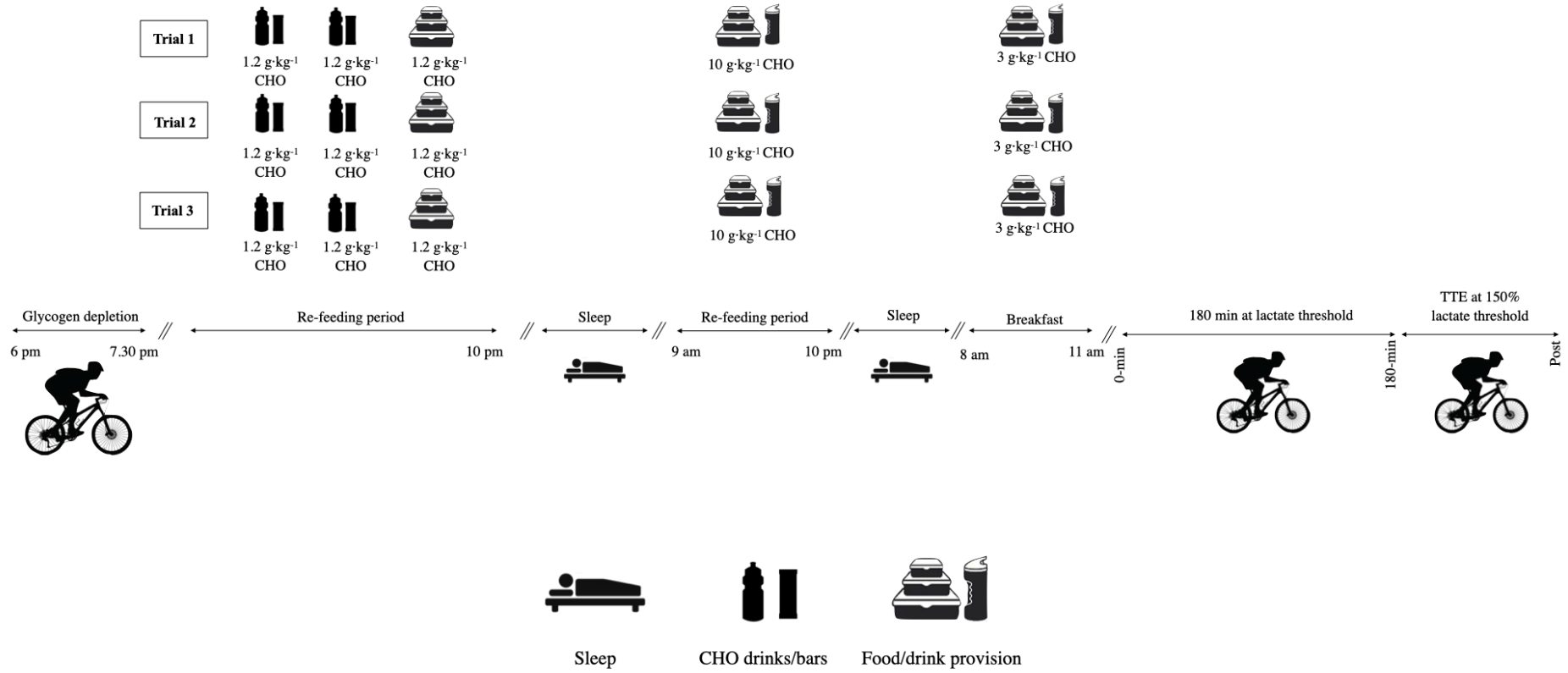


**Figure 4.1** Schematic overview of the experimental protocol. On the evening of day 1, subjects completed a glycogen depletion protocol followed by 3-h of best recovery nutritional practice. Throughout the entirety of day 2, subjects consumed a high carbohydrate (CHO) diet. During the main experimental trial on day 3, subjects performed a simulated endurance cycling protocol after consuming a high CHO pre-exercise meal 3-h before the trial. The cycling protocol consisted of a 180-min submaximal cycle at lactate threshold followed immediately by a cycling capacity test to exhaustion (TTE) at 150% lactate threshold.

### 4.3.3 Assessment of maximal oxygen uptake and lactate threshold

At least 7 days prior to experimental trials, all subjects performed a two-part incremental cycle test to determine peak oxygen consumption ($\dot{V}O_{2max}$), LT and peak power output (PPO) on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands). The two-part incremental cycling test has been detailed previously in Section 3.2.4.

### 4.3.4 Experimental protocols:

#### 4.3.4.1 Day 1: Glycogen depletion protocol

At 36-h before the main experimental trial (i.e., evening of Day 1), subjects reported to the laboratory at approximately 18:00 to perform a bout of intermittent glycogen depleting cycling, as previously completed in our laboratory (Taylor *et al.,* 2013; Impey *et al.,* 2016). Immediately before beginning the glycogen depletion protocol nude body mass was recorded (SECA, Hamburg, Germany) and a heart rate (HR) monitor (Polar H7, Kempele, Finland) was fitted. Subjects then performed an intermittent glycogen-depleting cycling protocol lasting 90-minutes. After a 15-min self-selected warm-up, participants cycled for 2-minutes at 90% PPO, followed immediately by a 2-minute recovery period at 50% PPO. They repeated this work to rest ratio until they could no longer complete 2-minutes cycling at 90% PPO, determined as an inability to maintain a cadence of 60 revs·min$^{-1}$. At this point exercise intensity remained the same but the work to rest ratio changed from 1-min at 90% PPO followed by the same 2-minute recovery period at 50% PPO. When participants could no longer maintain this work to rest ratio the exercise intensity was lowered to 80% PPO with the same durations as before being undertaken. When 80% PPO could no longer be maintained it was lowered to 70% and finally 60% PPO. However, if the elapsed time of the intermittent exercise had reached 90-minutes the exercise was terminated. The activity pattern and total TTE for the glycogen depleting ride was recorded for each participant during their first trial and replicated for the remainder of the trials. This intermittent pattern of exercise was employed so as to induce glycogen depletion in both type I and II fibres. HR and RPE were recorded at 2-minute intervals and body mass was also measured immediately after the exercise bout. Water intake was consumed ad libitum throughout exercise in the initial trial and was recorded and repeated for subsequent trials.

#### 4.3.4.2 Day 2: Carbohydrate loading regimen

To facilitate the goal of elevating muscle glycogen concentrations in preparation for the prolonged cycling protocol, subjects initially completed a CHO loading protocol in accordance with current competition nutritional guidelines (Thomas *et al.,* 2016) and the daily energy intake of elite road cyclists during (Heikura *et al.,* 2019; Muros *et al.,* 2019; Morton, unpublished observations; Sánchez-Muñoz *et al.,* 2016). Subjects were provided with high carbohydrate snacks and fluids (SiS GO Energy, SiS REGO Protein, SiS GO Bars, Science in Sport, Blackburn, UK) to consume immediately post glycogen depletion exercise (CHO: 1.2 $g \cdot kg^{-1}$ BM; PRO: 0.4 $g \cdot kg^{-1}$ BM; Fat: 0 $g \cdot kg^{-1}$ BM), at 1-hour post (CHO: 1.2 $g \cdot kg^{-1}$ BM; PRO: 0.1 $g \cdot kg^{-1}$ BM; Fat: 0 $g \cdot kg^{-1}$ BM) and 2-hours post (CHO: 1.2 $g \cdot kg^{-1}$ BM; PRO: 0.1 $g \cdot kg^{-1}$ BM; Fat: 0 $g \cdot kg^{-1}$ BM) (see Table 3.3). Over the course of the following day (i.e., Day 2), participants were provided with a pre-packaged standardised high CHO diet to consume throughout the entirety of that day (CHO: 10 $g \cdot kg^{-1}$ BM; PRO: 2 $g \cdot kg^{-1}$ BM; Fat: 1 $g \cdot kg^{-1}$ BM) (see Table 3.3) and refrained from any strenuous physical activity and alcohol consumption during this day to promote muscle and liver glycogen resynthesis (Bartlett *et al.,* 2013). Fluid intake was advised to water only and was allowed *ad libitum*.

#### 4.3.4.3 Day 3: Experimental trials

On the morning of the main experimental trials at ~08:00, subjects reported to the laboratory in a fasted state and were immediately provided with a standardized high-CHO "Race-day" breakfast (CHO: 3 $g \cdot kg^{-1}$ BM; PRO: 40 g; Fat: 35 g; comprising porridge oats, semi-skimmed milk, honey, orange juice, bread, jam, banana and eggs) that was representative of an elite real-world cyclists 'Race-day" breakfast (see Table 3.3) (Heikura *et al.,* 2019; Muros *et al.,* 2019; Morton, unpublished observations; Sánchez-Muñoz *et al.,* 2016). At 3-h post-prandial and immediately before the beginning of the cycling protocol, a capillary blood sample (fingertip) was obtained for blood lactate concentration analysis by micro-assay (Lactate Pro 2, ArkRay Inc., Kyoto, Japan). Subjects were then fitted with a heart rate monitor (Polar Electro, Finland) and nude body mass (SECA, Hamburg, Germany) was recorded. Resting values for expired gas were also recorded at this time-point and were measured via a mouthpiece connected to an automated gas analyser machine Moxus Modular Metabolic System (AEI Technologies, IL, USA) for a 5-minute period**.** The gas analyser was calibrated before each experimental trial according to the manufacturer's guidelines (Beltrami *et al.,* 2014). Participants then completed

a 10-minute warm-up at 100 W and began the 180-min cycle preload at LT. LT was chosen as the relative exercise intensity as it has been reported to be a better method of matching metabolic stress between participants when compared to exercising at a percentage of $\dot{V}O_{2max}$ (Baldwin *et al.,* 2000). During the 180-min steady state submaximal exercise protocol capillary blood lactate samples, heart rate, RPE and cycling cadence were obtained at 30-minute intervals throughout. Expired gas was also collected for 5-minutes at 30-minute intervals (Moxus Modular Metabolic System, AEI Technologies, IL, USA) in order to calculate cycling gross efficiency and substrate utilisation. Furthermore, subjects were provided with 125 ml of a lemon flavoured electrolyte drink containing 0 g CHO (SiS GO Hydro, Science in Sport, Blackburn, UK) at 20-minute intervals from 0 to 180 min with 250 ml consumed at 60 and 120 min which totalled 500 $ml \cdot h^{-1}$ of this solution. Immediately following the 180-min submaximal cycle, participants undertook the cycling capacity test whereby they cycled at 150% LT to volitional exhaustion. No music was played, and no physiological measurements were taken during the cycling capacity test. The only available information to the participants was the fixed power output and cadence. Every effort was made to ensure subjects were not disturbed throughout the performance trials. Upon cessation of the capacity test, a capillary blood lactate was recorded followed by a measurement of post-exercise body mass. No performance results were shown to the subjects until the completion of the study. All exercise tests were performed at the same time of day (± 1h) under normal laboratory conditions (20 – 23˚C and 50 – 60% humidity) using the same electrically braked cycle ergometer (Lode Excaliber Sport, Groningen, Netherlands), blood lactate micro-assay (Lactate Pro 2, ArkRay Inc., Kyoto, Japan) and automated gas analyser machine Moxus Modular Metabolic System (AEI Technologies, IL, USA). During exercise trials, subjects were cooled with a floor standing fan to minimise thermal stress. Each experimental trial was separated by 7-10 days and subjects were asked to continue with their habitual training schedule during this period which equated to 02:58 ± 01:07 (hh:min) and 77 ± 33 km (excluding the 90-min glycogen depletion and ~180-min cycling protocol).

### 4.3.5 Estimates of whole-body substrate oxidation, total energy expenditure and cycling gross efficiency

Rates of whole-body CHO and fat oxidation ($g \cdot min^{-1}$) were calculated using the equations of Jeukendrup and Wallis (2005) as described in Section 3.2.5. Cycling gross efficiency was

calculated according to Hopker *et al.* (2009) as described in Section 3.2.6. Total energy expenditure was estimated for each trial assuming an energy yield of 17.57 kJ and 39.33 kJ for 1 g of CHO and fat, respectively.

### 4.3.6 Statistical analysis

All statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS version 26). Descriptive statistics were produced for all data sets to check for normal distribution indicated by Shapiro-Wilk (accepted if $P > 0.05$). Changes in exercise capacity and total CHO and fat oxidation were analysed using a one-way repeated-measures general linear model (GLM), whereas comparisons of average physiological responses, metabolic responses and blood lactate concentrations were analysed with a two-way repeated-measures GLM, where the within factors were time and trial. If Mauchley's test of sphericity indicated a minimum level of violation, as assessed by a Greenhouse Geisser epsilon ($\varepsilon$) of > 0.75, data were corrected using Huyn-Feldt $\varepsilon$. If Mauchley's test of sphericity was violated, data were corrected using Greenhouse Geisser $\varepsilon$. Where a significant main effect was observed, pairwise comparisons were analysed according to Bonferroni post hoc tests to locate specific differences. Individual CVs were calculated for each participant by dividing the SD by the mean, multiplied by one hundred and then averaged to obtain an overall CV for TTE. 95% confidence intervals for TTE were calculated using the method described by Bland and Altman (1986). All data in text, figures, and tables are presented as means ± SD, with $P$ values < 0.05 indicating statistical significance.

## 4.4 Results

### 4.4.1 Physiological responses to submaximal exercise protocol

Comparisons of subjects' HR and RPE are displayed in Figure 4.2, respectively, whilst comparisons of $\dot{V}O_2$, $\dot{V}CO_2$, $\%\dot{V}O_{2max}$, $\dot{V}O_2$ ($ml \cdot kg \cdot min^{-1}$), cycling efficiency and cycling cadence are displayed in Table 4.1. Both HR and RPE were significantly different between trials (HR: $P = 0.007$; RPE: $P = 0.018$) with a specific reduction between Trial 1 and 3 (HR: $P = 0.01$; RPE: $P = 0.026$) whereas no differences occurred between Trial 1 and 2 (HR: $P = 0.657$; RPE: $P = 0.195$) or Trial 2 and 3 (HR: $P = 0.214$; RPE: $P = 0.687$), respectively. RPE progressively increased during exercise ($P = 0.001$) whilst exercise duration had no effect upon HR ($P = 0.221$). In addition, $\dot{V}O_2$ ($P = 0.454$), $\dot{V}CO_2$ ($P = 0.495$), $\%\dot{V}O_{2max}$ ($P = 0.473$), $\dot{V}O_2$

(ml·kg·min$^{-1}$) ($P$ = 0.437), cycling cadence ($P$ = 0.226) and cycling efficiency ($P$ = 0.487) were all not significantly different between trials with both V̇CO2 ($P$ = 0.001) and cycling efficiency ($P$ = 0.031) progressively decreasing during exercise. In contrast, $\dot{V}O_2$ ($P$ = 0.525), $\%\dot{V}O_{2max}$ ($P$ = 0.561), $\dot{V}O_2$ (ml·kg·min$^{-1}$) ($P$ = 0.523) and cycling cadence ($P$ = 0.2) did not change during exercise (see Table 4.1).

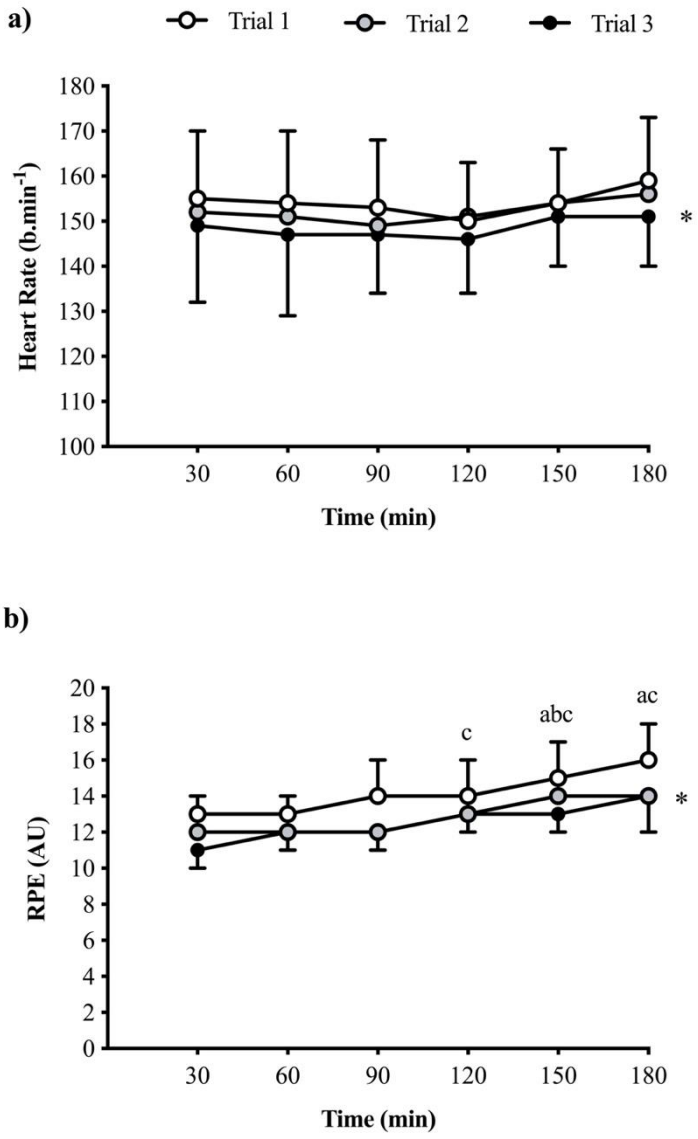


**Figure 4.2** (a) Heart rate and (b) RPE responses during the 180-min submaximal cycling protocol. *denotes significant difference from Trial 1, $P$<0.05. [a]denotes significant difference from 30-min, [b]denotes significant difference from 60-min and [c]denotes significant difference from 90-min, $P$<0.05. Data is presented as mean ± SD.

**Table 4.1** Cycling cadence, $\dot{V}O_2$, $\dot{V}CO_2$, $\dot{V}O_2$ ml·kg·min$^{-1}$, exercise intensity (%$\dot{V}O_{2max}$) and cycling efficiency during the 180-min submaximal cycling protocol.

| | Time (min) | | | | | |
|---|---|---|---|---|---|---|
| | 30 | 60 | 90 | 120 | 150 | 180 |
| **Cadence (rpm)** | | | | | | |
| Trial 1 | 84 ± 6 | 83 ± 7 | 81 ± 7 | 84 ± 8 | 81 ± 10 | 77 ± 9 |
| Trial 2 | 85 ± 7 | 87 ± 7 | 84 ± 7 | 84 ± 9 | 87 ± 11 | 83 ± 12 |
| Trial 3 | 84 ± 6 | 84 ± 8 | 82 ± 7 | 82 ± 7 | 85 ± 7 | 83 ± 9 |
| **$\dot{V}O_2$ (ml·min$^{-1}$)** | | | | | | |
| Trial 1 | 2915 ± 328 | 2923 ± 333 | 2968 ± 338 | 2958 ± 330 | 2961 ± 311 | 2870 ± 247 |
| Trial 2 | 2905 ± 357 | 2930 ± 330 | 2958 ± 331 | 3009 ± 352 | 3030 ± 319 | 2978 ± 307 |
| Trial 3 | 2941 ± 344 | 2968 ± 357 | 2933 ± 288 | 2895 ± 307 | 2963 ± 290 | 2933 ± 310 |
| **$\dot{V}CO_2$ (ml·min$^{-1}$)** | | | | | | |
| Trial 1 | 2765 ± 318 | 2709 ± 322 | 2678 ± 316 | 2589 ± 306 | 2532 ± 314[c] | 2420 ± 219 |
| Trial 2 | 2743 ± 350 | 2699 ± 326 | 2676 ± 341 | 2653 ± 346 | 2615 ± 303[c] | 2497 ± 282 |
| Trial 3 | 2781 ± 344 | 2747 ± 346 | 2672 ± 267 | 2552 ± 275 | 2570 ± 299[c] | 2466 ± 288 |
| **$\dot{V}O_2$ (ml·kg·min$^{-1}$)** | | | | | | |
| Trial 1 | 38.5 ± 4.7 | 38.6 ± 4.9 | 39.3 ± 4.8 | 39.0 ± 4.5 | 39.1 ± 4.6 | 37.8 ± 3.0 |
| Trial 2 | 38.4 ± 5.2 | 38.8 ± 5.2 | 39.1 ± 4.9 | 39.8 ± 5.2 | 40.1 ± 5.2 | 39.4 ± 4.9 |
| Trial 3 | 38.9 ± 4.8 | 39.2 ± 5.0 | 38.8 ± 4.1 | 38.3 ± 4.2 | 39.2 ± 4.4 | 38.8 ± 4.3 |
| **% $\dot{V}O_{2max}$** | | | | | | |
| Trial 1 | 64 ± 6 | 64 ± 5 | 65 ± 5 | 65 ± 4 | 65 ± 4 | 63 ± 3 |
| Trial 2 | 64 ± 7 | 64 ± 7 | 65 ± 5 | 66 ± 5 | 67 ± 5 | 65 ± 5 |
| Trial 3 | 65 ± 5 | 65 ± 6 | 64 ± 4 | 64 ± 4 | 65 ± 4 | 64 ± 5 |
| **Efficiency (%)** | | | | | | |
| Trial 1 | 19.8 ± 1.0 | 19.8 ± 0.9 | 19.7 ± 0.9 | 19.4 ± 0.9 | 19.5 ± 1.1 | 19.7 ± 1.5 |
| Trial 2 | 20.0 ± 1.4 | 19.7 ± 1.3 | 19.7 ± 1.3 | 19.0 ± 0.9 | 18.9 ± 1.3 | 18.8 ± 1.2 |
| Trial 3 | 19.6 ± 0.9 | 19.6 ± 1.0 | 19.5 ± 1.1 | 19.7 ± 1.1 | 19.2 ± 1.2 | 19.1 ± 0.9 |

[c]denotes significant difference from 90-min, ($P < 0.05$). Data is presented as mean ± SD.

### 4.4.2 Metabolic responses to exercise

Mean CHO (2.32 ± 0.51, 2.35 ± 0.45, 2.37 ± 0.49 g·min$^{-1}$; $P$ = 0.759) and fat oxidation rates (0.52 ± 0.20, 0.53 ± 0.20, 0.51 ± 0.20 g·min$^{-1}$; $P$ = 0.699) during exercise did not differ between Trial 1, 2 and 3, respectively, although rates of CHO oxidation decreased ($P$ < 0.001) and fat oxidation increased throughout exercise ($P$ < 0.001). Additionally, no difference in total CHO ($P$ = 0.757) and fat oxidation ($P$ = 0.708) was observed (Figure 4.3). Respiratory exchange ratio (RER) was progressively decreased across the duration of exercise ($P$ < 0.001; Figure 4.3) with no difference between Trial 1, 2 and 3, respectively ($P$ = 0.691). No difference in energy expenditure was observed across exercise duration ($P$ = 0.266; Figure 4.3) or between exercise trials ($P$ = 0.408). Capillary blood lactate was significantly different between trials ($P$ = 0.025) and was progressively reduced across exercise duration ($P$ = 0.004). Specifically, mean capillary blood lactate was significantly lower in Trial 3 compared to Trial 1 ($P$ = 0.039; Figure 4.3) with no significant difference between Trial 1 and 2 ($P$ = 0.691) or Trial 2 and 3 ($P$ = 0.384).

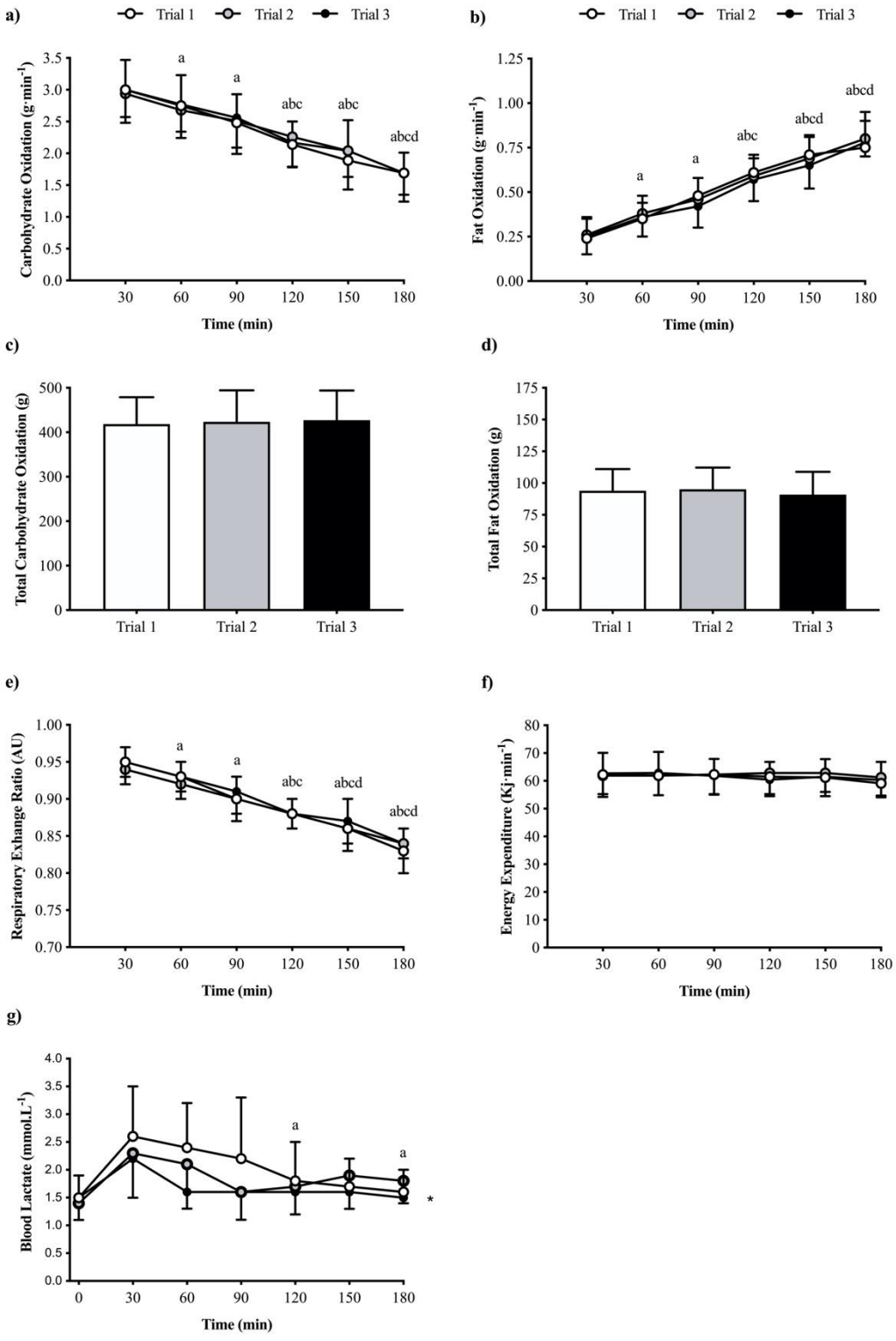


**Figure 4.3** carbohydrate oxidation (a), fat oxidation (b), total carbohydrate oxidation (c), total fat oxidation (d), respiratory exchange ratio (RER) (e), energy expenditure (f) and capillary blood lactate (g) during the 180-min submaximal cycling protocol. Data is presented as mean ± SD. *denotes significant difference from Trial 1. [a]denotes significant difference from 30-min, [b]denotes significant difference from 60-min, [c]denotes significant difference from 90-min, [d]denotes significant difference from 120-min, ($P < 0.05$).

### 4.4.3 Time to exhaustion exercise capacity test

Exercise capacity after 180 minutes of submaximal cycling exercise was significantly different between trials (main condition effect, $P = 0.016$) with an increase in capacity between Trial 3 (386 ± 262 s) and Trial 1 (260 ± 247; $P = 0.042$: 95% CI for differences = 5 to 246 s; Figure 4.4), whilst no differences in capacity were observed between Trial 1 and 2 (324 ± 262; $P = 0.268$: 95% CI for differences = -37 to 164 s, Figure 4.4) or Trial 2 and 3 ($P = 0.515$; 95% CI for differences = -65 to 189 s, Figure 4.4). Table 4.2, panel (a), shows the total time to exhaustion across the whole exercise protocol for the 3 trials completed by the eight participating cyclists. Individual CVs range from 0.4-1.7%, with a mean ± SD value of 0.8 ± 0.4%. Panel (b) shows the time to exhaustion at 150% LT for the exercise capacity component. Individual CVs range from 9-72%, with a mean ± SD value of 34 ± 19%. Figure 4.5 shows the limits of agreement graph for the for time to exhaustion at 150% LT. However, the magnitude of variability indicated by the 95% confidence intervals for the standard deviation of differences between trials is high in comparison to the mean time to exhaustion across all trials (mean ± 1.96 × SD differences for Trials 1 to 3 = 0.0 ± 153.4 s; mean time to exhaustion = 323.1 ± 250.1 s).

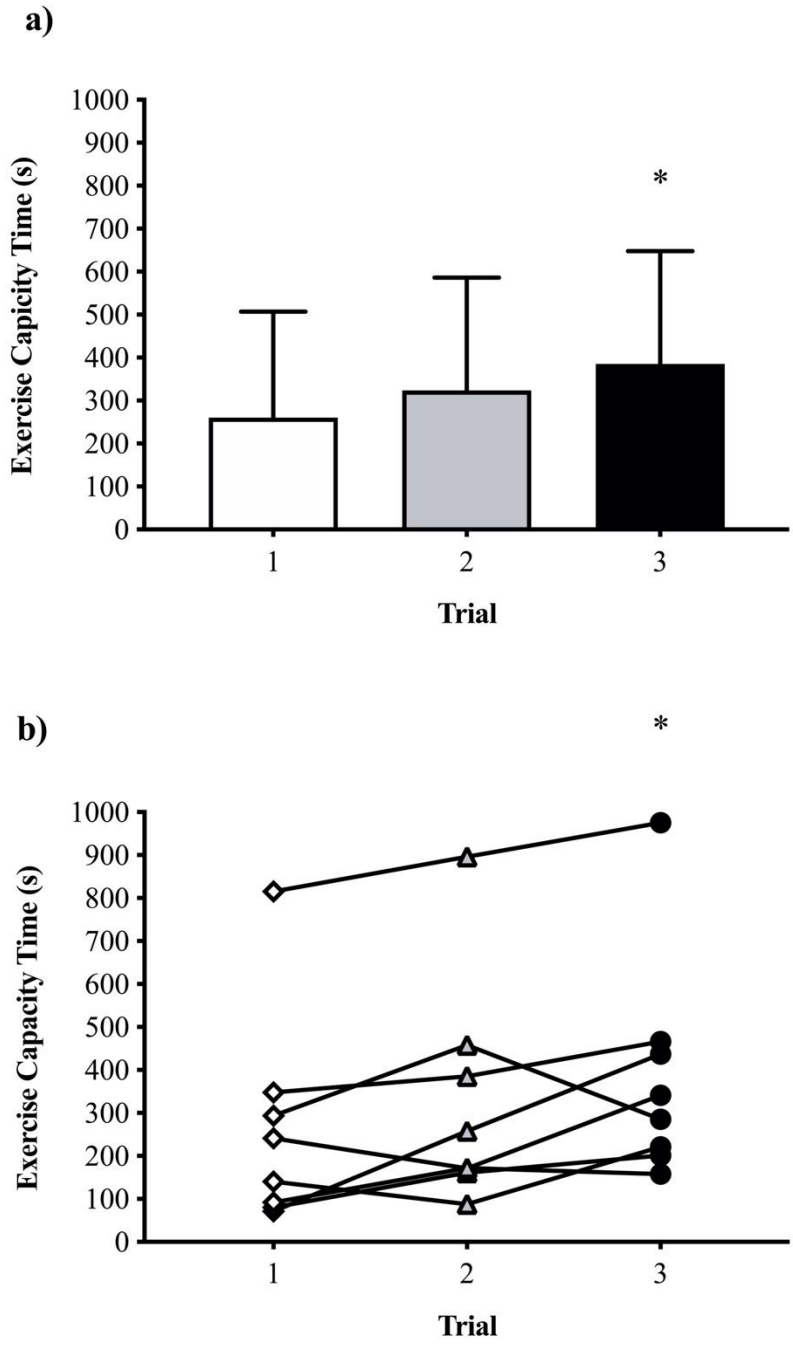


**Figure 4.4** Cycling capacity time (time to exhaustion) for Trial 1, 2 and 3 (a) and individual data points (N=8) (b). Data presented are mean ± standard deviation. *denotes significant difference from Trial 1, $P<0.05$.

**Table 4.2** The individual and mean duration and coefficient of variation (CV) of the cycling protocol with (a) the total time to exhaustion during the cycling capacity test including the 180-min submaximal cycling protocol and (b) the time to exhaustion during the cycling capacity component of the exercise protocol.

**(a) Total time to exhaustion (s)**

| Cyclist | Trial 1 | Trial 2 | Trial 3 | Mean | SD | CV (%) | CV Between Trial 2 and 3 (%) |
|---|---|---|---|---|---|---|---|
| 1 | 11094 | 11258 | 11085 | 11146 | 97 | 0.9 | 1.1 |
| 2 | 11615 | 11696 | 11775 | 11695 | 80 | 0.7 | 0.5 |
| 3 | 10871 | 11058 | 11237 | 11055 | 183 | 1.7 | 1.1 |
| 4 | 11148 | 11185 | 11266 | 11200 | 60 | 0.5 | 0.5 |
| 5 | 10940 | 10888 | 11021 | 10950 | 67 | 0.6 | 0.9 |
| 6 | 10880 | 10961 | 11001 | 10947 | 62 | 0.6 | 0.3 |
| 7 | 11041 | 10971 | 11141 | 11051 | 85 | 0.8 | 1.1 |
| 8 | 10892 | 10972 | 10958 | 10941 | 43 | 0.4 | 0.1 |
| **Mean** | **11060** | **11124** | **11186** | **11123** | **85** | **0.8** | **0.7** |
| **SD** | **247** | **262** | **262** | **250** | | **0.4** | **0.4** |

**(b) Time to exhaustion during the cycling capacity test alone (s)**

| Cyclist | Trial 1 | Trial 2 | Trial 3 | Mean | SD | CV (%) | CV Between Trial 2 and 3 (%) |
|---|---|---|---|---|---|---|---|
| **1** | 294 | 458 | 285 | 345 | 97 | 28 | 33 |
| **2** | 815 | 896 | 975 | 895 | 80 | 9 | 6 |
| **3** | 71 | 258 | 437 | 255 | 183 | 72 | 36 |
| **4** | 348 | 385 | 466 | 400 | 60 | 15 | 13 |
| **5** | 140 | 88 | 221 | 150 | 67 | 45 | 60 |
| **6** | 80 | 161 | 201 | 147 | 62 | 42 | 15 |
| **7** | 241 | 171 | 341 | 251 | 85 | 34 | 47 |
| **8** | 92 | 172 | 158 | 141 | 43 | 30 | 6 |
| **Mean** | **260** | **324** | **386** | **323** | **85** | **34** | **27** |
| **SD** | **247** | **262** | **262** | **250** | | **19** | **20** |

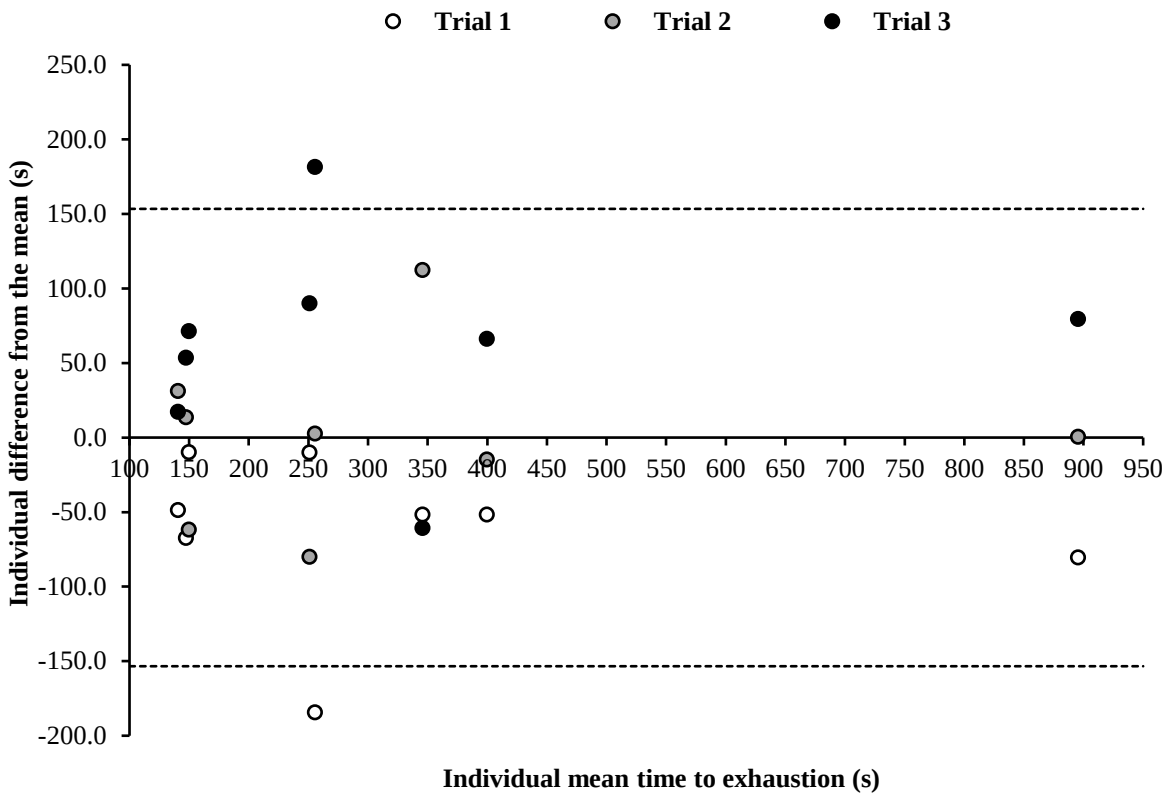


**Figure 4.5** Limits of agreement for time to exhaustion at 150% lactate threshold.

### 4.5 Discussion

The aim of the present study was to develop a prolonged submaximal endurance cycling protocol and exercise capacity test that could be utilised in future chapters to assess the dose-response effects of CHO feeding on muscle fuel selection and exercise capacity. When compared with Trial 1, Trial 3 exhibited a significant improvement in exercise capacity alongside a significant reduction in certain physiological and metabolic parameters such as HR, RPE and capillary blood lactate. Furthermore, no differences were evident for any of the metabolic, physiological or exercise capacity measures between Trial 1 and 2 or Trial 2 and 3. These findings suggest that at least one full familiarisation session should be completed prior to studying the effects of a specific CHO feeding strategy on metabolism and performance in subsequent chapters.

The experimental design was deliberately devised in an attempt to promote ecological validity whereby trained male cyclists undertook an exercise protocol that was intended to reflect the physiological and performance demands of professional road cycling. Indeed, our protocol was designed to replicate the demands of ‘mountain’ stages which have been characterised as

mostly requiring submaximal, constant power output over longer periods (Padilla *et al.*, 2007; Sanders *et al.*, 2018; Van Erp *et al.*, 2020; Vogt *et al.*, 2007) followed by high-intensity efforts towards the end of the stage. To this end, the first 180 minutes of the protocol replicated riders typically riding conservatively within the 'peloton' for long periods at submaximal power outputs in attempts to try and preserve energy, followed by a TTE test reflecting the final part of a mountain-based stage whereby riders will 'attack' or try to 'hold the wheel' of an attacking opponent on mountain climbs or summit finishes (Padilla *et al.*, 2007; Vogt *et al.*, 2007). These moments are considered to be one of the critical defining moments within multi-day stage races as general classification riders attempt to gain time over major contenders or minimise any time lost. Unlike previous studies, the current protocol was also undertaken in conditions of best competition nutritional practice (Thomas *et al.*, 2016) and in accordance with the dietary habits of elite cyclists (Heikura *et al.*, 2019; Muros *et al.*, 2019; Sánchez-Muñoz *et al.*, 2016), to help assess the 'true' magnitude of any metabolic or performance differences (Jeacocke & Burke, 2010) in future chapters. Indeed, subjects adhered to a CHO loading protocol and consumption of a pre-exercise high-CHO meal in order to commence each trial with high CHO availability. In this way, such dietary standardisation prior to the exercise protocols will have induced similar pre-exercise muscle glycogen concentrations between trials, this itself a key methodological factor considering that differing levels of starting muscle glycogen between trials would likely affect power output and self-selected pace during the latter stages of the protocol (Widrick *et al.*, 1993). Additionally, our approach to dietary standardisation will have also standardised the metabolic responses between trials given that differences in CHO availability can significantly alter metabolic responses to exercise (Bosch *et al.*, 1993; Horowitz *et al.*, 1997).

In relation to the physiological and metabolic responses between exercise trials, our data demonstrate that blood lactate, heart rate and RPE were all sensitive to reductions by the third trial compared to the first, in turn, suggesting a potential learning or training effect induced by the exercise protocol. It is, of course, difficult to compare the present data with previous studies examining reliability estimates given differences in exercise intensities, durations and differences in skeletal muscle recruitment and metabolic parameters between variable intensity and constant load prolonged cycling (Palmer *et al.*, 1999). It is noteworthy, however, that reductions in muscle lactate concentration during exercise have previously been reported to occur within as little as 5-days of daily endurance cycling training consisting of 2 h at 59% of pre-training $\dot{V}O_{2peak}$ (Phillips *et al.*, 1996a). When considered this way, it may be possible that

some early physiological adaptations regulating metabolic control may have occurred, thus presenting as reduced metabolic stress (i.e., blood lactate) and central stress (i.e., RPE). This further highlights the need to ensure highly trained participants are recruited in future studies to minimise any associated effects of training. Moreover, the observed reductions in the RPE within the present study also lends itself to a potential learning effect highlighting the need for a complete familiarisation trial prior to any experimental trials in future studies (Laursen *et al.* 2003). In spite of the differences in the above parameters, all other metabolic responses were observed to be consistent between trials with similar rates of energy expenditure and substrate utilisation evident. Nonetheless, we acknowledge that future protocol development studies would also benefit from additional measures of metabolism such as muscle glycogen and circulating glucose concentration. Indeed, Phillips et al. (1996b) also observed that as little as 5 days of cycling training significantly reduces muscle glycogen utilisation and maintain euglycemia during exercise. Notwithstanding these limitations, the present study clearly demonstrates that our chosen training status of participants are readily capable of completing the metabolic challenge associated with the exercise intensity and duration of this exercise protocol, even in the absence of CHO feeding during exercise.

When considering the reliability of the exercise capacity test utilised within the current research design, it is apparent that there was a level of systematic bias between the trials as demonstrated by the significant increase in exercise capacity during Trial 3 compared to Trial 1, whereas no differences were evident between Trial 1 and 2 or Trial 2 and 3. In this way, our data are similar to other researchers that have examined reliability using a 3 trial design in that a learning effect seems to occur after the first trial. For example, although Schabort *et al.* (1998) reported a CV of 1.7% when male endurance-trained cyclists completed three trials consisting of a 100 km TT, these authors also reported that TT 1 was significantly slower than TT 2 by ~3:30 min:s, whereas there was no significant difference between TT 2 and 3. Additionally, Laursen *et al.* (2003) demonstrated a CV of 3.0% across three separate 40 km cycling TTs undertaken by highly trained male cyclists. However, they also reported a trend towards a significantly slower performance by ~1:30 min:s in TT 1 compared to TT 2 and 3 whereas a similar mean finishing time was observed between TT 2 and 3, with the performance times of TT 2 and 3 also reporting a markedly lower CV of 0.9%. Collectively, such findings highlight the importance of an appropriate familiarisation trial in subsequent studies even with the inclusion of trained cyclists.

Although we observed excellent reliability (CV, 0.8%) when considering the total duration of the entire exercise protocol (i.e., 180-min submaximal steady cycle plus the TTE), it is noteworthy that large individual variation was evident when examining the exercise capacity test alone (CV, 34%). Such estimates of reliability are similar to those reported by other investigators using high-intensity protocols (Jeukendrup *et al.*, 1996; Krebs & Power, 1989; McLellan *et al.*, 1995; O'Hara *et al.*, 2011) and again demonstrate, that TTE tests (especially when high-intensity in nature) are associated with more variability than TT type performance tests. Specifically, O'Hara *et al.* (2011) reported a CV of 31.7% for the TTE (90% $W_{max}$, ~310 W) aspect of their high intensity endurance cycling protocol that was preceded by 20 minutes of progressive incremental cycling exercise (from 70-85 $W_{max}$) and ten repeated 90 s sprints (at 90% $W_{max}$ separated by 180 s at 55 $W_{max}$), with trained cyclists undertaking the protocol on four separate occasions. Interestingly, the 90% $W_{max}$ TTE intensity within that study corresponded to a similar power output to the TTE aspect of the present protocol (150% LT, ~300 W).

Nonetheless, we consider the present data of practical relevance as it now provides a baseline of variability in performance in the absence of any intervention related to CHO feeding. Additionally, the typical time to fatigue induced by our exercise protocol (i.e., <10 minutes) is in accordance with the typical duration of attacks or holding a wheel that often occurs during mountain stages of Grand Tour cycling. For example, Van Erp *et al.* (2020) recently reported in a case report, that the key mountains whereby a rider competed for the overall general classification victory within a GT were typically characterised by a duration of 20-30 minutes. However, within the overall duration of these mountain passes, shorter attacking or responding efforts of <10 minutes were undertaken when attacking rivals or responding to rival attacks.

In conclusion, the present chapter demonstrates that in conditions of high pre-exercise CHO availability, trained male cyclists are capable of completing the 180-min submaximal steady-state exercise protocol and that fatigue during the subsequent exercise capacity test typically occurs within <10 minutes. However, the significant reductions in capillary lactate, RPE and HR during the submaximal protocol between Trial 1 and 3 in combination with the significant extension in exercise capacity also between Trial 1 and Trial 3 suggest that at least one full familiarisation session should be completed prior to studying the effects of a specific CHO feeding strategy on metabolism and performance in trained male cyclists.

# Chapter Five:

# Carbohydrate feeding improves cycling capacity in trained male cyclists in a dose-dependent manner

The aim of this chapter was to test the hypothesis that exogenous carbohydrate ingestion improves cycling exercise capacity in a dose dependent manner. This study was presented orally at the European College of Sports Science (ECSS) Dublin, Ireland, 2018.

## 5.1. Abstract

**Purpose:** To test the hypothesis that carbohydrate feeding during exercise improves cycling exercise capacity in a dose dependent manner. **Methods:** In a randomised, repeated measures crossover design, eight endurance trained male cyclists ingested graded quantities of carbohydrate (0, 45 or 90 $g \cdot h^{-1}$) comprising a mixture of solids, gels and fluids during 180-min of submaximal cycling at lactate threshold (208 ± 11 W) followed by a cycling capacity test to exhaustion at 150% lactate threshold (312 ± 18 W). Subjects commenced each trial after a 36-h exercise and dietary standardization protocol comprising prior glycogen depleting exercise, a CHO loading regimen (~11.6 $g \cdot kg^{-1}$) and a CHO rich pre-exercise meal (2 $g \cdot kg^{-1}$) consumed at 3 h before exercise. Muscle biopsies were obtained from the vastus lateralis muscle immediately pre- and post-completion of the 180-min submaximal protocol. **Results:** In accordance with CHO feeding during exercise, mean plasma glucose ($P < 0.01$) and CHO oxidation ($P < 0.01$) were different between all pairwise comparisons such that the 90 $g \cdot h^{-1}$ (5.97 ± 0.49 and 2.29 ± 0.49) trial > 45 $g \cdot h^{-1}$ (5.66 ± 0.34 and 2.14 ± 0.48) > 0 $g \cdot h^{-1}$ (4.53 ± 0.33 and 1.75 ± 0.60 $mmol \cdot L^{-1}$ and $g.min^{-1}$, respectively). Pre-exercise muscle glycogen ($P = 0.137$) was comparable between trials (767 ± 87, 742 ± 109 and 698 ± 98 $mmol \cdot kg\ dw^{-1}$ for 0, 45 and 90 $g \cdot h^{-1}$, respectively) and exercise decreased ($P < 0.01$) glycogen with no effects ($P = 0.783$) of CHO feeding (252 ± 85, 269 ± 88 and 293 ± 109 $mmol \cdot kg^{-1}$ dw for 0, 45 and 90 $g \cdot h^{-1}$, respectively). Cycling capacity time was increased in a dose dependent manner such that 90 $g \cdot h^{-1}$ (233 ± 133 s) > 45 $g \cdot h^{-1}$ (156 ± 66 s; $P = 0.06$) > 0 $g \cdot h^{-1}$ (108 ± 54 s; $P = 0.029$). **Conclusion:** In conditions of high pre-exercise CHO availability (i.e., after CHO loading and consumption of a pre-exercise CHO meal), CHO feeding improves cycling exercise capacity in a dose dependent manner. Such ergogenic effects are independent of muscle glycogen sparing (as assessed in whole muscle homogenate) and are likely related to a combination of liver glycogen sparing, increased plasma glucose availability and higher rates of CHO oxidation.

## 5.2. Introduction

It is widely accepted that carbohydrate (CHO) feeding during endurance exercise has an ergogenic effect upon both exercise performance and capacity (Cermak & Van Loon, 2013; Stellingwerff & Cox, 2014). CHO feeding is thought to improve performance through a combination of multiple mechanisms consisting of muscle (Bosch et al., 1996; Björkman *et al.,* 1984; Erickson *et al.,* 1987; Hargreaves *et al.,* 1984; Tsintzas *et al.,* 1995; Tsintzas *et al.,* 1996) and liver glycogen sparing (Gonzalez *et al.,* 2015), maintenance of plasma glucose and elevated CHO oxidation rates (Coyle *et al.,* 1986) and direct effects upon the central nervous system (CNS) (Carter, Jeukendrup & Jones, 2004). Accordingly, it is now an established practice for elite endurance athletes to consume CHO during exercise, with current guidelines recommending CHO ingestion rates up to 90 $g \cdot h^{-1}$ when exercise is greater than 2.5-3 h in duration (Thomas *et al.,* 2016). Nonetheless, despite over 40 years of research demonstrating ergogenic effects of CHO feeding (Stellingwerff and Cox, 2014), the optimal dose (Smith *et al.*, 2013), feeding frequency (Mears *et al.*, 2020), CHO source (Currell and Jeukendrup, 2008; Rowlands *et al.*, 2015) and format (Pfeiffer *et al.,* 2010a; Pfeiffer *et al.*, 2010b; Guillochon and Rowlands, 2017) is still not known.

As such, the dose-response relationship of CHO ingestion during exercise upon exercise capacity and/or performance has received considerably less focus within the literature and still remains a topic of debate. The most comprehensive study to date that investigated the dose-response effects of CHO ingestion, estimated (through statistical modelling) that 78 $g \cdot h^{-1}$ CHO was considered the optimal dose to maximise performance when participants consumed CHO during a 2-h constant load cycle at ~70% $\dot{V}O_{2peak}$ followed by a 20-km TT (Smith *et al.*, 2013). Within this study, CHO doses between 10 and 120 $g \cdot h^{-1}$ (10 $g \cdot h^{-1}$ increments) were examined with each dose consisting of a 1:1:1 ratio of glucose, fructose and maltodextrin. It is must be noted, however, that the magnitude of performance improvement was curvilinear in nature given that the authors reported only a small 1.7% improvement in performance from 30 – 80 $g \cdot h^{-1}$ and a trivial 0.7% improvement in performance from 40 to 80 $g \cdot h^{-1}$. Furthermore, when higher doses only of CHO have been investigated ($\geq$60 $g \cdot h^{-1}$), King *et al.* (2018) observed no linear dose-response relationship as the ingestion of 90 $g \cdot h^{-1}$ of a glucose-fructose (2:1) solution resulted in the highest mean power output during a 30-min self-paced TT proceeding a 2-h submaximal ride compared to 60 and 75 $g \cdot h^{-1}$ of a glucose only solution and a 112.5 $g \cdot h^{-1}$ of a glucose-fructose (2:1) solution. In addition, when the same group extended exercise duration

beyond 3 hours they also reported that the ingestion of 90 g·h$^{-1}$ of a CHO beverage in the form of glucose and fructose (2:1) increased power output during a 30-minute self-paced TT by 6.8% compared with 100 g·h$^{-1}$ (2:1, glucose:fructose) and 4.0% compared to 80 g·h$^{-1}$ (2:1, glucose:fructose) (King *et al.*, 2019). Collectively, such data suggest that when higher doses of CHO, ~75-90 g·h$^{-1}$ in the form of 2:1 glucose-fructose blends are consumed during 2-3 h of submaximal exercise, subsequent ~15-30 min TT performance is maximised, with higher doses beyond 90 g·h$^{-1}$ seeming to have no further benefit and potentially negatively impacting performance.

It is noteworthy, however, that the practical application of such experimental data is often limited, owing to the fact that such studies typically provide CHO in the form of a beverage only and that exercise protocols were not undertaken in conditions associated with best nutritional practice i.e. after CHO loading and consumption of a pre-exercise meal. Such research designs clearly contrast with the common practices adopted by elite endurance athletes whereby races are commenced with "loaded" muscle and liver glycogen stores and a mixture of solids, fluids and gels are usually consumed as an in-race fuelling strategy (Fell and Morton, 2016; Burke and Hawley, 2018; Burke *et al.*, 2018). Additionally, the exercise durations utilized in such laboratory protocols (i.e., 2 hours) as well as the chosen performance tests (i.e., 15-30-min TT) are not always representative of the winning moves that often occur in elite level cycling. In such situations, riders will typically ride in the peloton at a submaximal intensity for 3-4 hours before 'attacking' or responding to 'attacks' on mountain climbs and summit finishes (Padilla *et al.*, 2007). In these instances, the ability to 'hold the wheel' can often be the difference between winning and losing and is representative of an exercise capacity type test.

With this in mind, the aim of the present study was to therefore test the hypothesis that CHO ingestion improves cycling exercise capacity in a dose dependent manner. To this end, trained male cyclists completed 180 minutes of steady state submaximal exercise (during which they consumed 0, 45 or 90 g·h$^{-1}$ of CHO) followed by a short duration high-intensity exercise capacity test that was intended to induce fatigue in <10 minutes (as devised from Study 1). Importantly, each experimental trial was commenced after a 36-h CHO loading protocol (12 g·kg$^{-1}$) and 3 hours after consumption of a pre-exercise meal that was rich in CHO (2 g·kg$^{-1}$). Furthermore, our chosen CHO feeding protocol during exercise comprised a mixture of fluids,

solids and gels so as to replicate the typical feeding strategies adopted by professional road cyclists.

## 5.3 Methods

### 5.3.1 Participants

Eight endurance-trained amateur male cyclists (mean ± SD: age, 31 ± 7 years; body mass 73.6 ± 6.1 kg; height, 177.0 ± 8.0 cm) took part in this study. Mean $\dot{V}O_{2peak,}$ peak power output (PPO) and power output at lactate threshold (LT) for the cohort were 60.5 ± 5.4 $mL \cdot kg^{-1} \cdot min^{-1}$, 383 ± 21 W and 208 ± 11 W, respectively. Subjects were defined as trained in accordance with the criteria of Jeukendrup, Craig & Hawley (2000) whereby the cyclists were competitive club level road cyclists as detailed by subject characteristics and training and racing data described in Table 3.1. None of the subjects had any history of musculoskeletal or neurological disease nor were they under any pharmacological treatment during the course of the testing period. All participants provided written informed consent before commencement of the study, which was approved by the local Ethics Committee of Liverpool John Moores University.

### 5.3.2 Experimental design

In a repeated-measures, randomised, crossover design separated by 7-8 days, subjects completed four prolonged endurance-based cycling exercise protocols which consisted of 180 minutes steady state submaximal exercise (undertaken at lactate threshold) followed by a cycling capacity test to exhaustion (undertaken at 150% of lactate threshold). The initial trial was a familiarisation to the exercise protocol and in the following three trials, subjects ingested CHO at a rate of 0, 45 or 90 grams per hour in the form of solids, gels and fluids during the exercise bouts. As such, these CHO ingestion regimens replicated the typical eating patterns of professional cyclists during competition (Heikura *et al.*, 2019; Muros *et al.*, 2019; Morton, unpublished observations; Sánchez-Muñoz *et al.*, 2016). At 36 - 40 h prior to all trials, all subjects performed a glycogen depletion cycling protocol followed by 36-h of high CHO intake (11.6 $g \cdot kg^{-1}$) so as to maximise muscle glycogen prior to commencing each experimental trial. Additionally, each trial was also commenced 3 h after a CHO rich meal (2 $g \cdot kg^{-1}$). Muscle biopsies were obtained from the vastus lateralis immediately before and after the 180-min submaximal ride where the post-exercise biopsy was obtained prior to commencing the exercise capacity protocol. An overview of the experimental protocol is shown in Figure 5.1.

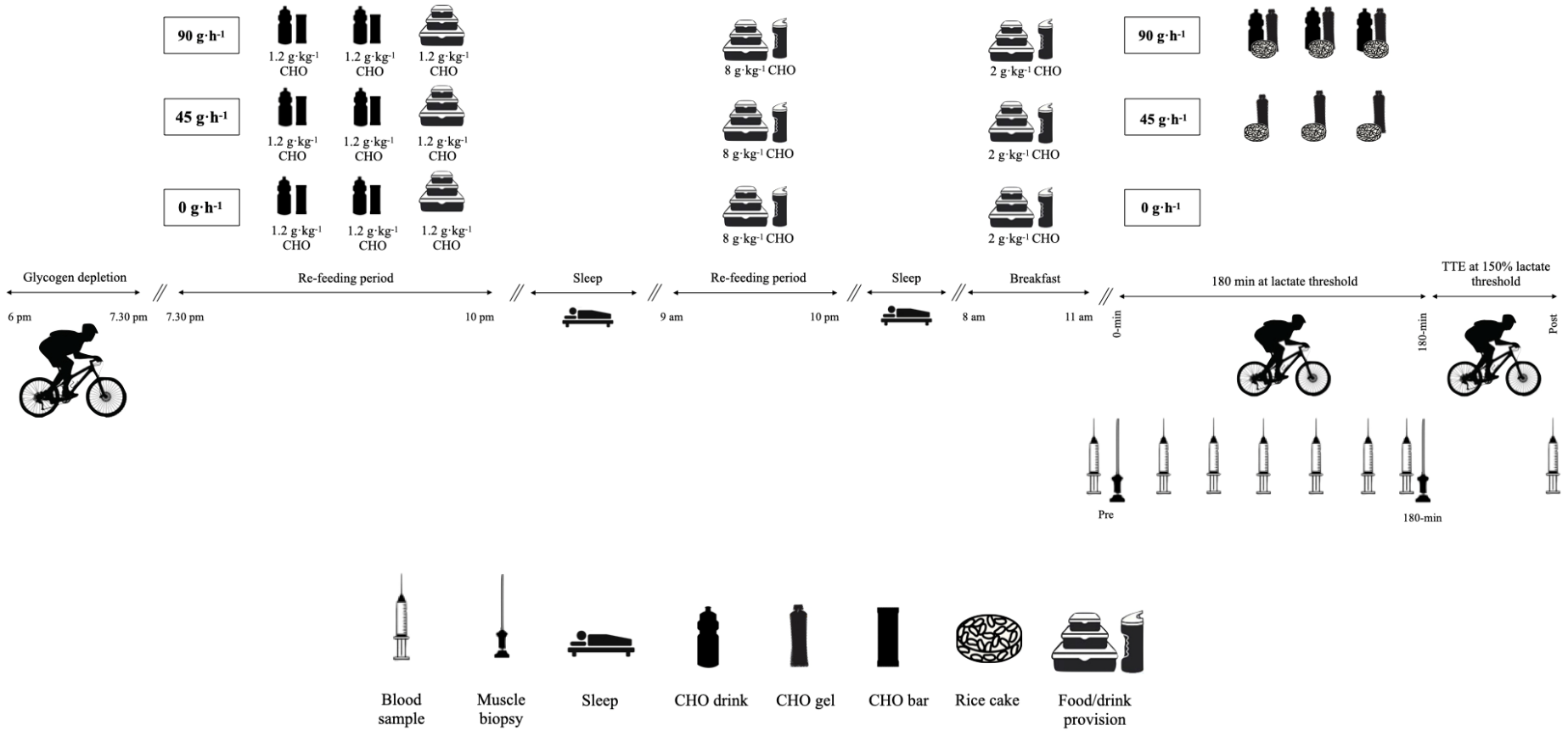


**Figure 5.1** Schematic overview of the experimental protocol employed in each trial. On the evening of day 1, participants completed a glycogen depleting cycling protocol followed by a high carbohydrate (CHO) diet in the hours following the depleting protocol. Throughout the entirety of day 2, participants consumed a high CHO diet. During the main experimental trial on day 3, subjects consumed a high CHO pre-exercise meal before undertaking a 180-min submaximal cycle whereby they consumed 0, 45 or 90 $g \cdot h^{-1}$ CHO followed by a cycling capacity test time to exhaustion (TTE). Muscle biopsies were obtained immediately pre-exercise and at the end of the 180-min submaximal cycle.

#### 5.3.2.1 Preliminary testing and familiarisation

At least 10 days prior to experimental trials, all subjects performed a two-part incremental cycle test to determine peak oxygen consumption ($\dot{V}O_{2max}$), lactate threshold (LT) and peak power output (PPO) on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands). The two-part incremental cycling test has been detailed previously in Section 3.2.4. Subjects also undertook full familiarisation of the experimental trial, at least 7 days prior to the main experimental trials where they refrained from CHO intake during the submaximal cycling protocol, thus replicating the same nutritional conditions as per the 0 $g \cdot h^{-1}$ trial. This familiarization trial served to verify subject's ability to complete the prescribed intensity of the 180-min submaximal ride (see Figure 5.2) as well as providing baseline data for the exercise capacity test. Additionally, data from Study 1 verified at least one full familiarization test should be completed to improve reliability of performance during the exercise capacity test.

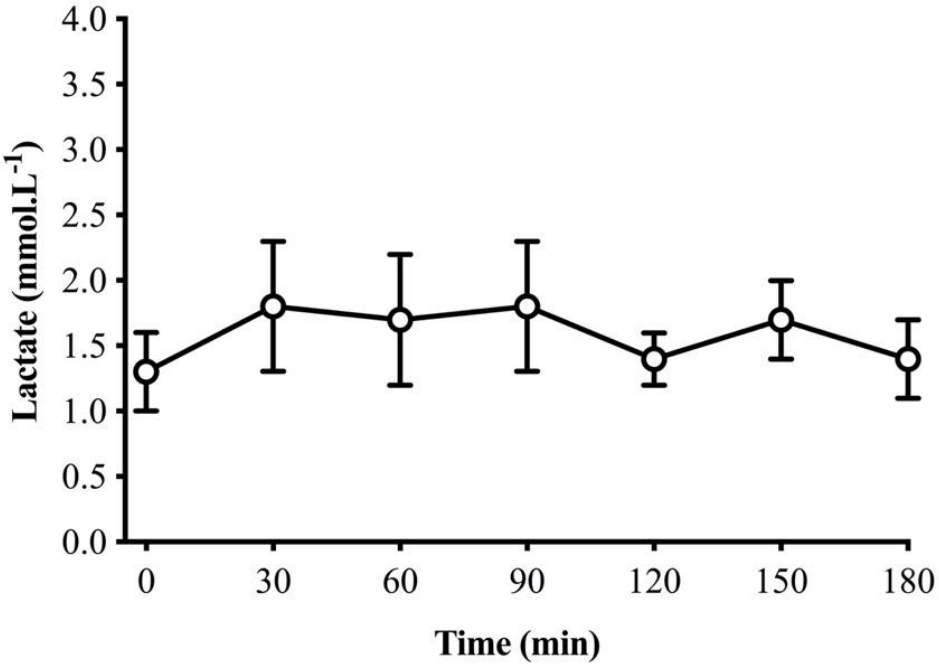


**Figure 5.2** Capillary blood lactate from the familiarisation trial to validate the individual subject's exercising intensity during the 180-min prolonged cycling aspect of the exercise protocol in accordance with the lactate threshold result from the preliminary submaximal lactate threshold test. Data presented as mean ± SD.

#### 5.3.2.2 Day 1: Glycogen depletion protocol

At 36-h before the main experimental trial (i.e., the evening of Day 1), subjects performed an intermittent glycogen-depleting cycling protocol lasting 90-minutes in duration. The glycogen depletion protocol has been previously described in Section 4.3.4.1.

#### 5.3.2.3 Day 2: Carbohydrate loading regimen

To facilitate the goal of elevating muscle glycogen concentrations in preparation for the prolonged cycling protocol in accordance with current competition nutritional guidelines (Thomas *et al.,* 2016) and the daily energy intake of elite road cyclists during (Heikura *et al.,* 2019; Muros *et al.,* 2019; Morton, unpublished observations; Sánchez-Muñoz *et al.,* 2016), subjects were provided with high carbohydrate snacks and fluids (SiS GO Energy, SiS REGO Protein, SiS GO Bars, Science in Sport, Blackburn, UK) to consume immediately post glycogen depletion exercise (CHO: 1.2 g·kg$^{-1}$ BM; PRO: 0.4 g·kg$^{-1}$ BM; Fat: 0 g·kg$^{-1}$ BM), at 1-hour post (CHO: 1.2 g·kg$^{-1}$ BM; PRO: 0.1 g·kg$^{-1}$ BM; Fat: 0 g·kg$^{-1}$ BM) and at 2-hours post (CHO: 1.2 g·kg$^{-1}$ BM; PRO: 0.1 g·kg$^{-1}$ BM; Fat: 0 g·kg$^{-1}$ BM) (see Table 3.4). Over the course of the following day (i.e., Day 2), participants were provided with a pre-packaged standardised high CHO diet to consume throughout the entirety of that day (CHO: 8 g·kg$^{-1}$ BM; PRO: 2 g·kg$^{-1}$ BM; Fat: 1 g·kg$^{-1}$ BM) (see Table 3.4) (Sánchez-Muñoz *et al.,* 2016; J.P., Morton, unpublished observations) and also refrained from any strenuous physical activity and alcohol consumption during this day to promote muscle and liver glycogen resynthesis (Bartlett *et al.,* 2013). Fluid intake was advised to water only and was allowed *ad libitum.*

#### 5.3.2.4 Day 3: Experimental trials

On the morning of the main experimental trials at ~08:00, subjects reported to the laboratory in a fasted state and were immediately provided with a standardized high-CHO "Race-day" breakfast (CHO: 2 g·kg$^{-1}$ BM; PRO: 60 g; Fat: 45g) that was representative of an elite real-world cyclists 'Race-day" breakfast (see Table 3.4) (Heikura *et al.,* 2019; Muros *et al.,* 2019; Morton, unpublished observations; Sánchez-Muñoz *et al.,* 2016). At 3-h post-prandial and immediately before the beginning of the cycling protocol, an indwelling cannula (Safety Lock 22G, BD Biosciences, West Sussex UK) was inserted into the antecubital vein in the anterior crease of the forearm and a resting blood sample drawn. After a resting blood sample was

taken, the cannula was flushed with ~5 mL of sterile saline (Kays Medical supplies, Liverpool, UK) to keep the cannula patent and sterile, this procedure was repeated after each subsequent blood draw. Subjects were then fitted with a heart rate monitor (Polar Electro, Finland) and nude body mass (SECA, Hamburg, Germany) was recorded. Resting values for expired gas were also recorded at this time-point and were measured via a mouthpiece connected to an automated gas analyser machine Moxus Modular Metabolic System (AEI Technologies, IL, USA) for a 5-minute period**.** The gas analyser was calibrated before each experimental trial according to the manufacturer's guidelines (Beltrami *et al.*, 2014). Participants then completed a 10-minute warm-up at 100 W and began the 180-min cycle preload at 100% LT (208 ± 11 W). LT was the chosen as the exercise intensity as it is a better method of matching metabolic stress between participants when compared to exercising at a percentage of $\dot{V}O_{2max}$ (Baldwin et al., 2000). Heart rate, RPE (Borg, 1970) and cycling cadence were obtained at 30-minute intervals throughout. Expired gas was collected for 5-minutes at 30-minute intervals (Moxus Modular Metabolic System, AEI Technologies, IL, USA) in order to calculate cycling efficiency (Hopker *et al.*, 2009) and substrate utilisation which was determined according to the equations of Jeukendrup and Wallis (2005). Venous blood samples were also collected at 30 min intervals throughout the 180 min submaximal exercise, with the first sample being taken immediately before the start of the exercise protocol. During the 180-min submaximal ride subjects consumed 0, 45 or 90 $g \cdot h^{-1}$ CHO. Specifically, during the 0 $g \cdot h^{-1}$ trial, subjects received 125 mL of a lemon flavoured drink containing no CHO (SiS GO Hydro, Science in Sport, Blackburn, UK) at 20 min intervals, beginning at 0 min (2 × 125 mL doses was provided at 60 and 120 min). During the 45 $g \cdot h^{-1}$ trial subjects consumed a solid homemade CHO food (rice cake, 23 g CHO, see Table 5.3 for nutritional composition of rice cake) every 30-min for the first 2-hrs of exercise followed by a commercially available CHO gel (22g Maltodextrin) every 30-min in the final hour of exercise (SiS GO Isotonic Gel, Science in Sport, Blackburn, UK) (see Table 5.1 for hourly nutritional composition). Additionally, a 125 mL of a lemon flavoured drink containing no CHO (SiS GO Hydro, Science in Sport, Blackburn, UK) was also consumed at 20 min intervals beginning at 0 min (2 × 125 mL doses was provided at 60 and 120 min). During the 90 $g \cdot h^{-1}$ trial, a rice cake was provided at 20-min intervals during the first 2-h of exercise followed by gels at 20-min intervals during the final hour of exercise. Additionally, 125 mL of a bespoke lemon flavoured 4% CHO drink (18g maltodextrin and 3g fructose, Science in Sport, Blackburn, UK) was consumed at 20 min intervals throughout the whole duration of the 90 $g \cdot h^{-1}$ trial (2 × 125 mL doses was provided at 60 and 120 min) (see Table 5.2 for hourly nutritional composition). Immediately following the 180-min submaximal

cycle and after the muscle biopsy (~10 min between the cessation of the 180-min cycle, having the muscle biopsy taken and returning to bike for the time to exhaustion test), participants began the TTE protocol whereby they cycled at 150% LT **(**312 ± 15 W) to volitional exhaustion. No music was played, and maximum HR was the only physiological measurement recorded during the TTE protocol. The only available information to the participants was the fixed power output and cadence. Every effort was made to ensure subjects were not disturbed throughout the performance trials. A venous blood sample and body mass recording was obtained immediately after the TTE. No performance results were shown to the subjects until the completion of the study. All exercise tests were performed at the same time of day (11:00 ± 1-h) under normal laboratory conditions (20-23$^{o}$C and 50-60% humidity) using the same electrically braked cycle ergometer (Lode Excaliber Sport, Groningen, Netherlands) and automated gas analyser machine Moxus Modular Metabolic System (AEI Technologies, IL, USA). During all exercise trials, subjects were cooled with a floor standing fan to minimise thermal stress. Each experimental trial was separated by 7-8 days and subjects were asked to continue with their habitual training schedule during this period which equated to 03:21 ± 02:14 (hh:min) and 95 ± 67 km (excluding the 90-min glycogen depletion and ~180-min cycling protocol).

**Table 5.1** Nutrient composition per hour of the 45 g·h$^{-1}$ trial. A carbohydrate (CHO) feed was provided every 30-min along with 125 mL of fluid every 20-min containing no CHO. During hours 1 and 2 the CHO feed consisted of a solid CHO source (rice cake) with CHO gels provided in hour 3.

| | Total (g) | | | | | | | | | | | Estimated Ratio |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | kcal | CHO | Protein | Fat | Fibre | Starch | Sugars | Glucose | Fructose | Sucrose | Maltodextrin | GLU:FRU |
| Hour 1 | 225 | 46 | 4 | 2 | 1 | 30 | 15 | 6 | 7 | 2 | 0 | 1:1.1 |
| Hour 2 | 225 | 46 | 4 | 2 | 1 | 30 | 15 | 6 | 7 | 2 | 0 | 1:1.1 |
| Hour 3 | 174 | 44 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 44 | 1:0 |
| Total | 625 | 136 | 8 | 4 | 2 | 60 | 30 | 12 | 14 | 4 | 44 | 4:1 |

**Table 5.2** Nutrient composition per hour of the 90 g·h$^{-1}$ trial. A carbohydrate (CHO) feed was provided every 20-min along with 125 mL of a 4% CHO fluid solution. During hours 1 and 2 the CHO feed consisted of a solid CHO source (rice cake) with CHO gels provided in hour 3.

| | Total (g) | | | | | | | | | | | Estimated Ratio |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | kcal | CHO | Protein | Fat | Fibre | Starch | Sugars | Glucose | Fructose | Sucrose | Maltodextrin | GLU:FRU |
| Hour 1 | 421 | 90 | 5 | 4 | 2 | 46 | 23 | 8 | 14 | 3 | 18 | 2:1 |
| Hour 2 | 421 | 90 | 5 | 4 | 2 | 46 | 23 | 8 | 14 | 3 | 18 | 2:1 |
| Hour 3 | 348 | 87 | 0 | 0 | 0 | 0 | 2 | 0 | 3 | 0 | 84 | 1:0 |
| Total | 1190 | 267 | 10 | 8 | 4 | 92 | 48 | 16 | 31 | 6 | 120 | 4:1 |

**Table 5.3** Nutrient composition of carbohydrate forms, solid (rice cake), gel and drink.

| | Total (g) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | kcal | CHO | Protein | Fat | Fibre | Starch | Sugars | Glucose | Fructose | Sucrose | Maltodextrin |
| Rice Cake | 113 | 23 | 2 | 1 | 1 | 15 | 8 | 3 | 4 | 1 | 0 |
| Gel | 87 | 22 | 0 | 0 | 0 | - | 1 | 0 | 0 | 0 | 22 |
| Drink | 83 | 21 | 0 | 0 | 0 | - | 2 | 0 | 3 | 0 | 18 |

### 5.3.3 Estimates of whole-body substrate oxidation, total energy expenditure and cycling gross efficiency

Rates of whole-body CHO and fat oxidation ($g \cdot min^{-1}$) were calculated at 30-min intervals from each steady-state gas sample collected throughout the 180-min submaximal cycle, according to the equations of Jeukendrup and Wallis (2005) as described in Section 3.2.5. Cycling gross efficiency was calculated according to Hopker *et al.* (2009) as described in Section 3.2.6, at the same time points. Total energy expenditure was estimated for each trial assuming an energy yield of 17.57 kJ and 39.33 kJ for 1 g of CHO and fat, respectively.

### 5.3.4 Blood analysis

Venous blood samples were collected and analysed for plasma glucose, lactate, FFA, glycerol and β-hydroxybutyrate (β-OHB) as described in Section 3.4.

### 5.3.5 Muscle biopsies

Skeletal muscle biopsies (~100 mg) were obtained from the vastus lateralis immediately pre-exercise and immediately after the 180-min submaximal cycle prior to the time to exhaustion test. Muscle biopsies were obtained from separate incision sites 2-3 cm apart using the Well-Blakesey conchotome technique under local anaesthesia (0.5% Marcaine) and immediately frozen in liquid nitrogen and stored at -80ºC for later analysis. ~20 mg was used for whole muscle glycogen analysis as described in Section 3.6.

### 5.3.6 Analysis of Muscle glycogen

Muscle glycogen concentration was determined according to the methods described by Van Loon *et al.* (2000) as described in Section 3.7.

### 5.3.7 Statistical Analysis

All statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS version 26). Descriptive statistics were produced for all data sets to check for normal distribution indicated by a Shapiro-Wilk (accepted if $P > 0.05$). Changes in exercise capacity, total hourly carbohydrate and fat oxidation, and post exercise capacity test blood parameters were analysed using a one-way repeated-measures general linear model (GLM), whereas

comparisons of average physiological responses, substrate utilisation, blood parameters, muscle glycogen concentrations and body mass changes were analysed with a two-way repeated-measures GLM, where the within factors were time and condition, and substrate and condition. If Mauchley's test of sphericity indicated a minimum level of violation, as assessed by a Greenhouse Geisser epsilon (ε) of > 0.75, data were corrected using Huyn-Feldt ε. If Mauchley's test of sphericity was violated, data were corrected using Greenhouse Geisser ε. Where a significant main effect was observed, pairwise comparisons were analysed according to LSD post hoc tests to locate specific differences. All data in text, figures, and tables are presented as means ± SD, with *P* values < 0.05 indicating statistical significance unless indicated otherwise.

## 5.4 Results

### 5.4.1 Physiological responses to exercise

Comparisons of subjects' HR, RPE, %$\dot{V}O_{2max}$, cycling cadence and cycling efficiency are displayed in Table 5.4. HR and RPE both displayed progressive increases during exercise (HR, $P = 0.027$; RPE, $P < 0.01$) however there was no significant differences between trials (HR, $P = 0.349$; RPE, $P = 0.328$). %$\dot{V}O_{2max}$ significantly increased during exercise ($P < 0.01$) whilst cycling cadence significantly declined ($P = 0.007$) with no differences between conditions (%$\dot{V}O_{2max}$, $P = 0.725$; Cycling cadence, $P = 747$). Cycling efficiency did not significantly change during exercise ($P = 0.191$) with no difference between conditions ($P = 0.487$).

**Table 5.4** Heart rate, RPE, cycling cadence, exercise intensity (%$\dot{V}O_{2max}$), cycling efficiency and energy expenditure during the 180-min submaximal steady-state cycling protocol.

| | Time (min) | | | | | |
|---|---|---|---|---|---|---|
| | 30 | 60 | 90 | 120 | 150 | 180 |
| **Heart Rate (b·min$^{-1}$)** | | | | | | |
| 0 g·h$^{-1}$ | 149 ± 13 | 151 ± 14 | 153 ± 16 | 155 ± 14$^{ac}$ | 160 ± 10$^{ac}$ | 160 ± 10$^{ac}$ |
| 45 g·h$^{-1}$ | 148 ± 10 | 150 ± 12 | 150 ± 9 | 153 ± 9$^{ac}$ | 157 ± 7$^{ac}$ | 159 ± 8$^{ac}$ |
| 90 g·h$^{-1}$ | 153 ± 11 | 156 ± 14 | 157 ± 11 | 159 ± 14$^{ac}$ | 159 ± 13$^{ac}$ | 159 ± 14$^{ac}$ |
| **RPE (AU)** | | | | | | |
| 0 g·h$^{-1}$ | 13 ± 2 | 14 ± 1$^{a}$ | 14 ± 2$^{ab}$ | 15 ± 1$^{abc}$ | 17 ± 2$^{abcd}$ | 18 ± 2$^{abcde}$ |
| 45 g·h$^{-1}$ | 13 ± 2 | 13 ± 2$^{a}$ | 14 ± 2$^{ab}$ | 15 ± 2$^{abc}$ | 16 ± 2$^{abcd}$ | 17 ± 2$^{abcde}$ |
| 90 g·h$^{-1}$ | 13 ± 2 | 14 ± 2$^{a}$ | 14 ± 2$^{ab}$ | 15 ± 2$^{abc}$ | 16 ± 2$^{abcd}$ | 16 ± 3$^{abcde}$ |
| **Cadence (rpm)** | | | | | | |
| 0 g·h$^{-1}$ | 87 ± 15 | 88 ± 15 | 88 ± 14 | 87 ± 13 | 81 ± 17$^{abcd}$ | 82 ± 14$^{abcd}$ |
| 45 g·h$^{-1}$ | 86 ± 13 | 87 ± 13 | 88 ± 14 | 88 ± 12 | 85 ± 15$^{abcd}$ | 82 ± 13$^{abcd}$ |
| 90 g·h$^{-1}$ | 89 ± 17 | 88 ± 16 | 87 ± 19 | 86 ± 21 | 85 ± 19$^{abcd}$ | 86 ± 18$^{abcd}$ |
| **% $\dot{V}O_{2max}$** | | | | | | |
| 0 g·h$^{-1}$ | 66 ± 4 | 66 ± 5 | 68 ± 5$^{a}$ | 68 ± 4$^{ab}$ | 69 ± 4$^{abc}$ | 69 ± 5$^{a}$ |
| 45 g·h$^{-1}$ | 67 ± 5 | 67 ± 6 | 68 ± 5$^{a}$ | 69 ± 4$^{ab}$ | 70 ± 5$^{abc}$ | 70 ± 5$^{a}$ |
| 90 g·h$^{-1}$ | 67 ± 3 | 68 ± 4 | 69 ± 4$^{a}$ | 69 ± 4$^{ab}$ | 69 ± 4$^{abc}$ | 67 ± 4$^{a}$ |
| **Efficiency (%)** | | | | | | |
| 0 g·h$^{-1}$ | 20.6 ± 1.1 | 20.5 ± 1.2 | 20.4 ± 1.2 | 20.2 ± 1.1 | 20.1 ± 0.7 | 20.5 ± 1.5 |
| 45 g·h$^{-1}$ | 20.3 ± 1.3 | 20.2 ± 1.4 | 20.1 ± 1.3 | 19.8 ± 1.2 | 19.8 ± 1.2 | 19.9 ± 1.2 |
| 90 g·h$^{-1}$ | 20.0 ± 1.4 | 20.0 ± 1.6 | 19.8 ± 1.6 | 19.7 ± 1.4 | 19.8 ± 1.7 | 20.1 ± 1.3 |
| **Energy Expenditure (kj·min$^{-1}$)** | | | | | | |
| 0 g·h$^{-1}$ | 61.5 ± 3.1 | 61.6 ± 3.8 | 62.0 ± 4.1 | 62.2 ± 3.8 | 62.6 ± 3.5 | 61.6 ± 5.4 |
| 45 g·h$^{-1}$ | 62.8 ± 4.1 | 62.8 ± 4.3 | 62.8 ± 4.6 | 63.5 ± 4.1 | 63.4 ± 4.2 | 63.2 ± 4.6 |
| 90 g·h$^{-1}$ | 62.9 ± 4.3 | 62.8 ± 4.9 | 63.4 ± 5.3 | 63.5 ± 5.4 | 63.3 ± 6.0 | 62.1 ± 5.0 |

[a]denotes significant difference from 30-min time point, [b]denotes significant difference from 60-min time point, [c]denotes significant difference from 90-min time point, [d]denotes significant difference from 120-min time point and [e]denotes significant difference from 150-min time point ($P < 0.05$). Data is presented as mean ± SD.

### 5.4.2 Skeletal muscle glycogen concentration

In accordance with the 36-h dietary standardisation protocol, subjects presented as CHO loaded where there was no difference in pre-exercise muscle glycogen concentration between trials ($P = 0.137$; 0 g·h$^{-1}$: 767 ± 87, 45 g·h$^{-1}$: 742 ± 109, 90 g·h$^{-1}$: 698 ± 98 mmol·kg$^{-1}$ dw). Exercise induced significant decreases in muscle glycogen to comparable levels immediately post the 180-min submaximal ride ($P < 0.01$; 0 g·h$^{-1}$: 252 ± 85, 45 g·h$^{-1}$: 269 ± 88, 90 g·h$^{-1}$: 293 ± 109 mmol·kg$^{-1}$ dw) and no differences were apparent between conditions ($P = 0.783$) (see Figure 5.3).

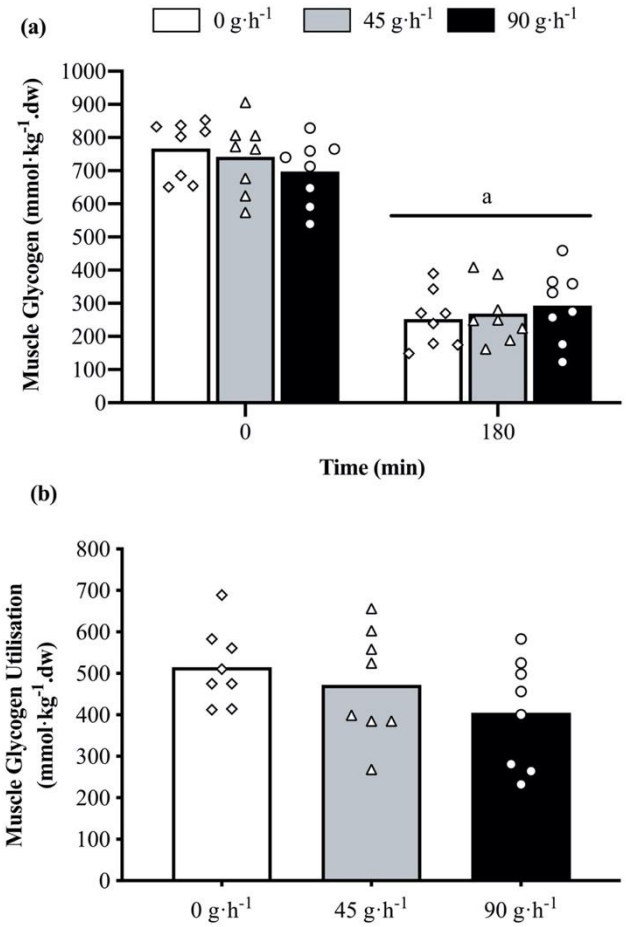


**Figure 5.3** (a) Skeletal muscle glycogen concentration before and immediately after the 180-min cycling protocol, (b) skeletal muscle glycogen utilisation from the 180-min cycling protocol for each nutritional condition. [a]denotes significant difference from 0-min, $P < 0.05$. Data are presented as means, and individual data points represent individual subjects.

### 5.4.3 Plasma metabolite responses

Plasma lactate ($P < 0.01$), NEFA ($P < 0.01$), glycerol ($P < 0.01$) and β-OHB ($P < 0.01$) all displayed significant changes during exercise (see Figure 5.4). In accordance with the increased dose of CHO, there was a significant difference between conditions for plasma glucose ($P < 0.01$), NEFA ($P = 0.005$), glycerol ($P < 0.01$) and β-OHB ($P = 0.002$) (see Figure 5.4). Specifically, plasma glucose was significantly higher during the 45 ($P < 0.01$) and 90 g·h$^{-1}$ trial ($P < 0.01$) compared to 0 g·h$^{-1}$ trial and 90 g·h$^{-1}$ was higher than 45 g·h$^{-1}$ ($P = 0.019$) (see Figure 5.4). Plasma NEFA was significantly higher in the 0 and 45 g·h$^{-1}$ dose compared to the 90 g·h$^{-1}$ dose ($P = 0.001$ and $P = 0.003$) (see Figure 5.4). Plasma glycerol was significantly higher in the 0 g·h$^{-1}$ trial compared to both the 45 ($P = 0.023$) and 90 g·h$^{-1}$ dose ($P < 0.01$) whilst the 45 g·h$^{-1}$ dose was also higher than the 90 g·h$^{-1}$ dose ($P = 0.020$) (see Figure 5.4). β-OHB followed a similar trend to that of plasma glycerol whereby it was significantly higher in the 0 g·h$^{-1}$ trial compared to both the 45 ($P = 0.013$) and 90 g·h$^{-1}$ dose ($P = 0.002$) and the 45 g·h$^{-1}$ dose was also higher than the 90 g·h$^{-1}$ dose ($P = 0.009$) (see Figure 5.4). Plasma glucose ($P = 0.001$), NEFA ($P = 0.002$), glycerol ($P < 0.01$) and β-OHB ($P < 0.01$) all displayed significant condition × time interaction effects.

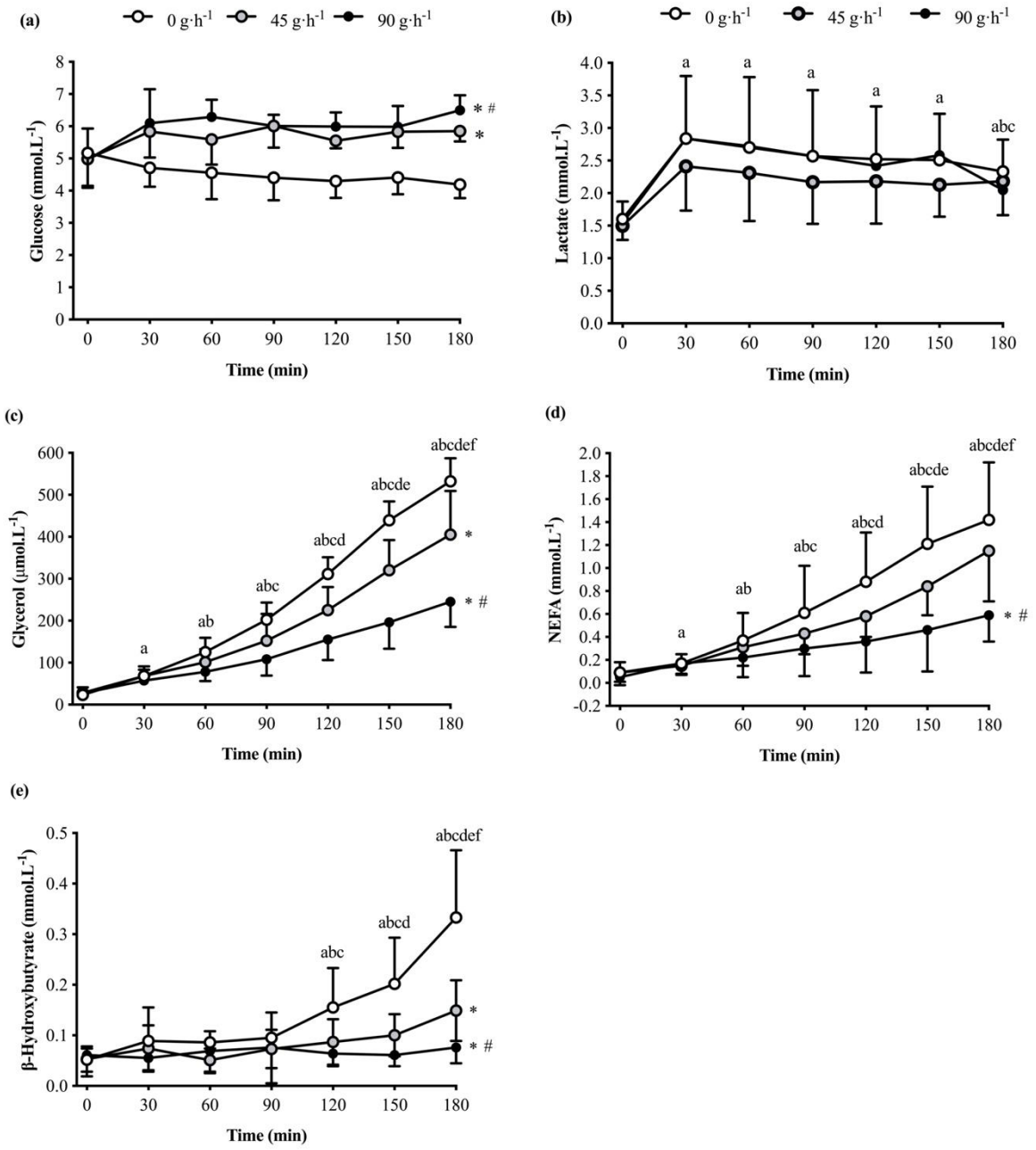


**Figure 5.4** (a) Plasma glucose, (b) lactate, (c) glycerol, (d) NEFA, (e) β-OHB response before and during the 180-min submaximal exercise. *denotes significant difference from 0 g·h$^{-1}$, #denotes significant difference from 45 g·h$^{-1}$, $P$<0.05. [a]denotes significant difference from 0-min, [b]denotes significant difference from 30-min, [c]denotes significant difference from 60-min, [d]denotes significant difference from 90-min, [e]denotes significant difference from 120-min and [f]denotes significant difference from 150-min, $P$<0.05. Data is presented as mean ± SD.

### 5.4.4 Substrate utilisation

Rates of whole-body CHO oxidation progressively decreased during exercise ($P$ < 0.01) with a significant difference also evident between conditions ($P$ = 0.005). Specifically, the 45 and

90 g·h$^{-1}$ trials maintained significantly higher CHO oxidation rates compared to the 0 g·h$^{-1}$ trial ($P$ = 0.030 and 0.004) whilst no difference was evident between the 90 and 45 g·h$^{-1}$ dose ($P$ = 0.369) (see Figure 5.5). Total CHO oxidation was significantly different between trials (P = 0.005) and was 315 ± 74, 386 ± 67 and 412 ± 69 g for 0, 45 and 90 g·h$^{-1}$, respectively, of which the 0 g·h$^{-1}$ trial was significantly different from the 45 and 90 g·h$^{-1}$ ($P$ = 0.030 and 0.004) (see Figure 5.5). Fat oxidation increased during exercise ($P$ < 0.01) with a significant difference between conditions ($P$ = 0.034) whereby the 90 g·h$^{-1}$ trial was significantly lower to the 0 g·h$^{-1}$ ($P$<0.01) whilst no differences were seen between any other trials (see Figure 5.5). Total fat oxidation was significantly different between trials (P = 0.035) and was 139 ± 23, 113 ± 17 and 104 ± 22 g for 0, 45 and 90 g·h$^{-1}$, respectively, of which the 0 g·h$^{-1}$ trial was significantly different from 90 g·h$^{-1}$ ($P$ < 0.01) (see Figure 5.5). RER significantly decreased during exercise ($P$ < 0.01) with a significant difference between conditions ($P$ = 0.019) (see Figure 5.5). Specifically, RER was significantly higher in the 45 and 90 g·h$^{-1}$ trial compared to the 0 g·h$^{-1}$ trial ($P$ = 0.041 and P = 0.002) (see Figure 5.5). Energy expenditure did not significantly change across exercise duration ($P$ = 0.420) or between conditions ($P$ = 0.386) (see Table 5.4). During hour 1 of exercise the contribution of CHO towards total energy expenditure was significantly greater than fat ($P$ < 0.001) and there was no significant interaction between condition and CHO contribution ($P$ = 0.07), see Figure 5.5). During hour 2 of exercise, CHO also contributed a greater proportion of energy expenditure than fat ($P$ = 0.029, see Figure 5.5) and furthermore, CHO contributed a greater energy yield in the 45 and 90 g·h$^{-1}$ trials compared with the 0 g·h$^{-1}$ trial ($P$ = 0.014 and $P$ = 0.005, respectively, see Figure 5.5). During hour 3, however, there was no significant difference between the contribution of CHO and fat towards total energy expenditure ($P$ = 0.06) though CHO again contributed a greater energy yield in the 45 and 90 g·h$^{-1}$ trials compared with the 0 g·h$^{-1}$ trial ($P$ = 0.015 and $P$ = 0.001, respectively, see Figure 5.5).

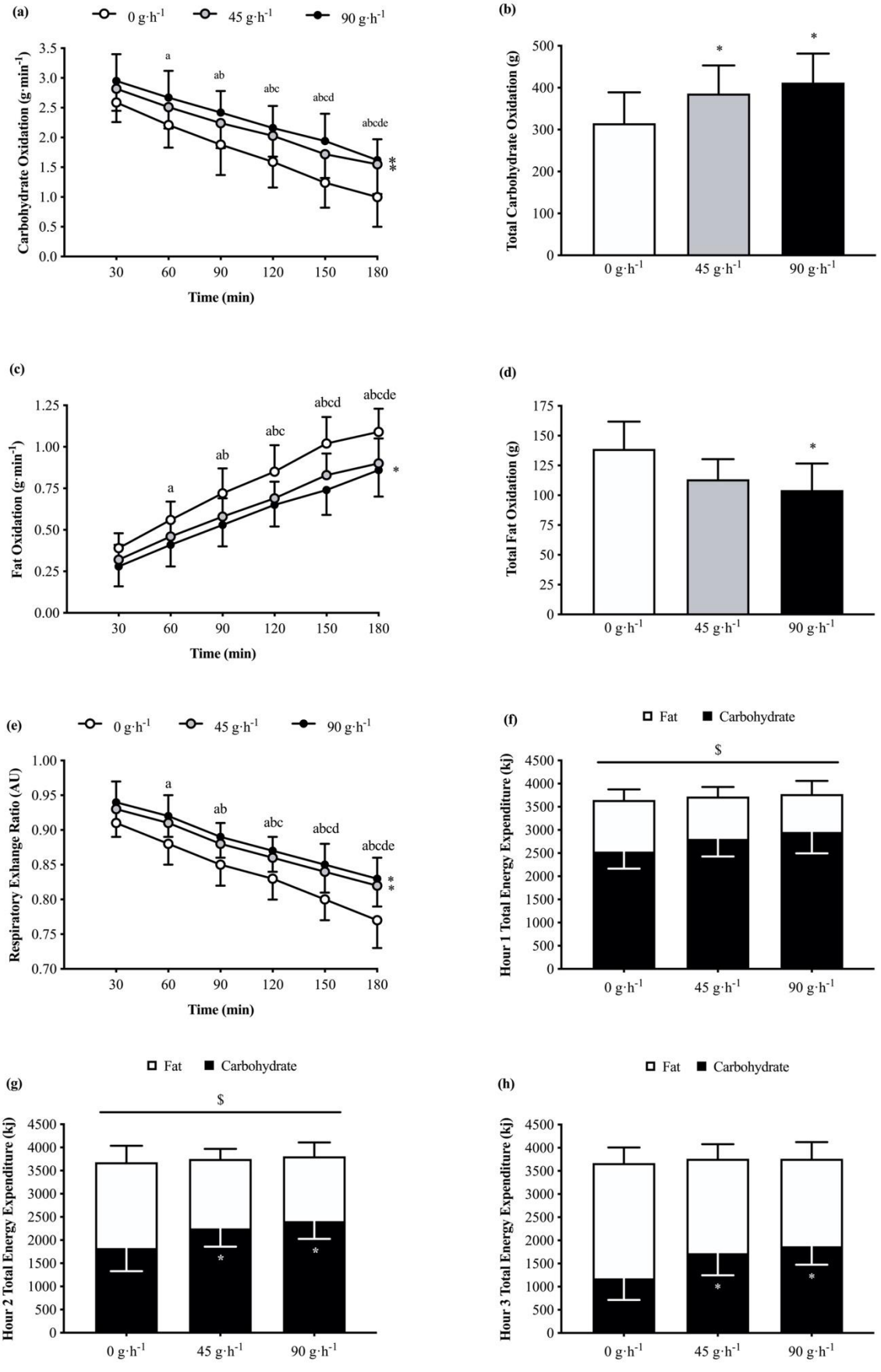
(a)
0 g·h-1
45 g·h-1
90 g·h-1
Carbohydrate Oxidation (g·min-1)
Time (min)
a
ab
abc
abcd
abcde
**
(b)
Total Carbohydrate Oxidation (g)
0 g·h-1
45 g·h-1
90 g·h-1
(c)
Fat Oxidation (g·min-1)
Time (min)
(d)
Total Fat Oxidation (g)
(e)
Respiratory Exhange Ratio (AU)
Time (min)
(f)
Fat
Carbohydrate
$
Hour 1 Total Energy Expenditure (kj)
(g)
Hour 2 Total Energy Expenditure (kj)
(h)
Hour 3 Total Energy Expenditure (kj)

**Figure 5.5 (**a) Carbohydrate oxidation rates during exercise, (b), total carbohydrate oxidation from exercise (c) fat oxidation rates during exercise, (d) total fat oxidation from exercise, (e) respiratory exchange ratio (RER) during exercise, (f) total energy expenditure during the first hour of exercise, (g) total energy expenditure during the second hour of exercise, (h) total energy expenditure during the third hour of exercise. $denotes significant main effect between carbohydrate and fat contribution to total energy expenditure, *denotes significant difference from 0 $g \cdot h^{-1}$, $P<0.05$. [a]denotes significant difference from 30-min, [b]denotes significant difference from 60-min, [c]denotes significant difference from 90-min, [d]denotes significant difference from 120-min, [e]denotes significant difference from 150-min, $P < 0.05$. Data is presented as mean ± SD.

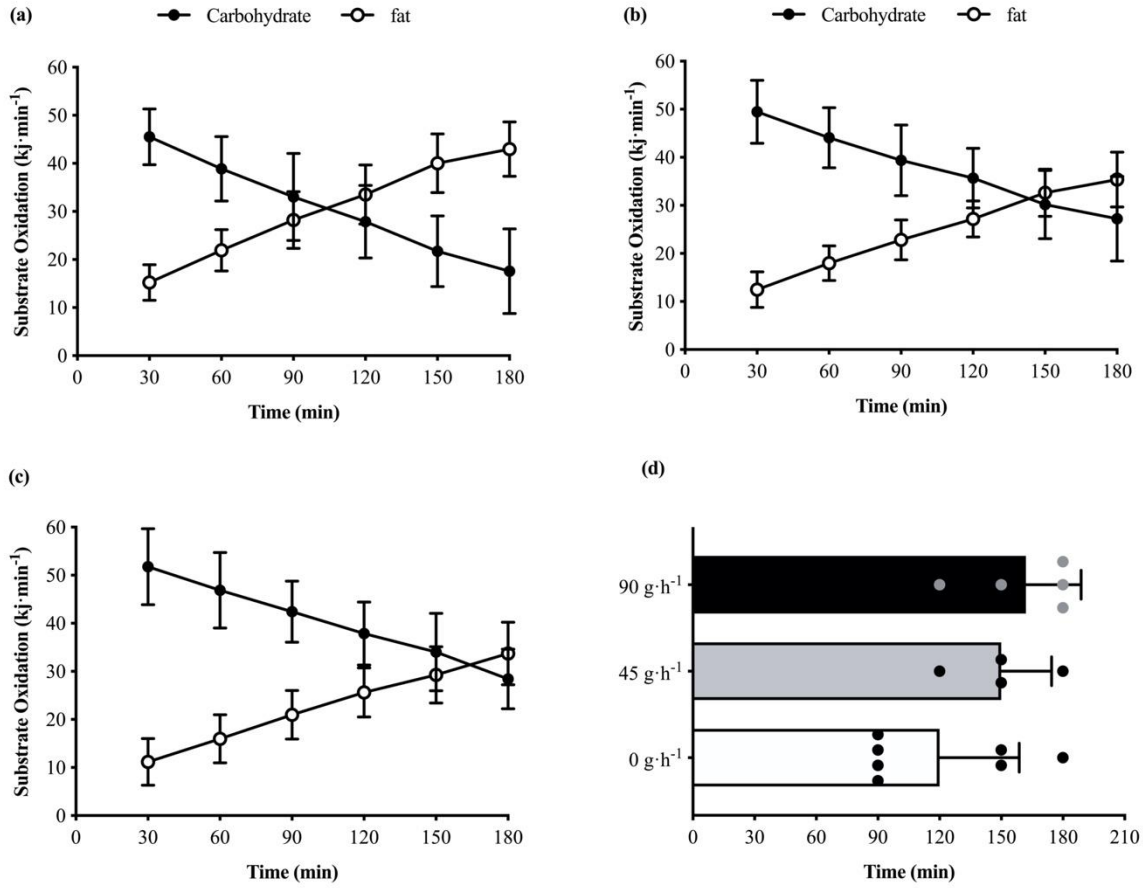


**Figure 5.6** Rates of energy provision from carbohydrate (CHO) and fat oxidation during the 0 (a), 45 (b) and 90 $g \cdot h^{-1}$ (c) trials. The mean (SD) and individual time points at which the crossover point occurred whereby fat provided the predominant contribution to energy expenditure (d), no individual data points are provided for those individuals who displayed no crossover contribution time point within a specific trial. Data is presented as mean ± SD.

### 5.4.5 Changes in body mass

Exercise induced a significant decrease in body mass from pre to post-exercise ($P < 0.01$) with body mass reducing from 74.0 ± 6.3 to 73.2 ± 6.0 kg (1.7 ± 0.4% dehydration) in the 0 g·h$^{-1}$; 73.7 ± 6.1 to 72.6 ± 5.9 kg (1.5 ± 0.4% dehydration) in the 45 g·h$^{-1}$ and 74.0 ± 5.6 to 73.2 ± 5.6 kg (1.0 ± 0.5% dehydration) in the 90 g·h$^{-1}$ with no significant difference between conditions ($P = 0.197$).

### 5.4.6 Time to exhaustion cycling capacity test

In accordance with the increase in CHO availability, cycling capacity was significantly increased in a dose dependent manner where the 90 g·h$^{-1}$ trial (233 ± 133 s) was significantly greater than the FAM (87 s ± 63 s; $P = 0.005$: 95% CI for differences = 62 to 232 s), 0 g·h$^{-1}$ (108 ± 54 s; $P = 0.013$: 95% CI for differences = 35 to 215 s) and 45 g·h$^{-1}$ (156 ± 66 s; $P = 0.06$: 95% CI for differences = -4 to 158 s) trials (see Figure 5.7). The 45 g·h$^{-1}$ dose was also significantly longer than the FAM ($P = 0.035$: 95% CI for differences = 6 to 133 s) and 0 g·h$^{-1}$ ($P = 0.029$: 95% CI for differences = 7 to 89 s) trial, whilst there was no significant difference between the 0 g·h$^{-1}$ and FAM ($P = 0.299$: 95% CI for differences = -24 to 68 s) (see Figure 5.7) trial. There was a significant difference in capacity times when data were analysed for a trial order effect ($P = 0.05$) but this was due to a significant increase in capacity time between FAM and the first experimental trial ($P = 0.005$: 95% CI for differences = 45 to 179 s) with no other pairwise differences present (see Figure 5.7). Plasma glucose ($P = 0.004$), NEFA ($P = 0.003$), glycerol ($P = 0.001$) and β-OHB ($P = 0.005$) were all significantly different between conditions at exhaustion whilst lactate ($P = 0.278$) and heart rate ($P = 0.294$) were not different between trials (see Figure 5.8). Specifically, plasma glucose was significantly higher in the 90 (6.38 ± 1.52 mmol·L$^{-1}$) compared to both the 0 (3.95 ± 0.48 mmol·L$^{-1}$, $P = 0.005$) and 45 g·h$^{-1}$ (5.03 ± 1.35 mmol·L$^{-1}$, $P = 0.001$) trials (see Figure 5.8). Plasma NEFA was significantly higher in the 0 (1.56 ± 0.15 mmol·L$^{-1}$) compared to both the 45 (0.75 ± 0.11 mmol·L$^{-1}$, $P = 0.023$) and 90 g·h$^{-1}$ (0.22 ± 0.07 mmol·L$^{-1}$, $P = 0.002$) trials whilst the 45 g·h$^{-1}$ condition was also significantly higher than the 90 g·h$^{-1}$ condition ($P = 0.013$) (see Figure 5.8). Plasma glycerol was significantly higher in the 0 (400 ± 25 mmol·L$^{-1}$) compared to the 90 g·h$^{-1}$ trial (117 ± 28 mmol·L$^{-1}$, $P = 0.001$) whilst the 45 g·h$^{-1}$ trial (286 ± 47 mmol·L$^{-1}$) was also significantly higher compared to the 90 g·h$^{-1}$ condition ($P = 0.006$) (see Figure 5.8). β-OHB was significantly higher in the 0 (0.56 ± 0.07 mmol·L$^{-1}$) compared to both the 45 (0.30 ± 0.04 mmol·L$^{-1}$, $P = 0.049$)

and 90 g·h$^{-1}$ (0.08 ± 0.03 mmol·L$^{-1}$, $P$ = 0.011) trials. 45 g·h$^{-1}$ condition was also significantly higher than the 90 g·h$^{-1}$ condition ($P$ = 0.028) (see Figure 5.8).

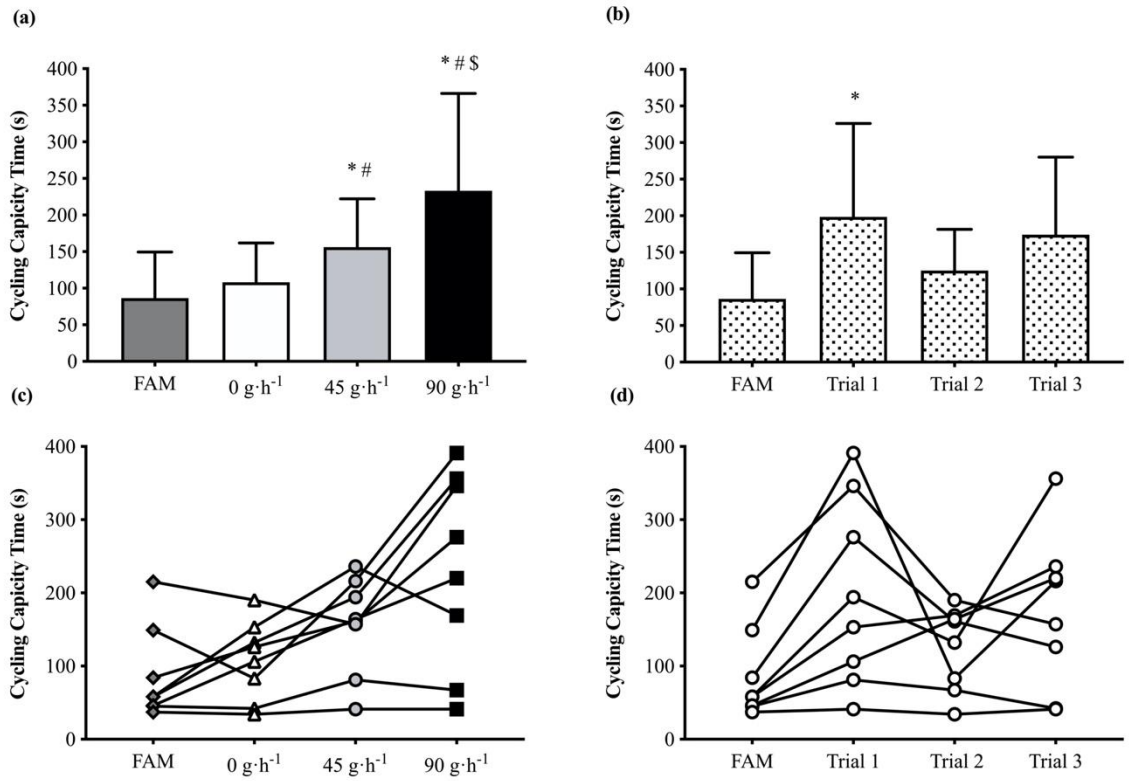


**Figure 5.7** Cycling capacity time (time to exhaustion) during the (a) familiarisation, 0, 45 and 90 g·h$^{-1}$ trials, (b) trial order cycling capacity time, (c) individual capacity time of subjects for experimental conditions and (d) individual capacity time of subjects for trial order. Data presented are mean ± standard deviation in (a) and (b), and individual data points in (c) and (d) represent individual subjects. *denotes significant difference from FAM, $P$ < 0.05, #denotes significant difference from 0 g·h$^{-1}$, $P$ < 0.05, $denotes significant difference from 45 g·h$^{-1}$, $P$ < 0.06. Data is presented as mean ± SD. Individual data points represent individual subjects, N = 8. FAM: familiarisation.

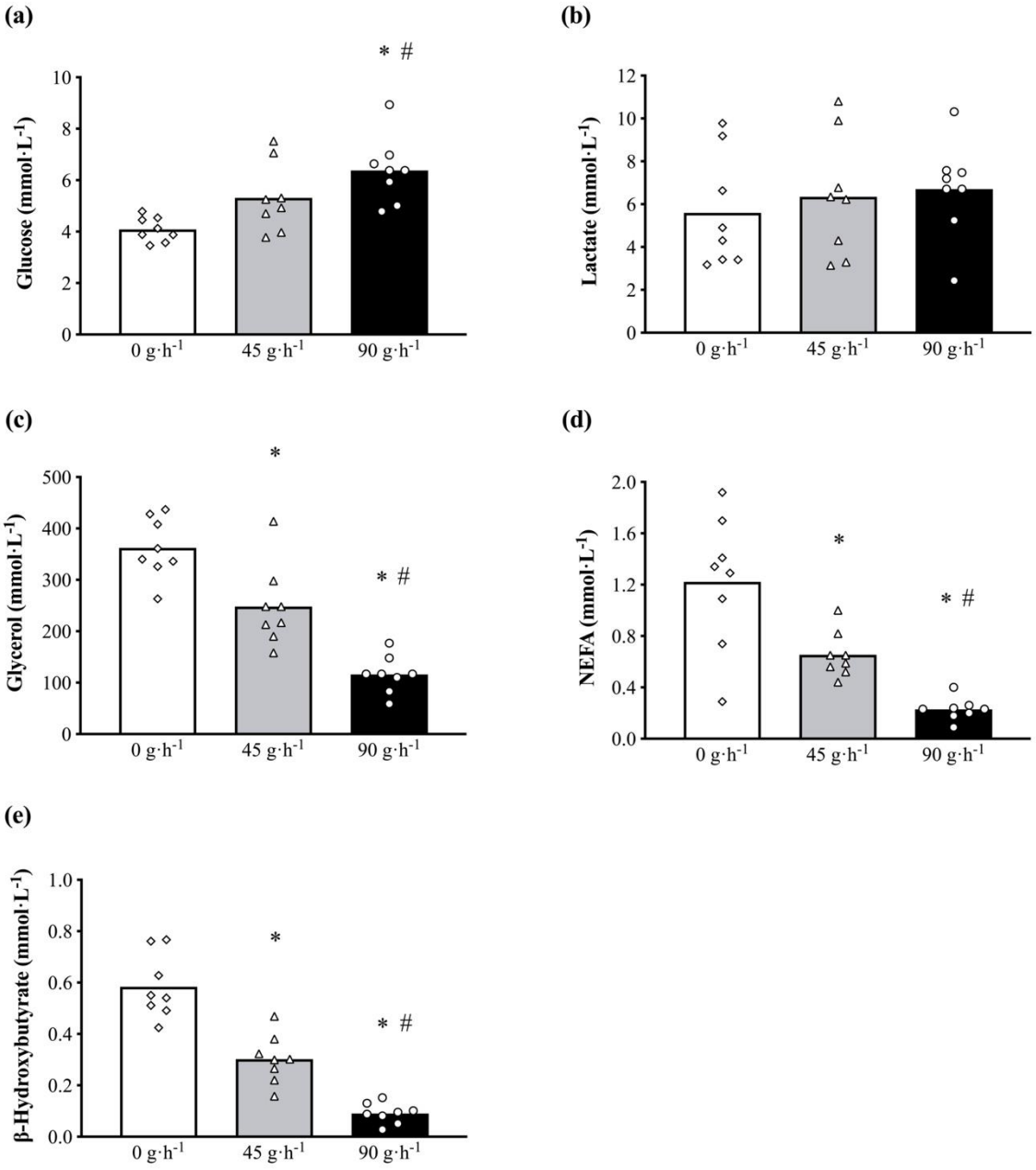


**Figure 5.8** (a) Plasma glucose, (b) lactate, (c) NEFA, (d) glycerol, (e) β-OHB response immediately post the cycling capacity test. *denotes significant difference from 0 g·h$^{-1}$, $denotes significant difference from 45 g·h$^{-1}$, $P < 0.05$. Data are presented as means, and individual data points represent individual subjects.

## 5.5 Discussion

Confirming our hypothesis, our data demonstrate that CHO feeding during steady-state submaximal cycling exercise improves subsequent high-intensity exercise capacity in a dose-dependent manner. Importantly, we utilised an exercise protocol that was intended to simulate the physiological demands of road cycling whereby riders typically ride in the "peloton" at a

submaximal intensity for the first few hours before "attacking" on mountain climbs / finishes in order to gain time over their opponents. From a practical perspective, we demonstrate that CHO consumption at a rate of 90 $g \cdot h^{-1}$ during 180-min of submaximal steady state exercise improves subsequent high-intensity exercise capacity when compared to both 45 (95% CI = -4 to 158 s) and 0 $g \cdot h^{-1}$ (95% CI = 35 to 215 s). As such, the magnitude of performance gain observed here can be considered to have practical relevance given that Grand Tour cycling stage races (where distances of up to ~3,500 km are contested over 21 days) can be won or lost by a matter of seconds (e.g. 59 seconds separated 1st and 2nd place of the 2020 Tour de France, https://www.letour.fr).

Unlike previous studies that examined the dose-response effects of CHO feeding after an overnight fast (King *et al.,* 2018; King *et al.,* 2019; Newell *et al.,* 2015; Smith *et al.,* 2010; Smith *et al.,* 2013;), we deliberately utilised an experimental design in which trained male cyclists commenced the steady state submaximal protocol after adhering to a CHO loading protocol and consumption of a pre-exercise meal rich in CHO. In this way, it was our intention that participants would commence exercise in conditions of both high muscle and liver glycogen stores. Indeed, although we did not measure liver glycogen concentrations, our resting muscle glycogen concentrations were comparable to those reported in a recent meta-analysis by Areta and Hopkins (2018) whereby trained male athletes ($\dot{V}O_{2max}$ of 60-70 $ml.kg.min^{-1}$) are expected to have resting muscle glycogen concentrations >700 $mmol \cdot kg^{-1}$ dw after consumption of a high CHO diet. Furthermore, whilst the majority of CHO feeding studies have provided CHO in the form of beverages only (see Stellingwerff and Cox, 2014), our chosen CHO feeding strategy also comprised a mixture of solids, gels and fluids so as to reflect the typical feeding patterns adopted by elite cyclists (Morton and Fell, 2016; Heikura *et al.,* 2019; Muros *et al.,* 2019; Sànchez-Muñoz *et al.,* 2016). In this way, our feeding protocol represented a mixture of CHO sources including maltodextrin, glucose, starch and fructose (see Table 5.1 and 5.2 for nutritional breakdown per hour of exercise) and participants consumed an average of ~500 mL fluid per hour, finishing each experimental trial 1.4 ± 0.5% dehydrated.

In relation to exercise capacity, we observed that CHO feeding improved exercise capacity in a dose dependent manner such that 90 $g \cdot h^{-1}$ > 45 $g \cdot h^{-1}$ > 0 $g \cdot h^{-1}$. Importantly, we also incorporated a full familiarisation trial in accordance with the findings of Study 1 and after accounting for this trial, we observed no trial order effect between the three experimental trials. Whilst it is difficult to directly compare between studies (given the differences in training

status, exercise protocol and CHO source and blend), our observation that 90 g·h$^{-1}$ is an ergogenic dose appears to agree with several other recent studies examining both single and multiple source blends. For example, Smith *et al.* (2010) observed that 15, 30 and 60 g·h$^{-1}$ of CHO (glucose provided in beverage format) during 2-h of cycling at 95% onset of blood lactate accumulation (OBLA) were all *very likely* at improving power output during a subsequent 20-km cycling time trial (TT) compared with 0 g·h$^{-1}$. Moreover, 60 g·h$^{-1}$ was *likely* to improve power output over and above that of the 15 and 30 g·h$^{-1}$ trial. Similarly, Newell *et al.* (2015) also demonstrated that consuming 39 and 64 g·h$^{-1}$ of a CHO (glucose) solution during 2-h submaximal steady-state cycling at 95% lactate threshold (LT) was equally effective in improving subsequent TT performance when compared to 20 or 0 g·h$^{--1}$. When taken together, such study designs demonstrate that consuming 30-60 g·h$^{-1}$ of a single source CHO during two hours of submaximal steady-state exercise is likely to induce a subsequent improvement in TT type protocols that are approximately 30 minutes in duration. However, when the ingestion of multiple source CHO blends is consumed (i.e., glucose/maltodextrin and fructose) across a wider range of 0 to 120 g·h$^{-1}$, Smith *et al.* (2013) estimated that 78 g·h$^{-1}$ CHO was the optimal dose to maximise performance. Using a multiple site research protocol, these researchers used a variety of doses of CHO (comprising a 1:1:1 ratio of glucose, fructose and maltodextrin solution consumed during 2 h steady state ride at 95% OBLA) and observed that the magnitude of performance improvement during a subsequent 20 km TT was curvilinear in nature. More recently, King *et al.* (2018) observed that ingestion of 90 g·h$^{-1}$ of a glucose-fructose (2:1 ratio) solution during a 2-h submaximal ride at 77% $\dot{V}O_{2max}$ produced the highest mean power output during a subsequent 30-min self-paced TT compared with 60 and 75 g·h$^{-1}$ of a glucose only solution and 112.5 g·h$^{-1}$ of a glucose-fructose (2:1 ratio) solution. When the same group extended exercise duration beyond 3 hours but of a lower exercise intensity (60% $\dot{V}O_{2max}$), the researchers reported that ingestion of 90 g·h$^{-1}$ of glucose and fructose (2:1 ratio) solution increased power output during a 30-minute self-paced TT by 6.8% and 4% when compared with 100 g·h$^{-1}$ and 80 g·h$^{-1}$, respectively (King *et al.,* 2019). When such studies are considered with the present data, it becomes apparent that where exercise duration exceeds 2.5-3 hours, the ergogenic effects of CHO feeding are likely most predominant with multiple source blends comprising 75-90 g·h$^{-1}$.

In relation to potential ergogenic mechanisms, our data suggest that CHO feeding improves exercise capacity in the absence of any muscle glycogen sparing (at least when assessed in whole muscle homogenate). This apparent lack of glycogen sparing agrees favourably with

several classical studies in the field that also demonstrate that CHO feeding during exercise does not induce a muscle glycogen sparing effect, at least when assessed in whole muscle homogenate (Arkinstall *et al.*, 2001; Bosch et al., 1994; Coyle *et al.*, 1986; De Bock *et al.*, 2007; Flynn *et al.*, 1987; Hargreaves & Briggs, 1988; McConell *et al.*, 1999; Mitchell *et al.*, 1989). We acknowledge, however, that our method of glycogen assessment is limited in that we did not quantify muscle glycogen in type I and type II muscle fibres. Indeed, previous studies examining fibre specific glycogen utilisation have observed a glycogen sparing effect in both type I (Stellingwerff *et al.*, 2007a) and type II fibres (De Bock *et al.*, 2007; Stellingwerff *et al.*, 2007). For example, Stellingwerff *et al.* (2007a) observed a glycogen sparing effect in both fibres when subjects consumed 50 $g \cdot h^{-1}$ of glucose during a 180-min cycling protocol completed at 50% $W_{max}$ in the absence of a pre-exercise meal. Whilst, in contrast, De Bock *et al.* (2007) only observed a glycogen sparing effect within type IIa fibres with the ingestion of 1 $g \cdot kg \cdot h^{-1}$ (~75 $g \cdot h^{-1}$) of maltodextrin during 2 h of exercise after the consumption of a high CHO pre-exercise meal (2.5 $g \cdot kg^{-1}$ in the CHO fed trial) compared to undertaking the exercise bout after an overnight fast with no CHO intake during the trial. As such, it is clear that future studies should examine the effects of the present CHO feeding strategy on fibre specific glycogen utilisation in order to definitively ascertain if alterations to muscle fuel selection are a contributory factor to the performance effect observed here.

In contrast to a muscle glycogen sparing effect, the observed exercise capacity improvements are most likely related to maintenance of high CHO availability, plasma glucose concentrations and ultimately, high rates of CHO oxidation. Such data appear consistent with classical studies (Coyle *et al.*, 1986) as well as more recent findings demonstrating that that CHO feeding exerts a liver glycogen sparing effect (Gonzalez *et al.*, 2015) and that performance improvements associated with CHO feeding are strongly correlated with maintenance of CHO availability and suppression of lipid oxidation (Coyle *et al.*, 1986; Newell *et al.*, 2018; Smith *et al.*, 2010). Indeed, although we acknowledge that our study design did not represent a true double-blind design (given that subjects consumed more solids in the 90 $g \cdot h^{-1}$ trial), it is noteworthy that examination of substrate availability and oxidation all support the hypothesis that maintenance of high rates of CHO oxidation (at the expense of lipid oxidation) underpinned the exercise capacity improvements observed here. Such a hypothesis is especially evident when examining the "crossover point" at which lipid oxidation comprised the largest proportion of energy production during exercise. Indeed, these data demonstrate that consuming 90 $g \cdot h^{-1}$ of CHO during exercise delayed the crossover point by ~10 and ~40 minutes when compared with the

45 and 0 g·h$^{-1}$ trial, respectively. Moreover, the specific improvement in exercise capacity time between the two CHO conditions (90 versus 45 g·h$^{-1}$) also seemed to be related to a greater maintenance of plasma glucose concentrations and an increased suppression of circulating plasma glycerol, NEFA and β-OHB during the 90 g·h$^{-1}$ trial compared to 45 g·h$^{-1}$ trial, indicative of a higher CHO availability and reduced reliance upon lipid oxidation during the 90 g·h$^{-1}$ trial. These differences in plasma parameters in relation to lipid oxidation seemed to be especially evident in the final hour of exercise in line with the delayed crossover point of lipid oxidation in the 90 g·h$^{-1}$ trial as previously noted. However, it is noteworthy, that despite these differences in blood parameters there was no difference between estimates of whole-body CHO and fat oxidation between CHO conditions. Nonetheless, these differences in plasma parameters where also evident immediately post-capacity test in that plasma glucose concentrations were higher and plasma glycerol, NEFA and β-OHB were lower in the 90 g·h$^{-1}$ trial compared to the 45 g·h$^{-1}$ trial. Such data further highlights a reduced reliance upon lipid oxidation during the exercise capacity test itself when participants were fed 90 g·h$^{-1}$ CHO during the preceding 180-min and seems to further support the hypothesis that a greater maintenance of plasma glucose and reduced reliance upon lipid oxidation underpin the improvements in exercise capacity with 90 g·h$^{-1}$ CHO compared to 45 g·h$^{-1}$ CHO in the present study. In addition, although rates of exogenous CHO oxidation or hepatic glucose output were not measured within the current study, the 90 g·h$^{-1}$ trial may have also been expected to produce higher rates of exogenous CHO oxidation alongside reducing hepatic glucose output given that previous work by Newell *et al.* (2018) reported that the ingestion of 64 g·h$^{-1}$ CHO increased exogenous CHO oxidation rates and reduced hepatic glucose output compared to 39 g·h$^{-1}$ CHO. This would have been expected given we provided considerably higher CHO (90 g·h$^{-1}$) with a larger difference between CHO doses (90 versus 45 g·h$^{-1}$) and in turn may have provided an additional rationale underpinning difference in exercise capacity outcomes.

In summary, we demonstrate that CHO feeding improves exercise capacity in trained male cyclists in a dose-dependent manner. Importantly, this ergogenic effect of CHO feeding was observed in conditions that are considered best nutritional practice (i.e., after CHO loading and consumption of a high CHO pre-exercise meal) and using an exercise duration (i.e., > 3 hours) and intensity (i.e., above lactate threshold) that has physiological relevance to professional road cyclists. Improvements in exercise capacity were observed in the absence of muscle glycogen sparing (at least when assessed in whole muscle homogenate) and are likely related to maintenance of CHO availability and high rates of CHO oxidation. Nonetheless, future studies

should examine glycogen utilisation within both type I and type II muscle fibres in response to the present feeding strategy. From a practical perspective, our data suggest that consuming 90 $g \cdot h^{-1}$ of CHO when riding in the peloton may subsequently improve the capacity to "hold a wheel" or "mount attacks" during the closing periods of mountain stages.

# Chapter 6:

# Carbohydrate feeding during prolonged submaximal cycling does not alter fibre type-specific utilisation of muscle glycogen and intramuscular triglyceride in trained male cyclists

The aim of this chapter was to examine the effects of CHO feeding on muscle fuel selection during endurance exercise. This chapter is based on further analysis of the muscle biopsies obtained in Study 2. This study was presented orally at European College of Sports Science (ECSS) Prague, Czech Republic, 2019 and was awarded third place in the Young Investigator Gatorade Sports Science Institute (GSSI) Nutrition Award session.

## 6.1 Abstract

**Purpose:** To test the hypothesis that carbohydrate (CHO) feeding alters muscle fuel selection during prolonged endurance exercise. **Methods:** In a repeated-measures, randomised, crossover design, eight endurance trained male cyclists ingested graded quantities of CHO (0, 45 or 90 $g \cdot h^{-1}$) comprising a mixture of solids, gels and fluids during 180-min of submaximal cycling at lactate threshold (208 ± 11 W). Subjects commenced each trial after a 36-h exercise and dietary standardisation protocol comprising prior glycogen depleting exercise, a CHO loading regimen (~11.6 $g \cdot kg^{-1}$) and a CHO rich pre-exercise meal (2 $g \cdot kg^{-1}$) consumed at 3 h before exercise. Muscle biopsies were obtained from the vastus lateralis muscle immediately pre- and post-completion of the 180-min submaximal protocol. **Results:** Exercise induced a significant reduction in glycogen ($P < 0.001$) with a difference between fibres being evident ($P < 0.001$). Specifically, muscle glycogen was reduced to a greater extent in type I compared to type II fibres ($P < 0.001$) and CHO feeding did not alter glycogen utilisation in either fibre type ($P = 0.309$). Similarly, exercise decreased muscle IMTG content in type I ($P < 0.001$) and type IIa fibres ($P = 0.002$), where the magnitude of utilisation was greater in type I fibres ($P < 0.001$). CHO feeding during exercise did not affect IMTG utilisation in either type I or IIa fibres (Type I: $P = 0.489$; Type IIa: $P = 0.841$). IMTG utilisation during exercise was a reflection of a decrease in lipid droplet (LD) number within the peripheral and central regions of both type I and IIa fibres whereas a reduction in LD size in both regions was exclusive to type I fibres. **Conclusion:** In conditions of high pre-exercise CHO availability (i.e., after CHO loading and consumption of a pre-exercise CHO meal), CHO feeding during exercise does not alter muscle glycogen or IMTG utilisation in either type I or type II fibres. Data suggest that the ergogenic effects of CHO feeding observed in Study 2 are therefore likely due to liver glycogen sparing, maintenance of plasma glucose availability and whole-body rates of CHO oxidation.

## 6.2 Introduction

Muscle glycogen and intramuscular triglycerides (IMTG) provide important energy substrates during endurance exercise (Coyle *et al.,* 1986; Van Loon *et al*., 2003; Watt *et al.,* 2002). Indeed, both substrates can become depleted during prolonged moderate intensity exercise, an effect that is especially evident in type I muscle fibres (De Bock *et al*., 2005; De Bock *et al*., 2008; Jevons *et al,* 2020; Stellingwerff *et al*., 2007; Van Loon *et al*., 2005; Van Proeyen *et al.,* 2011b). From an exercise performance perspective, the depletion of muscle glycogen is particularly relevant given that reduced glycogen availability is associated with the onset of fatigue (Bergström & Hultman, 1966; Bergström *et al.,* 1967; Hermansen *et al.,* 1967; Impey et al., 2016; Jensen *et al.,* 2020). In this regard, "muscle glycogen sparing" was initially proposed as a potential mechanism underpinning the ergogenic effects of CHO feeding during exercise (Bergstrom and Hultman, 1967; Bosch et al., 1996; Björkman *et al.,* 1984; Erickson *et al.,* 1987; Hargreaves *et al.,* 1984; Tsintzas *et al.,* 1995; Tsintzas *et al.,* 1996). It is noteworthy, however, that we (Chapter 5, Study 2) and others (Bosch et al., 1994; Coyle *et al.,* 1986; De Bock *et al.,* 2007; Flynn *et al.,* 1987; Hargreaves & Briggs, 1988; McConell *et al.,* 2000; Mitchell *et al.,* 1989) have observed that glycogen sparing is not always evident, especially when assessed in whole muscle homogenate. Nonetheless, when glycogen utilisation has been examined within specific muscle fibres, CHO feeding during prolonged cycling exercise has induced glycogen sparing in both type I (Stellingwerff *et al.,* 2007a) and type II muscle fibres (De Bock *et al.,* 2007; Stellingwerff *et al.,* 2007a).

In contrast to muscle glycogen, the effects of CHO feeding on IMTG utilisation during exercise has been relatively less well studied and is complicated by important methodological differences between studies. De Bock *et al.* (2005) initially reported an attenuated use of IMTG in type I muscle fibres when CHO was consumed before (~150 g) and during 2 h moderate intensity cycling (1 g·kg·h$^{-1}$), an effect attributed to the anti-lipolytic action of insulin (and decreased circulating adrenaline concentrations) and down regulation of hormone sensitive lipase (HSL) (Watt *et al.,* 2004). In contrast, Stellingwerff *et al.* (2007a) observed no effect of CHO feeding (0.7 g·kg·h$^{-1}$) during 180-min of exercise upon the net utilisation of IMTG stores in either type I or II muscle fibres. Such discrepancies in fibre-specific muscle glycogen and IMTG use in the aforementioned studies may be due to the difference in CHO feeding protocols between studies. Indeed, although Stellingwerff *et al.* (2007a) provided ~0.7 g·kg·h$^{-1}$ (~50 g·h$^{-1}$) of glucose during 180-min of exercise in the absence of a pre-exercise meal (i.e., a total of

~150 g CHO), the protocols adopted by De Bock *et al.* (2005, 2007) provided 1 g·kg·h$^{-1}$ of maltodextrin during 2 h of exercise in conditions where CHO was also fed before exercise (~2.5 g·kg$^{-1}$ of CHO ) in the CHO fed trial (thus providing a total of ~300 g of CHO in the CHO fed trial) whereas the control trial commenced after an overnight fast. As such, it is possible that any potential effects of CHO feeding "during" exercise on the metabolic regulation of muscle fuel metabolism (especially IMTG utilisation) may be negated in conditions where CHO has already been consumed in a pre-exercise meal. When taken together, such studies clearly demonstrate the importance of isolating the effects of CHO feeding to that consumed during exercise (as opposed to pre-exercise) as well as the requirement to assess fibre type specific substrate utilisation.

With this in mind, the aim of the present study was to test the hypothesis that CHO feeding during exercise alters muscle fuel selection in both type I and type II muscle fibres. To this end, we conducted further analysis on the muscle biopsies collected in Study 2 whereby trained male cyclists completed 180 minutes of steady state submaximal exercise during which they consumed 0, 45 or 90 g·h$^{-1}$ of CHO. Importantly, each experimental trial was commenced after a 36-h CHO loading protocol (11.6 g·kg$^{-1}$) and 3 hours after consumption of a pre-exercise meal that was rich in CHO (2 g·kg$^{-1}$).

## 6.3 Methods

### 6.3.1 Participants

Eight endurance-trained amateur male cyclists (mean ± SD: age, 31 ± 7 years; body mass 73.6 ± 6.1 kg; height, 177.0 ± 8.0 cm) took part in this study. Mean $\dot{V}O_{2max,}$ peak power output (PPO) and power output at lactate threshold (LT) for the cohort were 60.5 ± 5.4 mL·kg$^{-1}$·min$^{-1}$, 383 ± 21 W and 208 ± 11 W, respectively. Subjects were defined as trained in accordance with the criteria of Jeukendrup, Craig & Hawley (2000) whereby the cyclists were competitive club level road cyclists as detailed by subject characteristics and training and racing data described in Table 3.1. None of the subjects had any history of musculoskeletal or neurological disease nor were they under any pharmacological treatment during the course of the testing period. All participants provided written informed consent before commencement of the study, which was approved by the local Ethics Committee of Liverpool John Moores University.

### 6.3.2 Experimental design

In a repeated-measures, randomised crossover design, subjects completed four prolonged endurance-based cycling exercise protocols which consisted of 180-min steady state submaximal exercise (undertaken at lactate threshold). The initial trial was a familiarisation to the exercise protocol and in the following three trials, subjects ingested CHO at a rate of 0, 45 or 90 g per hour in the form of solids, gels and fluids during the exercise bouts. As such, these CHO ingestion regimens replicated the typical eating patterns of professional cyclists during competition (Heikura *et al.*, 2019; Muros *et al.*, 2019; Morton, unpublished observations; Sánchez-Muñoz *et al.*, 2016). At 36 - 40 h prior to all trials, all subjects performed a glycogen depletion cycling protocol followed by 36-h of high CHO intake (11.6 $g \cdot kg^{-1}$) so as to maximise muscle glycogen prior to commencing each experimental trial. Additionally, each trial was also commenced 3 h after a CHO rich meal (2 $g \cdot kg^{-1}$). Muscle biopsies were obtained from the vastus lateralis immediately before and after the 180-min submaximal exercise protocol. An overview of the experimental protocol is shown in Figure 6.1.

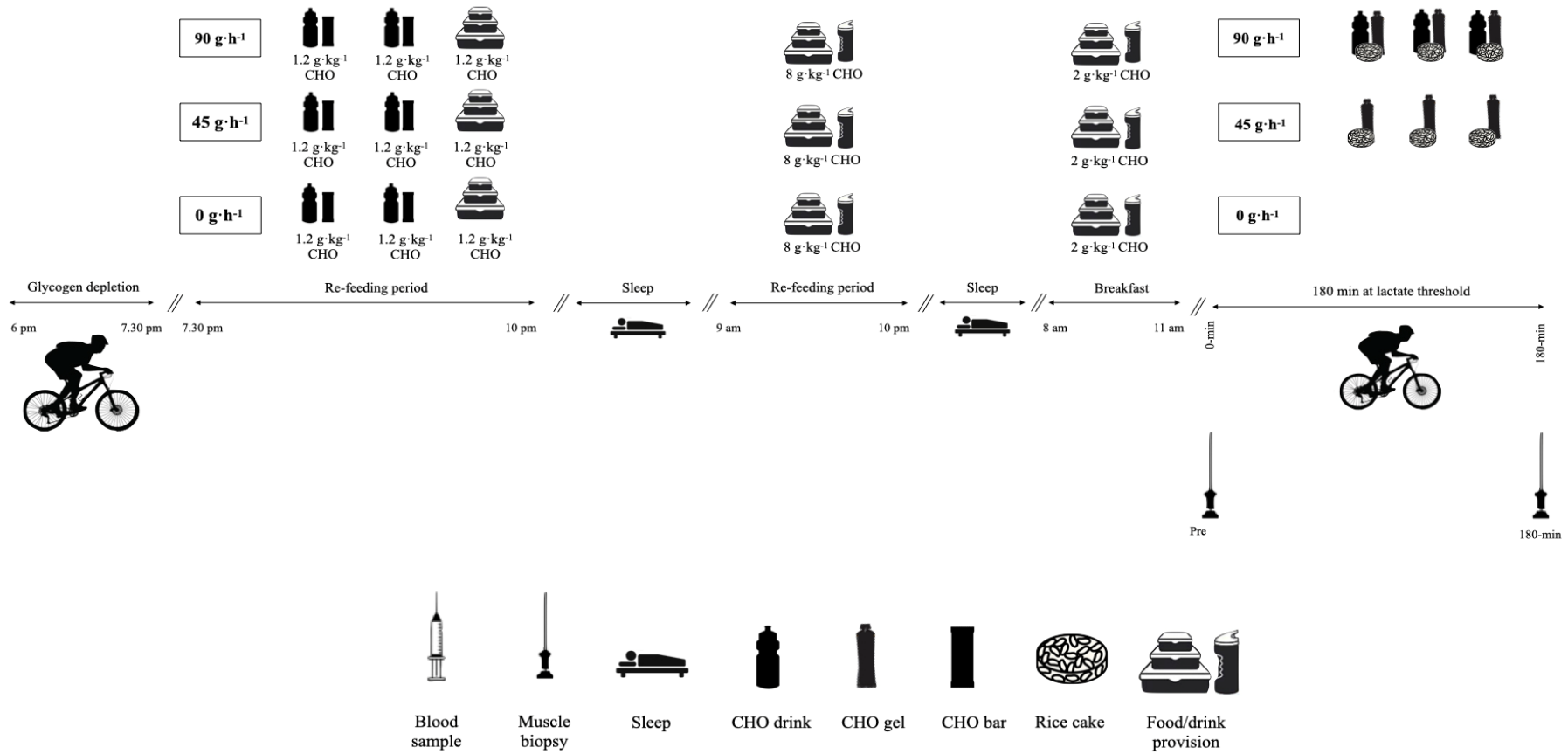


**Figure 6.1** Schematic overview of the experimental protocol. Subjects completed an evening bout of glycogen depleting cycling exercise followed by the consumption of a high carbohydrate (CHO) diet for 36 h prior to the main experimental trial. Following a high CHO pre-exercise meal, subjects completed a bout of submaximal cycling, during which graded quantities of CHO were ingested. Muscle biopsies were obtained immediately before and after exercise.

#### 6.3.2.1 Preliminary testing and familiarisation

At least 10 days prior to experimental trials, all subjects performed a two-part incremental cycle test to determine peak oxygen consumption ($\dot{V}O_{2max}$), lactate threshold (LT) and peak power output (PPO) on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands). The two-part incremental cycling test has been detailed previously in Section 3.2.4. Subjects also undertook full familiarisation of the experimental trial, at least 7 days prior to the main experimental trials where they refrained from CHO intake during the submaximal cycling protocol, thus replicating the same nutritional conditions as per the 0 $g \cdot h^{-1}$ trial.

#### 6.3.2.2 Day1: Glycogen depletion exercise

At 36-h before the main experimental trial (i.e., the evening of Day 1), subjects performed an intermittent glycogen-depleting cycling protocol lasting 90-minutes in duration. The glycogen depletion protocol has been previously described in Section 4.3.4.1.

#### 6.3.2.3 Day 2: Carbohydrate loading regimen

To facilitate the goal of elevating muscle glycogen concentrations in preparation for the prolonged cycling protocol in accordance with current competition nutritional guidelines (Thomas *et al.,* 2016) and the daily energy intake of elite road cyclists during (Heikura *et al.,* 2019; Muros *et al.,* 2019; Morton, unpublished observations; Sánchez-Muñoz *et al.,* 2016), subjects were provided with high carbohydrate snacks and fluids (SiS GO Energy, SiS REGO Protein, SiS GO Bars, Science in Sport, Blackburn, UK) to consume immediately post glycogen depletion exercise (CHO: 1.2 $g \cdot kg^{-1}$ BM; PRO: 0.4 $g \cdot kg^{-1}$ BM; Fat: 0 $g \cdot kg^{-1}$ BM), at 1-hour post (CHO: 1.2 $g \cdot kg^{-1}$ BM; PRO: 0.1 $g \cdot kg^{-1}$ BM; Fat: 0 $g \cdot kg^{-1}$ BM) and at 2-hours post (CHO: 1.2 $g \cdot kg^{-1}$ BM; PRO: 0.1 $g \cdot kg^{-1}$ BM; Fat: 0 $g \cdot kg^{-1}$ BM) (see Table 3.4). Over the course of the following day (i.e., Day 2), participants were provided with a pre-packaged standardised high CHO diet to consume throughout the entirety of that day (CHO: 8 $g \cdot kg^{-1}$ BM; PRO: 2 $g \cdot kg^{-1}$ BM; Fat: 1 $g \cdot kg^{-1}$ BM) (see Table 3.4) (Sánchez-Muñoz *et al.,* 2016; J.P., Morton, unpublished observations) and refrained from any strenuous physical activity and alcohol consumption during this day to promote muscle and liver glycogen resynthesis (Bartlett *et al.,* 2013). Fluid intake was allowed *ad libitum*.

#### 6.3.2.4 Day 3: Experimental trials

On the morning of the main experimental trials at ~08:00, subjects reported to the laboratory in a fasted state and were immediately provided with a standardized high-CHO "Race-day" breakfast (CHO: 2 $g \cdot kg^{-1}$ BM; PRO: 60 g; Fat: 45g) that was representative of an elite real-world cyclists 'Race-day" breakfast (see Table 3.4) (Heikura *et al.,* 2019; Muros *et al.,* 2019; Morton, unpublished observations; Sánchez-Muñoz *et al.,* 2016). Immediately prior to exercise, a muscle biopsy sample was taken from the vastus lateralis. The submaximal cycling protocol consisted of 180 min of cycling at 100% LT (208 ± 11 W) at a self-selected cadence, during which carbohydrate was ingested at a rate of either 0 $g \cdot h^{-1}$, 45 $g \cdot h^{-1}$or 90 $g \cdot h^{-1}$ (see Table 5.1 and 5.2 for full nutritional breakdown). In both 45 and 90 $g \cdot h^{-1}$ trials, carbohydrate was provided in the form of gels (GO Energy, Science in Sport, Nelson, UK) and rice cakes (21 g CHO per rice cake) whilst subjects in the 90 $g \cdot h^{-1}$ trial were also provided with a specially formulated maltodextrin-based drink containing 22 g CHO per 500 ml (see Table 5.3 for full nutritional breakdown). Fluid intake during exercise was matched at 500 $ml \cdot h^{-1}$ across all three trials, whereby subjects in the 0 and 45 $g \cdot h^{-1}$ trials consumed water flavoured with electrolyte tablets (Go Hydro, Science in Sport, Nelson, UK) and subjects in the 90 $g \cdot h^{-1}$ trial consumed the aforementioned maltodextrin drink.

### 6.3.3 Muscle biopsies

Skeletal muscle biopsies (~100 mg) were obtained from the vastus lateralis immediately pre- and post-exercise. Muscle biopsies were obtained as described in Section 3.6.

### 6.3.4 Quantitative immunofluorescence microscopy

Quantitative immunofluorescence microscopy was used to investigate the effects of exercise and CHO feeding on fibre-specific IMTG content. Details of the specific quantitative measurement methods and image capture, processing and analysis are described in detail in Sections 3.8, 3.8.1, 3.8.2 and 3.8.4, respectively.

### 6.3.5 Semi-quantitative fibre type specific muscle glycogen analysis

Semi-quantitative muscle glycogen content was assessed through the use of brightfield periodic acid-Schiff stain (PAS) in combination with an immunofluorescence myosin heavy chain (MHC) stain. Details of the specific quantitative measurement methods and image capture,

processing and analysis are described in detail in Sections 3.9, 3.9.1, 3.9.2 and 3.9.3, respectively.

### 6.3.6 Statistical analysis

All statistical analyses were performed with the statistical package for the Social Sciences (SPSS version 26). Descriptive statistics were produced for all data sets to check for normal distribution indicated by Shapiro-Wilk (accepted if $P > 0.05$). Linear mixed modelling was used to examine the dependent variable, skeletal muscle glycogen content, at pre- and post-exercise with data separated into the three different experimental conditions (0, 45 and 90 $g \cdot h^{-1}$ CHO). All main effects and interactions were tested using a linear mixed-effects model, with random intercepts to account for repeated measurements within subjects to examine differences between experimental conditions, fibre type and time. Subsequent Bonferroni adjustment post hoc analysis was used to examine main effects and interactions. Comparisons of pre-exercise IMTG content (% area stained) and LD morphology were analysed with a three-way repeated-measures general linear model (GLM), where the within factors were condition, region and fibre. Changes in IMTG content (% area stained) and LD morphology over time between conditions within each fibre, were analysed with a three-way repeated-measures GLM, where the within factors were condition, region and time. Moreover, the relative distribution of IMTG content between subcellular regions over time were analysed with a three-way repeated-measures general linear model, where the within factors were condition, region and time. Comparisons of IMTG breakdown were analysed with a three-way repeated-measures GLM, where the within factors were condition, region and fibre. If Mauchley's test of sphericity indicated a minimum level of violation, as assessed by a Greenhouse Geisser epsilon ($\varepsilon$) of > 0.75, data were corrected using Huyn-Feldt $\varepsilon$. If Mauchley's test of sphericity was violated, data were corrected using Greenhouse Geisser $\varepsilon$. Where a significant main effect was observed, pairwise comparisons were analysed according to Bonferroni post hoc tests to locate specific differences. All data in text, figures, and tables are presented as means ± SD for skeletal, with $P$ values < 0.05 indicating statistical significance.

## 6.4 Results

### 6.4.1 Skeletal muscle glycogen content

Subjects commenced exercise with similar muscle glycogen concentrations across all three conditions (main condition effect, $P = 0.801$; Figure 6.2) and between fibres (main fibre effect, $P = 0.860$; Figure 6.2). Exercise induced a significant reduction in glycogen (main time effect, $P < 0.001$) with a difference between fibres being evident (main fibre effect, $P < 0.001$). Specifically, muscle glycogen was reduced to a greater extent in type I compared to type II fibres (time × fibre interaction, $P < 0.001$; Figure 6.2) and CHO feeding did not alter glycogen utilisation in either fibre type (main condition effect, $P = 0.309$; Figure 6.2). Additionally, muscle glycogen content was reduced by 41 ± 16, 35 ± 12 and 30 ± 18% in type I fibres and by 7 ± 8, 11 ± 11 and 9 ± 14% in type II fibres for the 0, 45 and 90 $g \cdot h^{-1}$ trial, respectively. Further representation of the relative scale of glycogen depletion within type I fibres illustrates that after 180 minutes of exercise, ~90, 60 and 75% of type I fibres in the 0, 45 and 90 $g \cdot h^{-1}$ were classified as partially empty and empty (see Figure 6.3). In type II fibres, ~8, 10 and 25% of fibres were classified as partially empty and empty in the 0, 45 and 90 $g \cdot h^{-1}$ trial (see Figure 6.3). Figure 6.4 depicts visual representation of glycogen content in type I and II fibres at 0-min and after 180-min of submaximal cycling exercise in the 0, 45 and 90 $g \cdot h^{-1}$ condition.

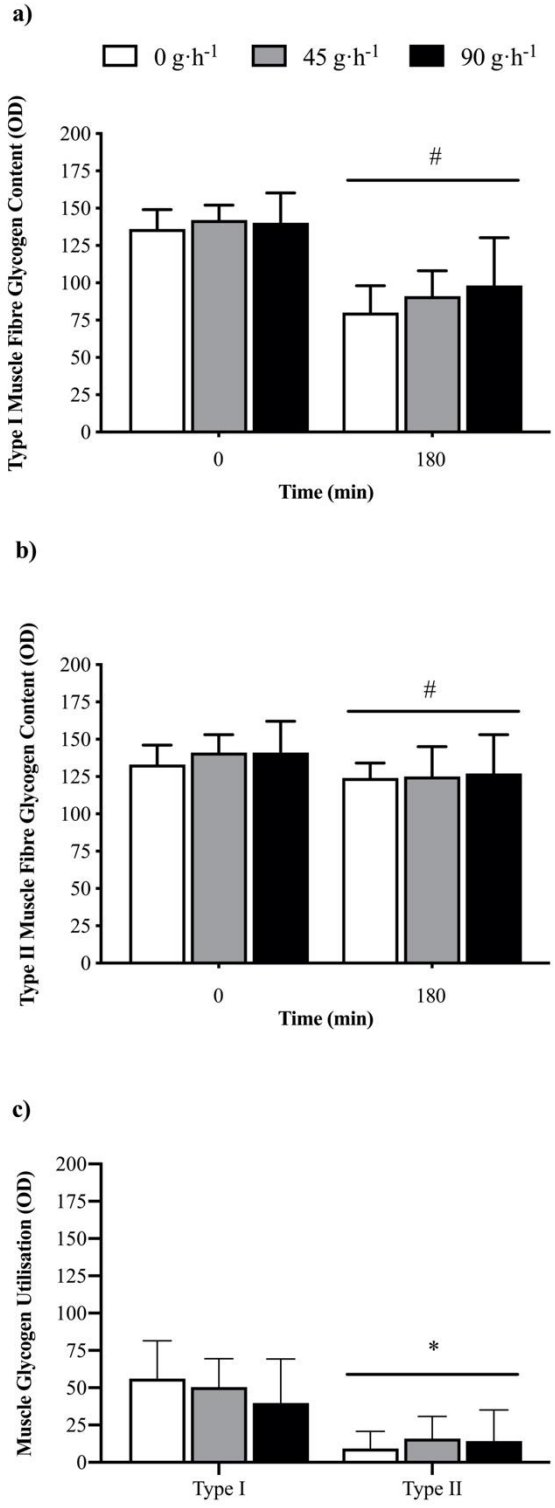


**Figure 6.2** Effect of exercise and dose of carbohydrate (CHO) intake on muscle fibre type-specific glycogen content. Muscle glycogen for type I (a) and type II fibres (b) before and after the 180-min submaximal cycling exercise protocol as determined on periodic acid Schiff-stained muscle cross sections and calculated net muscle glycogen utilisation (c). 0 g·h$^{-1}$ (clear bars), 45 g·h$^{-1}$ (grey bars) or 90 g·h$^{-1}$ (black bars) of CHO was consumed during the exercise bout. #$P$ < 0.05, significantly different from pre-exercise. *$P$ < 0.05, significantly different from type I fibres. Data is presented as mean ± SD, carbohydrate (CHO).

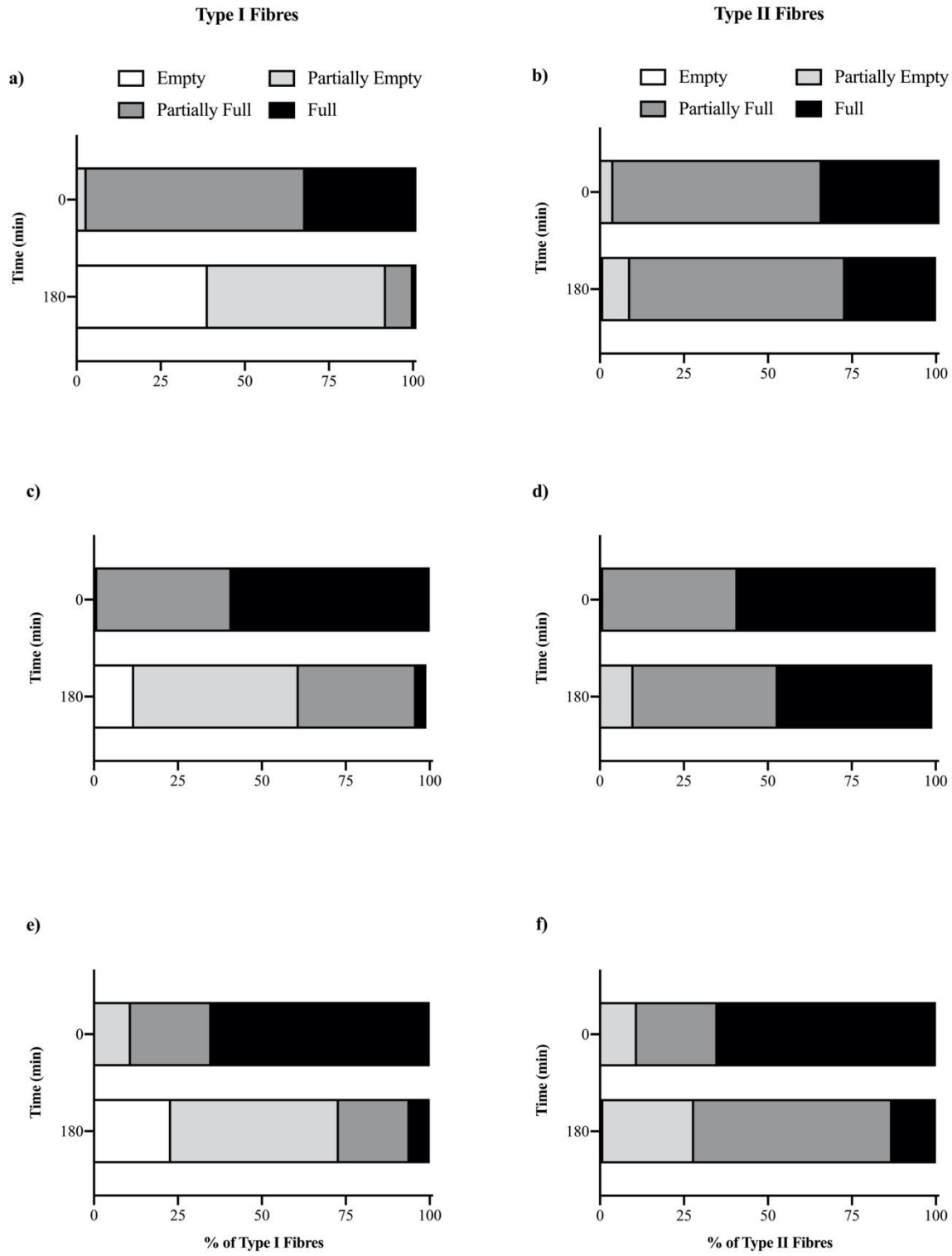


**Figure 6.3** Relative glycogen content in 0 and 180-min sections in a) 0 $g \cdot h^{-1}$ type I, b) 0 $g \cdot h^{-1}$ type II, c) 45 $g \cdot h^{-1}$ type I, d) 45 $g \cdot h^{-1}$ type II, e) 90 $g \cdot h^{-1}$ type I, f) 90 $g \cdot h^{-1}$ type II muscle fibres. Data is presented as percentage of fibres categorised within optical density increments of 25% from the lowest recorded optical density to the highest value across all samples (see Section 3.9.3).

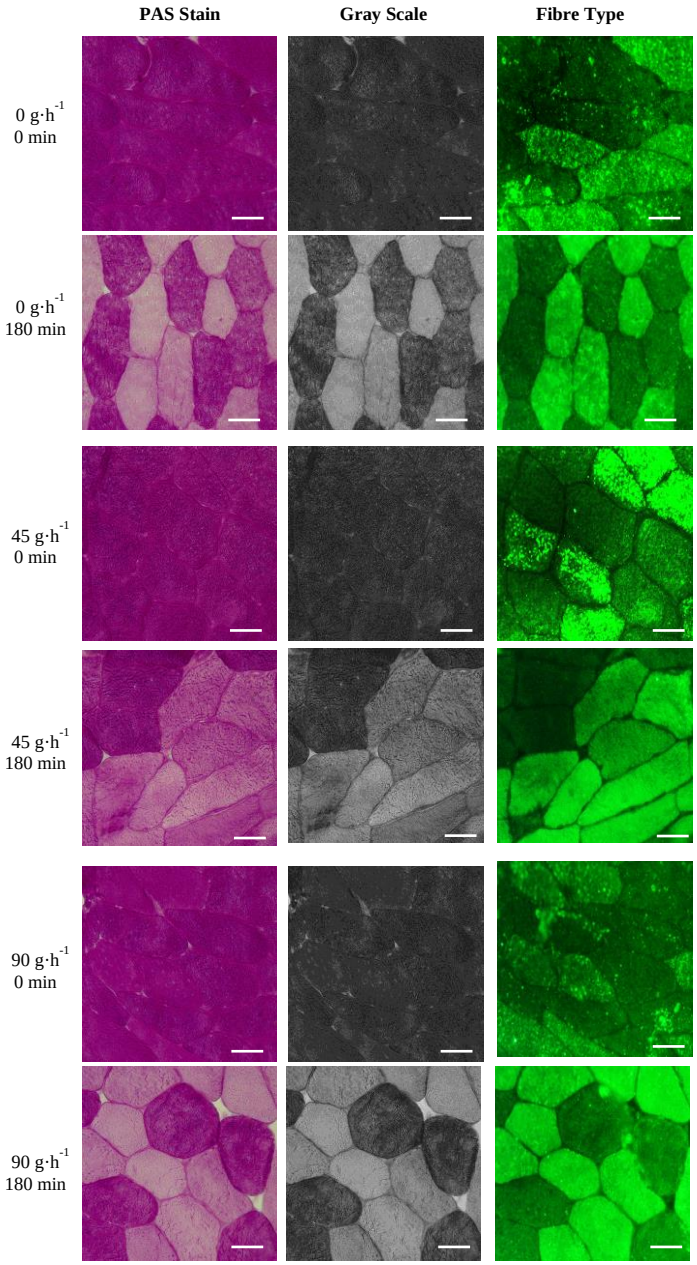


**Figure 6.4** Representative images of muscle glycogen in response to prolonged endurance exercise with and without carbohydrate (CHO) feeding during exercise. Sections were stained for glycogen (periodic acid-Schiff (PAS)), fibre type (primary antibody MHCI – A4.840c; secondary antibody goat anti-mouse IgM 488, green). Images depict glycogen content in type I and II fibres at 0 and 180-min in the 0, 45 and 90 $g \cdot h^{-1}$ condition. Scale bars represent 50 µm.

### 6.4.2 Skeletal muscle lipid analysis

#### 6.4.2.1 Pre exercise IMTG content and LD morphology

Before exercise, fibre-type specific muscle IMTG content (expressed as percentage of fibre stained) was similar between trials (main condition effect, $P = 0.944$), although, type I muscle fibres had greater IMTG content compared to type IIa fibres (main fibre effect, $P < 0.001$; Table 6.1), and IMTG content was greater in the periphery of the cell (within the 2 μm border) compared with the central region (main region effect; $P = 0.003$; Table 6.2). Overall though, the majority of IMTG was observed in the central compared with the peripheral region of the cell (main region effect, $P < 0.001$; Table 6.2). Considering the number and size of LDs, there was a greater number of LDs (main fibre effect, $P = 0.001$; Table 6.1) with a larger average size (main fibre effect, $P = 0.012$; Table 6.1) in type I fibres compared with type IIa fibres. Thus, pre-exercise fibre type differences in IMTG content were predominantly explained by differences in LD number and size.

**Table 6.1** Pre-exercise IMTG content and LD morphology.

| | 0 g·h$^{-1}$ | | | | 45 g·h$^{-1}$ | | | | 90 g·h$^{-1}$ | | | | *P* Value | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Type I Fibres | | Type IIa Fibres | | Type I Fibres | | Type IIa Fibres | | Type I Fibres | | Type IIa Fibres | | | | |
| | Peripheral | Central | Peripheral | Central | Peripheral | Central | Peripheral | Central | Peripheral | Central | Peripheral | Central | Condition | Fibre | Region |
| IMTG Content (% area stained) | 7.60 ± 2.16* | 5.40 ± 2.20* | 5.03 ± 1.63 | 3.68 ± 2.16 | 7.65 ± 2.16* | 5.82 ± 2.93* | 5.24 ± 2.43 | 3.42 ± 2.18 | 7.39 ± 2.54* | 5.60 ± 1.77* | 4.60 ± 1.18 | 3.65 ± 1.02 | *P* = 0.944 | *P* < 0.001 | *P* = 0.003 |
| LD Size (μm$^2$) | 0.365 ± 0.076* | 0.364 ± 0.118* | 0.326 ± 0.117 | 0.324 ± 0.098 | 0.346 ± 0.056* | 0.348 ± 0.072* | 0.289 ± 0.062 | 0.301 ± 0.086 | 0.351 ± 0.067* | 0.390 ± 0.138* | 0.304 ± 0.048 | 0.384 ± 0.066 | *P* = 0.6 | *P* = 0.012 | *P* = 0.112 |
| LD number (LD/μm$^2$) | 0.209 ± 0.054* | 0.143 ± 0.028* | 0.156 ± 0.041 | 0.104 ± 0.037 | 0.215 ± 0.048* | 0.159 ± 0.063* | 0.165 ± 0.062 | 0.104 ± 0.059 | 0.209 ± 0.077* | 0.150 ± 0.050* | 0.148 ± 0.025 | 0.096 ± 0.027 | *P* = 0.781 | *P* = 0.001 | *P* < 0.001 |

Intramuscular triglycerides (IMTG) content and lipid droplet (LD) number are expressed relative to the area of the peripheral or central region. *Significantly greater in type I fibres ($P < 0.05$). Data are means ± SD.

#### 6.4.2.2 Effects of carbohydrate feeding and exercise on IMTG content and LD morphology

The 180-min submaximal cycling protocol resulted in a significant reduction in IMTG content in type I (main time effect; $P < 0.001$; Figure 6.5) and type IIa fibres (main time effect; $P = 0.002$; Figure 6.5), the magnitude of which was not affected by CHO feeding during exercise (main condition effect; Type I: $P = 0.489$; Type IIa: $P = 0.841$; Figure 6.5). As such, there was no difference in the net breakdown of absolute IMTG between trials (main condition effect; $P = 0.152$; Figure 6.5). A greater net IMTG breakdown was also observed in type I compared to type IIa fibres (main fibre effect; type I: 2.66 ± 1.61, type II: 1.61 ± 1.03 % area stained, $P < 0.001$; Figure 6.5). Moreover, when examining exercise-induced changes on a subcellular-specific basis, IMTG content was significantly reduced in both the peripheral (region × time interaction; type I: $P < 0.001$; type IIa: $P = 0.016$, Figure 6.5) and central regions (region × time interaction; type I: $P < 0.001$; type IIa: $P = 0.001$, Figure 6.5) of both fibres after 180-min of cycling exercise. Additionally, the relative contribution of IMTG across the subcellular regions within type I fibres decreased in the central region after 180-min of exercise (region × time interaction; $P = 0.001$, Table 6.2) with a reciprocal increase in the relative distribution of IMTG within the peripheral region (region × time interaction; $P = 0.001$, Table 6.2). In contrast, the relative distribution within type IIa fibres remained similar after 180-min of exercise (region × time interaction; $P = 0.141$, Table 6.2). When examining changes in LD morphology after prolonged endurance exercise and between nutritional experimental conditions, LD number reduced significantly in both type I (main time effect; $P < 0.001$, Figure 6.6) and type IIa muscle fibres (main time effect; $P = 0.001$, Figure 6.6) with no effect of CHO feeding in either fibre type (main condition effect; type I: $P = 0.417$; type IIa: $P = 0.975$, Figure 6.6). LD number decreased in both the central and peripheral regions within both type I (region × time interaction; $P < 0.001$, Figure 6.6) and type IIa fibres (region × time interaction; $P = 0.001$, Figure 6.6). However, LD size was significantly smaller after exercise in type I muscle fibres only (main time effect; $P = 0.037$, Figure 6.5) with reductions in both the peripheral (region × time interaction; $P = 0.011$, Figure 6.6) and central (region × time interaction; $P = 0.005$, Figure 6.6) regions. Thus, IMTG utilisation during exercise could be explained by a decrease in LD number within the peripheral and central regions of both type I and IIa fibres but a reduction in LD size occurred exclusively in type I fibres. Figure 6.7 depicts visual representation of IMTG content in type I and IIa fibres at 0-min and after 180-min of submaximal cycling exercise in the 0, 45 and 90 $g \cdot h^{-1}$ condition.

**Table 6.2** Relative distribution of IMTG between subcellular regions before and after the 180-min submaximal cycling exercise protocol.

| | | % of IMTG | | | |
|---|---|---|---|---|---|
| | | Type I Fibres | | Type IIa Fibres | |
| | | Peripheral | Central* | Peripheral | Central* |
| **0-min** | | | | | |
| | 0 g·h$^{-1}$ | 16 ± 6 | 84 ± 6 | 19 ± 7 | 81 ± 7 |
| | 45 g·h$^{-1}$ | 16 ± 5 | 84 ± 5 | 18 ± 6 | 82 ± 6 |
| | 90 g·h$^{-1}$ | 14 ± 3 | 86 ± 3 | 14 ± 4 | 86 ± 4 |
| **180-min** | | | | | |
| | 0 g·h$^{-1}$ | 22 ± 15$^{\#}$ | 78 ± 15$^{\#}$ | 18 ± 7 | 82 ± 7 |
| | 45 g·h$^{-1}$ | 25 ± 13$^{\#}$ | 75 ± 13$^{\#}$ | 22 ± 13 | 78 ± 13 |
| | 90 g·h$^{-1}$ | 19 ± 7$^{\#}$ | 81 ± 7$^{\#}$ | 15 ± 5 | 85 ± 5 |

Data are means ± SD. IMTG, intramuscular triglycerides. *Significant effect of region across all time points ($P < 0.05$). #Significantly different from 0-min time point within same condition ($P < 0.05$).

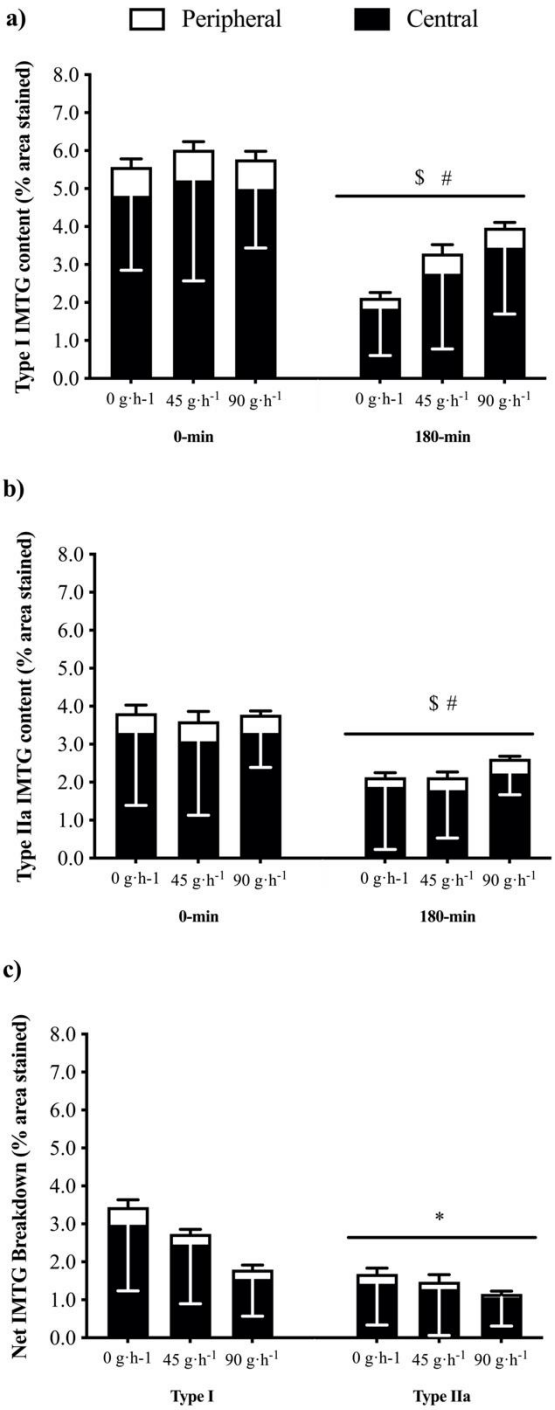


**Figure 6.5** Fibre type and subcellular-specific changes in intramuscular triglycerides (IMTG) content in response to prolonged endurance cycling exercise and carbohydrate (CHO) intake. IMTG content in each region was normalized to total cell area. Shown are IMTG content in type I (a) and type IIa muscle fibres (b) and net IMTG breakdown (c). #Significant main effect reduction in IMTG content from 0-min ($P < 0.05$). $Significant region × time interaction from 0-min ($P < 0.05$). *Significantly different from Type I fibres. Data is presented as mean ± SD. Intramuscular triglycerides, IMTG.

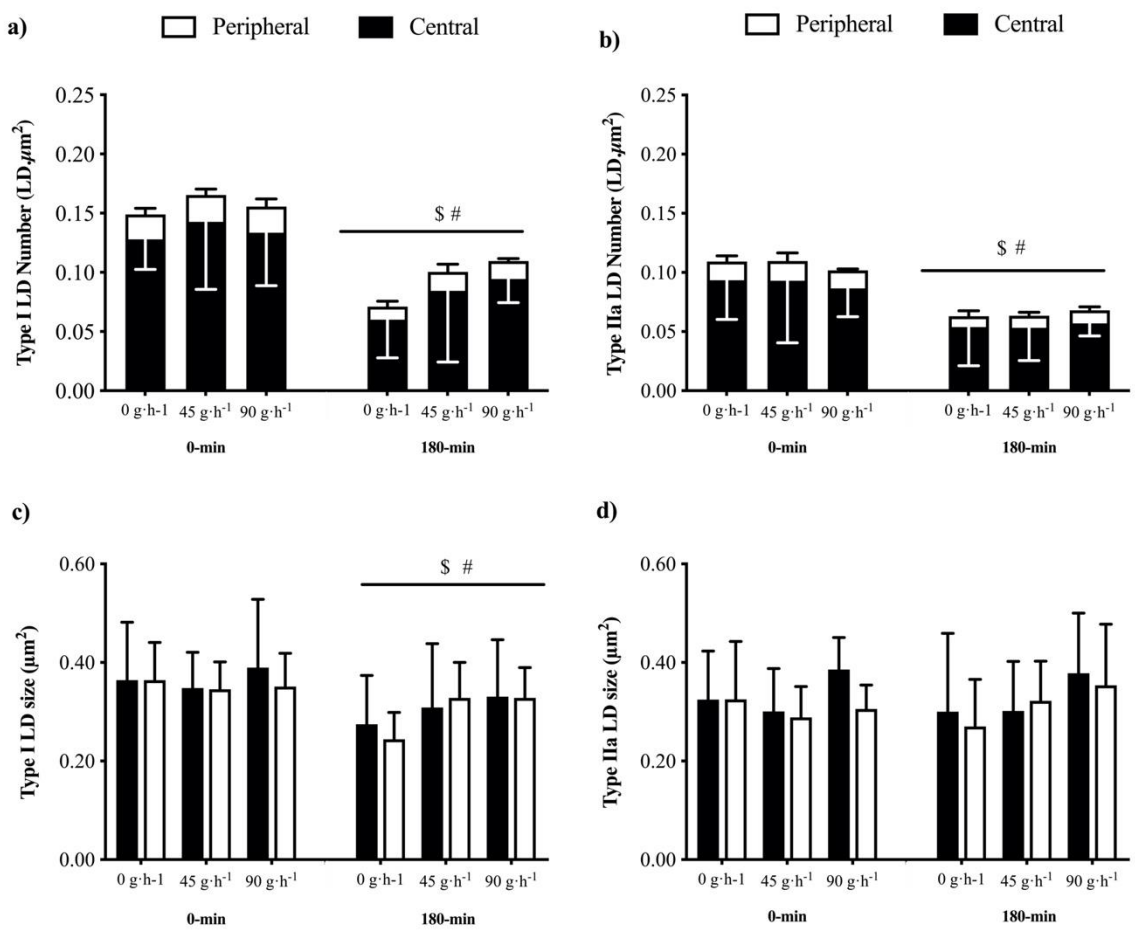


**Figure 6.6** Fibre type and subcellular-specific changes in lipid droplet (LD) morphology in response to prolonged endurance cycling exercise and carbohydrate (CHO) intake. LD number in each region was normalized to total cell area. Shown are LD number in type I (a) and type IIa muscle fibres (b), LD size in type I (c) and type IIa muscle fibres (d). #Significant main effect reduction from 0-min ($P < 0.05$). $Significant region × time interaction from 0-min ($P < 0.05$). Data is presented as mean ± SD.

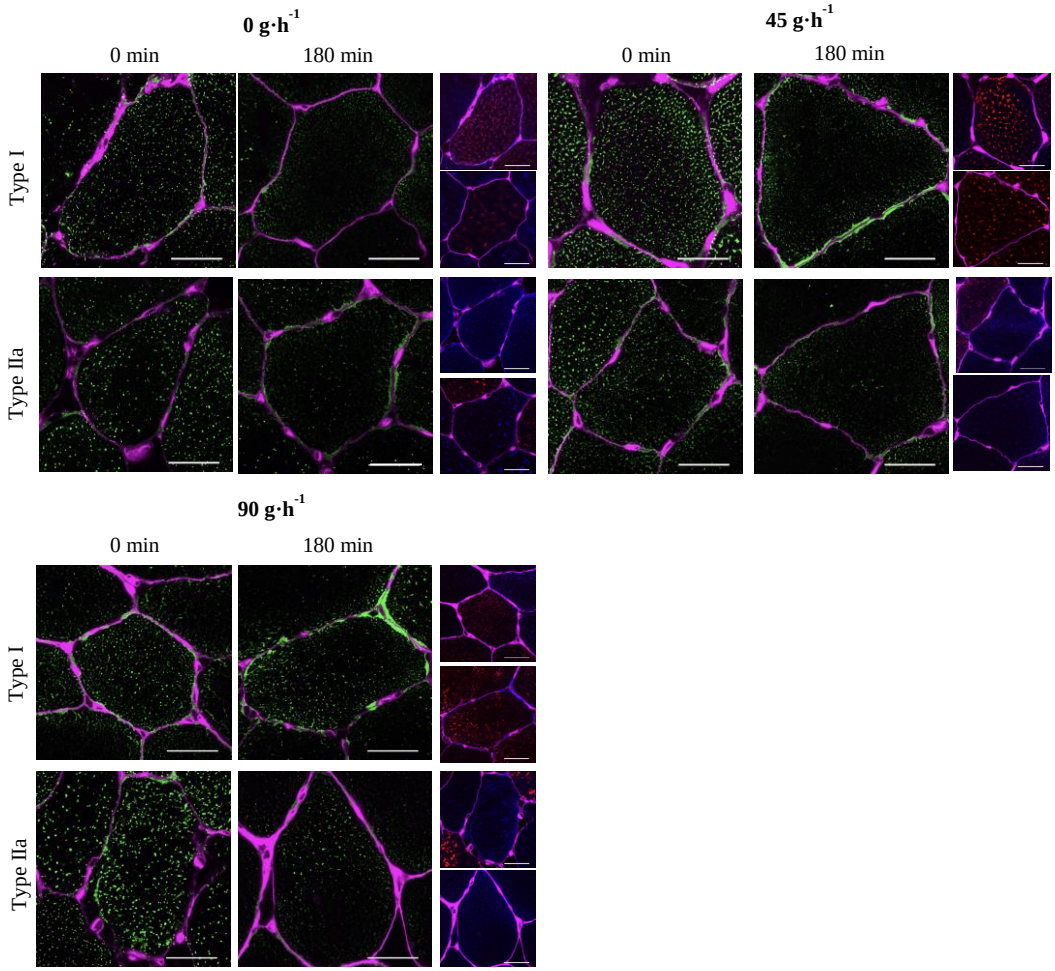


**Figure 6.7** Representative immunofluorescence images of intramuscular triglycerides (IMTG) in response to prolonged endurance exercise with and without carbohydrate (CHO) feeding during exercise in type I and IIa muscle fibres. Sections were stained for IMTG (stained using BODIPY 493/503; green), fibre type (Type I: primary antibody MHCI – A4.840c; secondary antibody goat anti-mouse IgM 546, red; Type IIa: MHCIIa – N2.261c; secondary antibody goat anti-mouse goat anti-mouse IgG 405, blue), and wheat germ agglutinin Alex Fluor 633 to identify cell border (stained pink). Images depict IMTG content in type I (stained red) and IIa fibres (stained blue) at 0-min and after 180-min of submaximal cycling exercise. Scale bars represent 30 µm.

## 6.5 Discussion

In contrast to our hypothesis, the present data demonstrate that CHO feeding during prolonged steady state submaximal cycling exercise does not alter muscle fuel selection in type I or type II muscle fibres. Importantly, our study design incorporated experimental conditions in which trained male cyclists commenced exercise in conditions considered best nutritional practice i.e. after glycogen loading and consumption of a CHO rich pre-exercise meal. Additionally, CHO

was consumed during exercise in graded quantities (i.e., 45 or 90 $g.h^{-1}$) that have previously been shown to improve exercise performance and capacity. As such, these data (and those presented in Chapter 4) suggest that the well documented ergogenic effect of CHO feeding during exercise does not occur as a result of sparing of glycogen or IMTG stores and is more likely related to liver glycogen sparing, maintenance of plasma glucose concentrations and whole-body rates of CHO oxidation.

In accordance with the trained status of our participants and the 36 h CHO loading protocol, baseline muscle glycogen content appeared to be highly elevated in both type I and type II muscle fibres, as evidenced by the dark PAS staining. Additionally, our exercise and dietary standardisation protocol was successful in ensuring comparable glycogen availability between trials (see Figure 6.2). Consistent with previous observations on moderate intensity exercise (Costill *et al.,* 1973; De Bock *et al.,* 2007; Gollnick *et al.,* 1972; Gollnick *et al.,* 1973; Jensen *et al.,* 2015; Stellingwerff *et al.,* 2007; Tsintzas *et al.,* 1995), we subsequently observed that our chosen 180-min submaximal exercise protocol induced greater relative glycogen depletion in type I fibres compared with type II muscle fibres (see Figure 6.2). Moreover, we also observed that the magnitude of glycogen utilisation in both fibre types was not affected by CHO feeding during exercise, thus demonstrating that a glycogen sparing effect did not occur. The absence of a glycogen sparing effect is in contrast to previous reports demonstrating that CHO feeding exerts glycogen sparing in type I (Stellingwerff *et al.,* 2007a) and type II fibres (Stellingwerff *et al.,* 2007; De Bock *et al.,* 2007). Such discrepancies between studies may be underpinned by methodological differences that are related to muscle glycogen availability, pre-exercise CHO feeding, exercise duration and timing of assessment of substrate metabolism. In relation to the latter, Stellingwerff *et al.* (2007a) observed that the glycogen sparing effect in type I and type II fibres (induced by consuming 50 $g \cdot h^{-1}$ of glucose during a 180-min cycling protocol completed at 50% $W_{max}$) was specifically due to a reduction in muscle glycogen utilisation in the first hour of exercise, as evidenced by stable isotope methodology. These researchers also used a dietary protocol in which subjects commenced exercise in the fasted state and after consuming ~6 $g \cdot kg^{-1}$ CHO per day for two days. Given the greater CHO availability associated with the present dietary protocol (i.e., a true CHO loading protocol as well as consumption of a pre-exercise high CHO meal), it is possible that any potential regulatory effects of consuming CHO "during" exercise are offset by the effects of pre-exercise muscle glycogen availability on absolute muscle glycogen utilisation (Arkinstall *et al.,* 2004; Hargreaves *et al.,* 1995). Nonetheless, despite using similar dietary conditions to that adopted

here (i.e., consumption of a pre-exercise CHO meal of 2.5 g·kg$^{-1}$ in the CHO fed trial), De Bock *et al.* (2007) observed a glycogen sparing effect in type IIa fibres as a result of consuming 1 g·kg·h$^{-1}$ (~75 g·h$^{-1}$) of maltodextrin during 2 h of exercise. This was demonstrated by a ~18% net reduction in glycogen within type IIa fibres in the CHO fed trial compared to a ~53% reduction when the trial was commenced after an overnight fast with no CHO ingested during the exercise bout. Given the well documented effects of exercise duration on glycogen utilisation (Areta and Hopkins, 2018) coupled with the selective recruitment of type II fibres as type I fibres become depleted (Gollnick *et al.,* 1973), it is possible that discrepancies between studies in this instance are simply a reflection of a greater exercise duration (i.e., 3 v 2 h cycling protocol). Collectively, such findings highlight the need for future studies to assess the time course of fibre-specific muscle glycogen use during prolonged endurance exercise. In addition, the assessment of glycogen utilisation within distinct subcellular pools within muscle fibres through the use of quantitative transmission electron microscopy is also warranted to provide a more complete understanding of glycogen utilisation with CHO feeding. This is especially relevant given the recent findings underpinning the importance of intramyofibrillar glycogen within type I fibres for endurance capacity and that supercompensation of the subsarcolemmal glycogen pool as a result of CHO loading reduces the utilisation of the intramyofibrillar glycogen pool during exercise (Jensen *et al*., 2020).

In agreement with previously documented storage patterns of IMTG (De Bock *et al.*, 2005; Essén *et al.*, 1975; Jevons *et al.*, 2020; Koopman *et al.*, 2001; Malenfant *et al.*, 2001; Shepherd *et al.*, 2013; Stellingwerff *et al.*, 2007; Van Loon *et al.*, 2003a; Van Loon *et al.*, 2003b), we also observed that resting IMTG stores were twofold greater in type I fibres compared to type IIa fibres (see Table 6.1). Additionally, IMTG content was also greater in the periphery of the cell compared with the central region, as also documented in previous studies utilising immunofluorescence analysis (Jevons *et al.*, 2020; Shaw *et al.*, 2008; Stellingwerff *et al.*, 2007a; Van Loon *et al.*, 2004). The fibre type specific differences in resting IMTG is also a function of both a greater number and size of LDs in type I fibres compared with type IIa fibres (see Table 6.1), consistent with recent observations from our laboratory in male endurance trained participants (Jevons *et al.*, 2020). Importantly, the resting storage pattern of IMTG was not different between trials thus demonstrating that the 36-h exercise and dietary protocol was successful in ensuring similar substrate availability prior to the main experimental trial.

As expected, our submaximal exercise protocol induced an approximate 50% decrease in IMTG content within type I fibres, a magnitude of utilisation that agrees well with previous studies also utilising cycling protocols lasting $\geq$ 3 h (Jevons *et al.*, 2020; Stellingwerff *et al.*, 2007a; Stellingwerff *et al.*, 2007b; Van Loon *et al.*, 2003). However, although many previous studies have reported IMTG degradation to be negligible within type II (Stellingwerff *et al.*, 2007a; Stellingwerff *et al.*, 2007b; Van Loon *et al.*, 2003) and IIa (De Bock *et al.*, 2005; De Bock *et al.*, 2008; Jevons *et al.*, 2020; Van Proeyen *et al.*, 2011b) fibres after prolonged moderate intensity exercise, we observed an approximate 30% utilisation of IMTG stores within type IIa fibres. Our data appear in agreement with previous observations that reported a ~45% depletion of IMTG in type IIa fibres during 2-h of prolonged cycling when examined after 6-weeks of consistent endurance training in the fasted state (Van Proeyen *et al.*, 2011b). From a subcellular-specific perspective, we also provide novel data by demonstrating that the exercise-induced decrease in IMTG content in both fibre types occurred within both the central and peripheral region. This pattern of utilisation is in contrast to previous authors who observed that the central (Jevons *et al.*, 2020) or peripheral (Stellingwerff *et al.*, 2007b) region only of type I fibres to display reduced IMTG content. The reduction in IMTG content observed in the present study was also due to a reduction in LD number in both type I and IIa fibres whereas a reduction in LD size was only evident in type I fibres. Our data are in agreement with Stellingwerff *et al.* (2007a) who also reported a reduction in both LD size and number in type I fibres. However, Jevons *et al.* (2020) observed that the decrease in IMTG content in type I fibres after a 4 h cycling protocol at ~56 $\dot{V}O_{2max}$ was the consequence of a reduction in LD number with no difference in LD size. When taken together, our data therefore present novel findings in relation to IMTG utilisation in type IIa fibres as well as subcellular-specific IMTG utilisation. Such differences between the present and aforementioned studies may be methodological in nature and a reflection of different immunohistochemical staining techniques. Indeed, most of these aforementioned studies assessed IMTG content through the use of Oil Red O (ORO) lipid dye (De Bock *et al.*, 2005; Stellingwerff *et al.*, 2007a; Stellingwerff *et al.*, 2007b; Van Loon *et al.*, 2003) whilst the present study utilised an alternative dye, BODIPY 493/503 (BODIPY). The different lipid dyes have important methodological considerations given ORO does not exclusively stain IMTG but also labels all neutral lipids (e.g., phospholipids within membranes) and recent comparisons between the two suggest that BODIPY detects greater estimates of IMTG content compared to ORO in all fibre types (Strauss *et al.*, 2020). Additionally, differences in the quantification of the subcellular utilisation of IMTG are also evident with the use of the different dyes, as comparisons showed

IMTG utilisation to be specific to the peripheral region when examined using ORO whilst utilisation was of a similar magnitude in the peripheral and central region with the use of BODIPY (Strauss *et al.*, 2020).

Similar to fibre-specific muscle glycogen utilisation, CHO feeding also had no effect upon the exercise induced reductions in IMTG utilisation within type I and IIa fibres (see Figure 6.5). These findings are consistent with previous suggestions that the ingestion of 50 $g \cdot h^{-1}$of glucose during exercise does not alter skeletal muscle IMTG utilisation during 180-min of moderate intensity cycling exercise (Stellingwerff *et al.*, 2007a). In contrast, De Bock *et al.* (2005) observed an attenuated use of IMTG exclusively in type I fibres with 2.5 $g \cdot kg^{-1}$ (~150g) and 1 $g \cdot kg \cdot h^{-1}$ (~75 $g \cdot h^{-1}$) of CHO intake before and during 2 h of a more intense cycling protocol (~75% of $\dot{V}O_{2max}$) compared to completing the exercise trial after an overnight fast with no CHO intake during the exercise bout. As discussed previously, it is possible that the consumption of a pre-exercise meal across all trials in the present study may have offset any potential regulatory effects of CHO feeding during the exercise bout itself. In this way, the metabolic conditions during the initial 60-90 minutes of exercise may not have been large enough between trials to alter the pattern of IMTG use (see NEFA data from Chapter 5). This is especially relevant given that IMTG utilisation appears to predominate during the first two hours of exercise with an attenuated use thereafter (Romijn *et al.*, 1993; Watt *et al.*, 2002). Indeed, although a progressive difference in plasma NEFA availability and delivery during the third hour of exercise (see NEFA data from Chapter 5) may have suppressed IMTG mobilisation and/or oxidation (Van Loon *et al.*, 2003a; Watt *et al.*, 2002; Van Loon *et al.*, 2005a; Van Loon *et al.*, 2005b; Watt *et al.*, 2004), it is possible that the predominant IMTG utilisation had already occurred during the first 1-2 h of exercise. Such a hypothesis is supported by the observation that whole body rates of fat oxidation did not differ between trials until the second and third hour of exercise (see Figure 5.5, Chapter 5).

In summary, we demonstrate that CHO feeding during exercise does not alter muscle glycogen or IMTG utilisation in type I or type II muscle fibres during prolonged submaximal steady state exercise. When taken together, our data demonstrate that the ergogenic effects of CHO feeding during exercise are more likely underpinned by mechanisms related to liver glycogen sparing, maintenance of plasma glucose concentrations and whole-body rates of CHO oxidation, as opposed to alterations to muscle fuel selection. However, given that glycogen is stored within distinct subcellular pools, there is a definitive need to further examine the effects of endurance

exercise and CHO feeding on subcellular glycogen utilisation before definitively concluding that CHO feeding does not alter muscle fuel selection.

# Chapter 7:

# The effects of carbohydrate feeding on muscle glycogen utilisation within subcellular storage pools

The aim of this chapter was to examine the effects of CHO feeding during prolonged endurance exercise on the fibre-type-specific content of the three subcellular storage pools of glycogen. Due to the unfortunate issues with the COVID-19 world pandemic and laboratory restrictions, complete analysis was unable to be finished to meet the aim of Study 4. However, preliminary data demonstrating the efficacy of the TEM technique is presented in the following chapter.

## 7.1 Abstract

**Purpose:** To examine the effect of CHO feeding during prolonged endurance exercise on muscle glycogen utilisation within subcellular storage pools. **Methods:** Using the muscle biopsies obtained from the experimental design in Study 2, we intended to assess muscle glycogen in three subcellular locations using transmission electron microscopy, i.e. intermyofibrillar (IMF), intramyofibrillar (INTRA) and subsarcolemmal (SS) glycogen pools. **Results:** At the time of laboratory closure, we had quantified glycogen from six muscle fibres in a resting sample from the vastus lateralis of one subject (Age: 33 y, body mass: 74.8 kg, height: 176 cm, $\dot{V}O_{2max}$: 59.4 $mL \cdot kg^{-1} \cdot min^{-1}$, peak power output: 375 W, lactate threshold: 200 W). Data demonstrate that type II fibres seemed to have greater glycogen volume in the IMF compared to type I fibres at rest, whilst it seemed similar in the INTRA and SS between fibres. Additionally, the relative storage of glycogen was greatest in the IMF pool (77.6 ± 9.7%) followed by the SS (19.8 ± 8.6%) and INTRA pools (3 ± 1.3 %). **Conclusion:** The subcellular distribution of glycogen in rested muscle appears consistent with previously published data though it is noteworthy that we observed greater glycogen volume in the SS pool. The enhanced storage within the SS pool observed here is likely underpinned by the more aggressive CHO loading protocol adopted here as compared with previous studies. Future work is now required to complete the full analysis and thus evaluate the effects of CHO feeding during prolonged endurance exercise on the utilisation of subcellular glycogen pools.

## 7.2 Introduction

It is now recognised that glycogen granules are located in distinct subcellular compartments when examined both qualitatively (Oberholzer *et al.,* 1976; Sjöström *et al.,* 1982; Fridén *et al.,* 1985; Fridén 1989) and semi-quantitively by transmission electron microscopy (TEM) (Marchand *et al.,* 2007; Nielsen *et al.,* 2011). Specifically, muscle glycogen is heterogeneously distributed between three separated compartments: below the sarcolemma (subsarcolemmal glycogen), between the myofibrils (intermyofibrillar glycogen) and within the myofibrils (intramyofibrillar glycogen) (Marchand *et al.,* 2002). Interestingly, these distinct pools are utilised at different rates during exercise whilst also exhibiting different functional roles (Friden *et al.,* 1989; Marchand *et al.,* 2002; Ørtenbland & Nielsen, 2015). For example, Jensen *et al.* (2020) recently observed that the intramyofibrillar (INTRA) glycogen volume of type I fibres before exercise and after one hour of exercise was associated with the best predictor of time to exhaustion despite having the smallest volume among the subcellular pools. Additionally, a local supercompensation of subsarcolemmal (SS) glycogen via prior CHO loading mediates a sparing of intramyofibrillar glycogen utilisation during the initial hour of exercise, thereby postponing exhaustion (Jensen *et al.,* 2020). Moreover, intramyofibrillar glycogen is also preferentially utilised during high-intensity exercise (Nielsen *et al.,* 2011) and restored in the recovery period following severe depletion of glycogen (Marchand *et al.,* 2007). From this data, it is clear that endurance exercise at moderate to high intensity exercise is not just dependent on the mixed muscle glycogen concentration within the muscles, but more specifically the subcellular distribution of glycogen within the fibre and between fibre types.

In relation to the resting distribution of subcellular muscle glycogen, stereological glycogen quantification of TEM images has revealed that highly trained endurance athletes generally deposit 8-11% as SS glycogen, 77-84% as intermyofibrillar (IMF) glycogen and 3-13% as INTRA glycogen. Furthermore, type I fibres contain 82% more INTRA glycogen and 31% more SS glycogen than type II fibres, with type II fibres containing 11% more IMF glycogen than type I fibres (Gejl *et al.*, 2014; Nielsen *et al.,* 2011; Nielsen *et al.,* 2012; Nielsen & Ørtenblad, 2013; Ørtenblad *et al.,* 2013). In this regard, measuring mixed muscle samples through biochemical assessment of glycogen in whole muscle homogenate and/or histological evaluation of fibre specific glycogen is limited in that it does not enhance our understanding of glycogen storage or utilisation within each subcellular pool. As such, this may partially explain the lack of a whole muscle glycogen sparing effect that has been repeatedly observed

with CHO feeding. Therefore, a more complete understanding of glycogen utilisation during prolonged endurance exercise with CHO feeding is warranted to examine the quantification of glycogen in the different subcellular pools and fibre types.

Accordingly, the present study was designed to investigate the effects of CHO feeding during prolonged endurance exercise on the fibre type-specific content of three subcellular glycogen pools. Our hypothesis was that INTRA glycogen is utilised preferentially during prolonged endurance cycling in type I fibres and that CHO feeding specifically reduces utilisation of this pool during exercise.

## 7.3 Methods

### 7.3.1 Participants

The subjects who participated within this study have been previously described (see Section 6.3.1). Basic subject characteristics are described in Table 3.1.

### 7.3.2 Experimental design

The experimental design has been previously described in Section 6.3.2.

#### 7.3.2.1 Preliminary testing

Participant preliminary testing has been previously described in Section 3.2.4.

#### 7.3.2.2 Day 1: Glycogen depletion exercise

At 36-h before the main experimental trial (i.e., the evening of Day 1), subjects performed an intermittent glycogen-depleting cycling protocol lasting 90-minutes in duration. The glycogen depletion protocol has been previously described in Section 4.3.4.1.

#### 7.3.2.3 Day 2: Carbohydrate loading regimen

The pre-exercise carbohydrate loading regimen has been previously described in Section 6.3.2.3.

#### 7.3.2.4 Day 3: Experimental trials

The experimental trials are similar to that described previously in Section 6.3.2.4 with an experimental schematic illustrated in Figure 6.1.

### 7.3.3 Muscle biopsies

Skeletal muscle biopsy samples (~20 mg) were obtained from the vastus lateralis immediately pre- and post-exercise. Muscle biopsies were obtained as described in Section 3.6.

### 7.3.4 Transmission electron microscopy

Muscle biopsy specimens were prepared for glycogen visualisation using TEM, as described previously in Section 3.10 to allow for glycogen particle analysis and six muscle fibres from a pre-exercise sample were initially analysed.

### 7.3.5 Glycogen quantification

The process for image capture, processing and data analysis has been described previously in Section 3.11.

## 7.4 Results

### 7.4.1 Pre-exercise subcellular glycogen pools

Due to the restrictions associated with COVID-19 World Pandemic, unfortunately complete analysis was unable to be finished. However, preliminary data from one analysed pre-exercise sample is presented in Table 7.1, 7.2 and Figure 7.1. Fibre type determination was undertaken based of absolute mitochondria fraction volume (see Table 7.1) with Fibres 1-4 likely being classified as type I fibres, with Fibres 5 and 6 likely being classified as type II fibres, due to the differences in total mitochondria volume fraction. Such assumptions were based on the fibre type quantification conducted in our laboratory in another unpublished study. Figure 7.1 reports the glycogen volume within the IMF, INTRA and SS subcellular glycogen pools prior to the 180-min submaximal cycling protocol, within both type I and II fibres. Type II fibres seemed to have greater glycogen volume in the IMF compared to type I, whilst it seemed similar in the INTRA and SS between fibres. In addition, the relative storage of glycogen is presented in Table 7.2 whereby the greatest storage was seen in the IMF pool followed by the SS and INTRA pools. Representative TEM images illustrating glycogen distributions are also shown in Figure 7.2.

**Table 7.1** Intermyofibrillar, subsarcolemmal and total absolute mitochondria fraction volume in the six analysed individual fibres which was used for fibre type determination.

| **Individual Fibre** | **Absolute Mitochondria Fraction Volume** | | |
|---|---|---|---|
| | **IMF** ($\mu m^3 \cdot \mu m^{-3}$) | **SS** ($\mu m^2 \cdot \mu m^{-3}$) | **Total** ($\mu m^3 \cdot \mu m^{-3}$) |
| 1 | 0.0882 | 0.5892 | 0.1176 |
| 2 | 0.0990 | 0.3477 | 0.1164 |
| 3 | 0.1129 | 0.2180 | 0.1238 |
| 4 | 0.0743 | 0.5574 | 0.1021 |
| 5 | 0.0389 | 0.0000 | 0.0389 |
| 6 | 0.0352 | 0.1409 | 0.0423 |

IMF: Intermyofibrillar; SS: Subsarcolemmal.

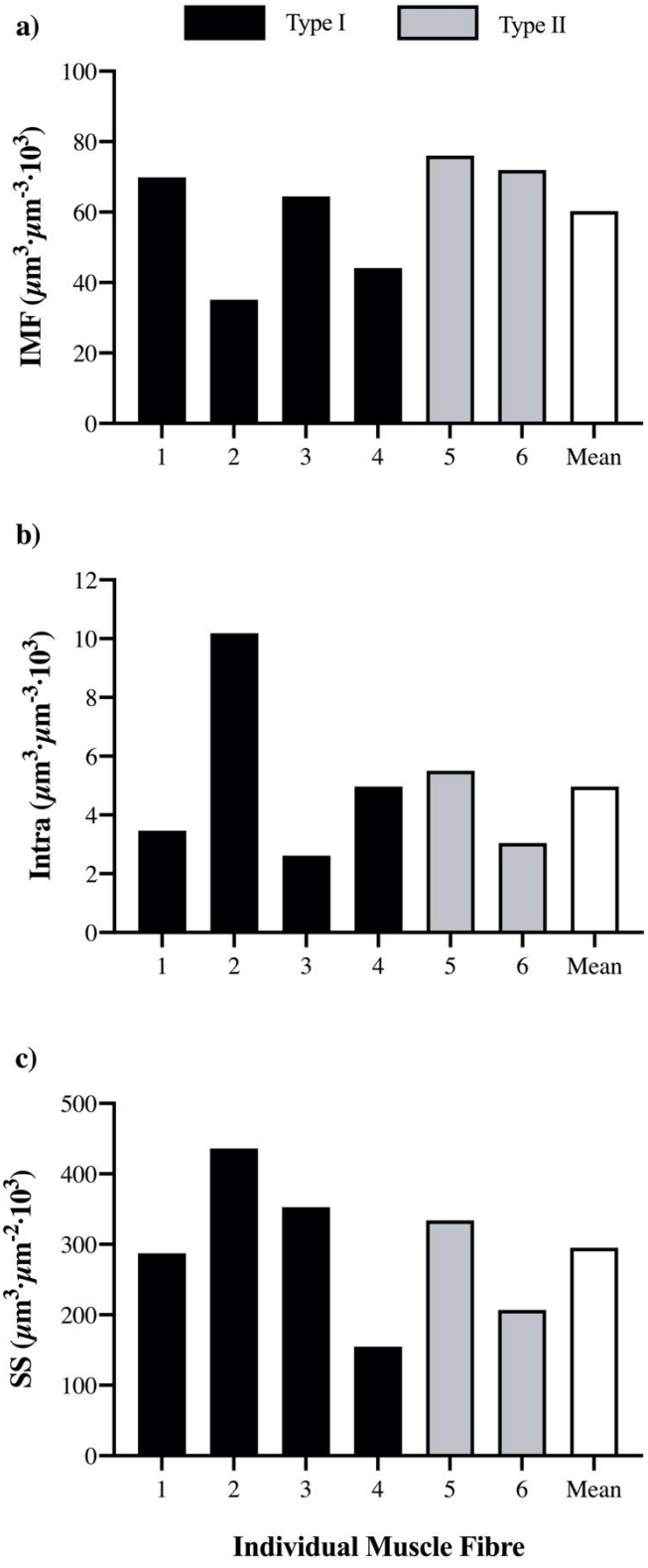


**Figure 7.1** Glycogen storage within the a) intermyofibrillar, b) intramyofribillar and c) subsarcolemmal storage pools within six muscle fibres of one participant at the pre-exercise time point after a high carbohydrate load and consumption of a high carbohydrate pre-exercise meal. Black bars represent the individual type I fibres, the grey bars represent the individual type II fibres and the clear bars represent the mean of the six individual fibres.

**Table 7.2** Relative glycogen storage within the intermyofibrillar, intramyofribillar and subsarcolemmal storage pools within six muscle fibres (4 Type I fibres and 2 Type II fibres) of one participant at the pre-exercise time point after a high carbohydrate (CHO) load and consumption of a high CHO pre-exercise meal.

| **Individual Fibre** | **Relative glycogen storage (%)** | | |
|---|---|---|---|
| | **IMF** | **Intra** | **SS** |
| 1 (Type I) | 81.5 | 1.8 | 16.7 |
| 2 (Type I) | 58.5 | 5.1 | 36.4 |
| 3 (Type I) | 77.1 | 1.8 | 21.1 |
| 4 (Type I) | 82.6 | 2.9 | 14.5 |
| 5 (Type II) | 80.0 | 2.5 | 17.6 |
| 6 (Type II) | 86.0 | 1.6 | 12.4 |
| **Mean** | **77.6** | **2.6** | **19.8** |
| **SD** | **9.7** | **1.3** | **8.6** |

IMF: Intermyofibrillar; Intra: Intramyofribillar; SS: Subsarcolemmal.

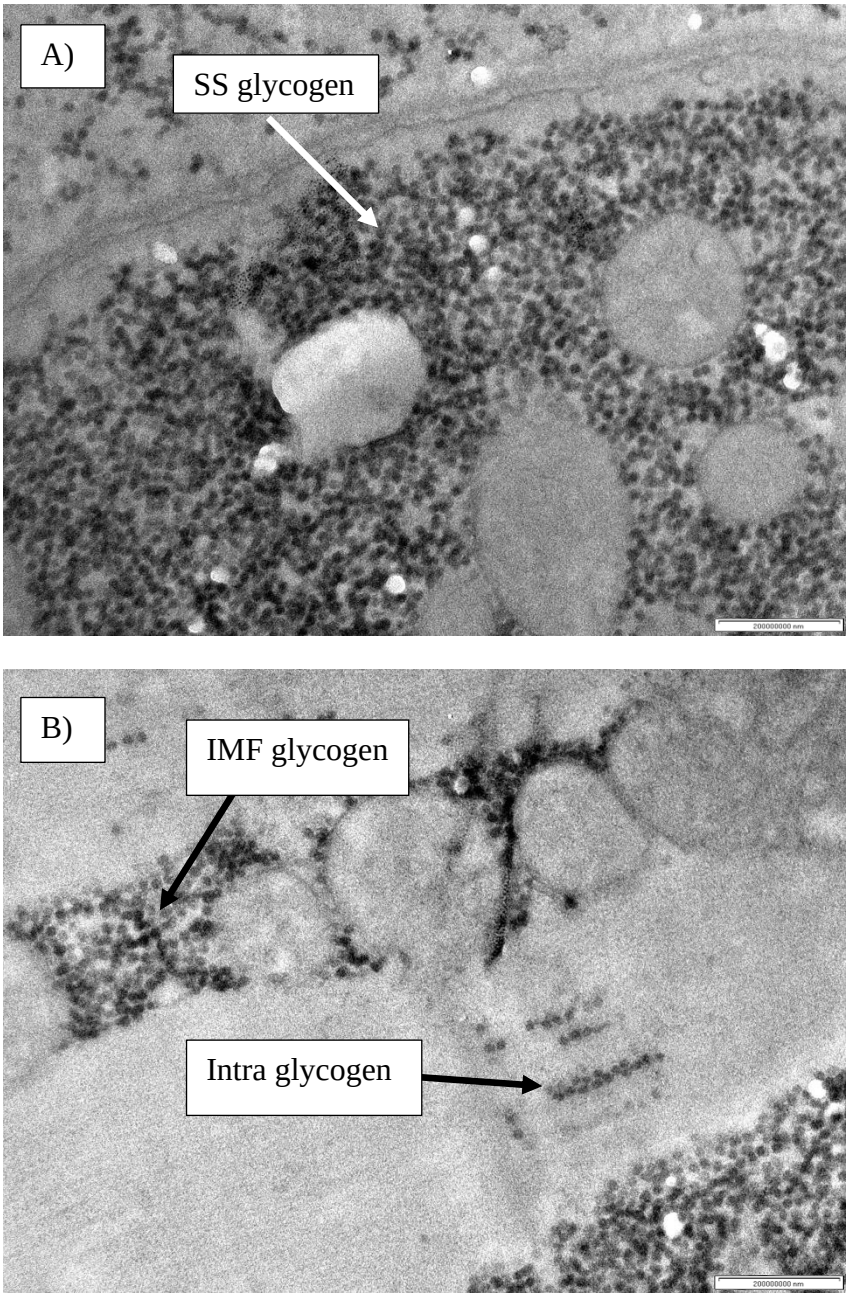


**Figure 7.2** Images illustrate (A) subsarcolemmal, (B) intermyofibrillar and intramyofribillar glycogen particles (taken at ×36,000 magnification). SS: subsarcolemmal, IMF: intermyofibrillar, Intra: intramyofribillar.

### 7.5 Discussion

Due to the limited analysis presented within this chapter it is difficult to compare the preliminary findings with previous studies. However, it seems our analysis from one pre-exercise sample reports higher amounts of glycogen volume within each of the three storage pools compared to previous observations examining subcellular muscle glycogen storage in

human muscle tissue in both untrained (Nielsen *et al.*, 2010a, 2010b) and trained (Gejl *et al.*, 2017; Jensen *et al.*, 2020; Nielsen *et al.*, 2011) participants. For example, our data demonstrate that glycogen storage in the IMF, INTRA and SS pools was approximately 44, 22 and 68% higher than that published in trained male subjects (Gejl *et al.*, 2017; Jensen *et al.*, 2020; Nielsen *et al.*, 2011). However, the relative distribution of glycogen within the subcellular pools in the current study (see Table 7.2) is similar to previous observations in trained participants whereby 8-11% is generally deposited as SS glycogen, 77-84% as IMF glycogen and 3-13% as INTRA glycogen (Gejl *et al.*, 2017; Nielsen *et al.*, 2011; Nielsen *et al.*, 2012). Additionally, we seemed to observe a greater glycogen volume in the IMF region of type II fibre compared to type I fibres, whilst INTRA and SS glycogen volume seemed similar between both fibres. However, it must be noted that this observation is based of only six individual fibres which limits any firm conclusions being made. Nonetheless, the distribution of glycogen deposited in the subcellular pools has been previously found to differ between fibre types at rest (Gejl *et al.*, 2017; Nielsen *et al.*, 2011). For example, type I fibres from musculus triceps brachii of elite cross-country skiers have been shown to contain twice as much INTRA glycogen as type II fibres, whereas glycogen storage is similar in other pools (Gejl *et al.*, 2017; Nielsen *et al.*, 2011).

The enhanced subcellular glycogen volume observed in the current study compared to previous investigations in endurance trained participants (Gejl *et al.*, 2017; Jensen *et al.*, 2020; Nielsen *et al.*, 2011) is most likely due to the higher CHO intake associated with our chosen CHO loading protocol. Indeed, subjects in the present study consumed a total of 14 $g \cdot kg^{-1}$ prior to biopsy sampling whereas previous studies have fed CHO at an upper intake of 8.0 $g \cdot kg^{-1}$ of CHO. For example, Gejl *et al.* (2017) provided participants with 8.0 $g \cdot kg^{-1}$ of CHO during the 24-h period prior to the cross-country skiing race simulation. Most recently, Jensen *et al.* (2020) provided 0.2 $g \cdot kg^{-1} \cdot day^{-1}$ and 8.0 $g \cdot kg^{-1} \cdot day^{-1}$ of CHO, respectively, in the "LOW" and "HIGH" CHO trials for 72-h prior to exercising to exhaustion. These latter researchers demonstrated that glycogen storage in the IMF, INTRA and SS pools were enhanced by approximately 42, 56 and 65% as a result of the higher dietary CHO intake. The greater glycogen storage in subcellular pools reported here is also supported by our assessment of mixed muscle glycogen concentration (see Chapter 5). Indeed, whereas we reported values > 700 $mmol.kg^{-1}$ dw, the previous researchers reported lower resting glycogen concentrations of approximately 225 and 500 $mmol.kg^{-1}$ dw, respectively, in the "LOW" and "HIGH" CHO trials within Jensen *et al.* (2020), and 575 $mmol.kg^{-1}$ dw within Gejl *et al.* (2017). When taken

together, our data appear to support recent findings in that increasing dietary CHO intake can augment glycogen storage in all subcellular pools (Jensen *et al.*, 2020), though we suggest that a true CHO loading protocol (i.e., utilisation of prior exercise to induce a supercompensation effect) can augment the magnitude of this response.

In summary, our preliminary analysis demonstrates that the relative distribution of glycogen between subcellular storage pools is comparable to that published previously in trained male subjects. However, we report greater glycogen volume in all three pools, likely a reflection of the CHO loading protocol adopted here. Further work is now required to evaluate the effects of our chosen exercise protocol on subcellular glycogen utilisation and further evaluate the hypothesis that CHO feeding may spare utilisation of specific subcellular pools.

# Chapter 8

# Case Study: The practical delivery of a periodised approach to daily carbohydrate and energy intake for a General Classification winner during a cycling Grand Tour

Having examined the effects of carbohydrate feeding on exercise capacity and substrate metabolism in the laboratory setting, the aim of this chapter was to practically deliver a periodised daily energy and CHO feeding strategy for a male professional World Tour road cyclist during a UCI Grand Tour.

## 8.1 Abstract

**Purpose:** To practically deliver a periodised daily CHO feeding strategy for a male professional World Tour road cyclist during a UCI Grand Tour. We quantified daily energy (EI) and macronutrient intake, exercise energy expenditure (EEE) and energy availability (EA) during the final 10 stages of the 2018 Giro d'Italia. **Methods:** Energy and macronutrient intake were assessed via a combination of weighed food intakes (off-bike) and dietary recall (on-bike fuelling). Exercise energy expenditure was estimated via power meter data. Body mass was measured upon waking each morning and within minutes of completion of each stage. **Results:** Mean daily absolute and relative EI was 3986 ± 1452 kcals (range: 1817 – 6413 kcals) and 58.6 ± 21.4 kcal·kg$^{-1}$ BM (range: 26.7 – 94.3 kcal·kg$^{-1}$ BM). Mean daily absolute and relative CHO intake was 739 ± 332 g (range: 240 – 1305 g) and 10.7 ± 4.8 g·kg$^{-1}$ BM (range: 3.5 – 18.9 g·kg$^{-1}$ BM). Absolute in-race CHO intake averaged 327 ± 93 g (range: 235 – 522 g) equating to an hourly intake of 70 ± 18 g·h$^{-1}$ (range: 52 – 100 g·h$^{-1}$). Daily EA was 2.3 ± 17.7 kcal·kg$^{-1}$ FFM (range: -21 kcal·kg$^{-1}$ to 31 kcal·kg$^{-1}$ FFM) and the athlete's waking body mass varied throughout the race (Stage 11, 12, 13, 17, 18 and 19 was 69.3, 68.3, 67.8, 68.1, 68.3 and 68.9, respectively). The highest daily EI (6413 kcal), EEE (6180 kJ), CHO intake (18.9 g·kg$^{-1}$) and in-race CHO intake (100 g·h$^{-1}$) occurred on the race winning Stage 19 where the athlete gained 03 minutes and 23 seconds to move into the race lead by 40 seconds. **Conclusion:** We provide the first report to assess daily energy and CHO intake of an elite professional Grand Tour winning cyclist. Data demonstrate that this race winning performance was supported by a periodised approach to daily energy and CHO intake.

## 8.2 Introduction

Within elite road cycling, three-week Grand Tours (GT) are some of the most well-recognised and physically demanding events in the racing calendar. There are three Grand Tours within a racing season, namely the Giro d'Italia, Tour de France and Vuelta a España, with each composed of 21 stages of almost consecutive daily racing that vary in terms of exercise intensity, duration and terrain (Lucia *et al.,* 2001). Such stages typically comprise a variety of flat terrains, medium mountains, high mountains (with summit finishes) and time trials (both individual and team). With the introduction of heart rate monitors and power meters during racing coupled with assessments of energy expenditure (via doubly labelled water), the extreme physiological demands and typical exercise intensities inherent to GT racing have become more readily published over recent years (Lucia *et al.,* 2003; Padilla *et al.,* 2001; Padilla *et al.,* 2008; Plasqui *et al.,* 2019; Sanders & Heijboer, 2018; Saris *et al.,* 1989; Van Erp *et al.,* 2020; Vogt *et al.,* 2007). In this regard, it is now recognised that GT stages (with the exception of time trials) incorporate daily racing durations of 4 - 6 hours (encompassing daily distances ranging from 110 – 225 km) with energy expenditure during exercise ranging from 3000-5000 kJ (Plasqui *et al.,* 2019; Van Erp *et al.,* 2020). Such workloads typically result in total daily energy expenditures equating to 7400 to 8365 kcal (31 to 35 MJ) (Plasqui *et al.,* 2019). Although the majority of power output during the stage is considered below threshold, it is also recognised that the race winning moments that occur on mountain stages typically comprise 30-minute high-intensity efforts above threshold (i.e., average power outputs >400 W equating to 6.0 $W \cdot kg^{-1}$ for a 70 kg rider) (Van Erp *et al.,* 2020).

Despite the increased knowledge of the physiological demands of GT racing, a detailed understanding of riders' daily and within day distribution of energy and macronutrient is limited. Classical observations from Saris *et al.* (1989) reported a mean daily energy intake of 24.7 $MJ \cdot day^{-1}$ (5903 $kcal \cdot day^{-1}$) during the Tour de France when assessed daily throughout the entirety of the Tour. Additionally, they also observed that the highest daily intake occurred on a mountain stage (32.4 $MJ \cdot day^{-1}$ or 7744 $kcal \cdot day^{-1}$) whereas the lowest EI was evident on a rest day (16.1 $MJ \cdot day^{-1}$ or 3848 $kcal \cdot day^{-1}$). Such data agree with later observations whereby a daily average energy intake of 23.5 $MJ \cdot day^{-1}$ (5617 $kcal \cdot day^{-1}$) was reported with daily CHO intakes of 12.6 $g \cdot kg^{-1}$ also reported when dietary assessment was conducted on 2 separate 24-h periods during the Vuelta a España, 1 flat stage (day 2, 178 km) and 2 mountain stages (day 14, 174 km; day 16, 148 km) (García-Rovés *et al.,* 1998). Moreover, in more recent

observations, Muros *et al.* (2019) reported an average daily energy and carbohydrate (CHO) intake of 5415 kcal and 12.5 $g \cdot kg^{-1}$, respectively, during the 2015 Vuelta a España when quantified from dietary assessment conducted each day throughout the entirety of the race. In relation to CHO feeding during races, collective observations from such studies demonstrate that CHO intake ranged from 25 – 94 $g \cdot h^{-1}$ (García-Rovés *et al.*, 1998; Muros *et al.*, 2019; Saris *et al.*, 1989).

Whilst such studies provide interesting insights as to habitual feeding strategies, there is a definitive need to better understand the within and between day variation of energy and macronutrient intake in accordance with variations in the stage demands. Additionally, the assessment of in-stage CHO feeding could also be expanded to report the different sources of CHO as to whether solids, fluids or gels represent the predominant fuelling strategy. Such an assessment would allow practitioners to better formulate stage specific and individualised nutritional guidelines that attempt to achieve the intricate balance between promoting fuelling and recovery whilst also maintaining or improving a rider's power to weight ratio where appropriate. Whilst this concept of nutritional periodisation has been communicated for training scenarios according to the "fuel for the work required" concept (Impey *et al.*, 2018), it is not yet clear if a periodised strategy could be successfully implemented in competitive racing scenarios.

Accordingly, the aim of the present chapter was to practically deliver a periodised daily CHO feeding strategy for a male professional World Tour road cyclist during the 2018 Giro d'Italia Grand Tour. In using this detailed case-study design, we quantified daily energy (EI) and macronutrient intake, exercise energy expenditure (EEE) and energy availability (EA) during the final 10 stages of the 2018 Giro d'Italia.

## 8.3 Interventions and Methods

### 8.3.1 Presentation of Athlete and Overview of Sporting History

At the time of data collection, the athlete was a 32-yr-old elite male cyclist competing in the UCI World Tour Series. The athlete is one of the one most decorated cyclists within the sport having won several stage races, including four editions of the Tour de France (in 2013, 2015, 2016 and 2017) and the Vuelta a España twice (2011 and 2017). In addition, the athlete has

also won two Olympic bronze medals in road time trials, in 2012 and 2016, and also took bronze in the 2017 World Championships (time trial).

### 8.3.2 Athlete Physiological Profile

Table 8.1 provides a summary of the physiological profile of the athlete which was conducted in an external independent laboratory one week before competing in the 2015 Vuelta a España.

**Table 8.1** Physiological profile of the athlete. Data were independently collected in August 2015 at the GlaxoSmithKline Human Performance Laboratory. A full laboratory report and testing procedures is provided at https://www.gskhpl.com/dyn/_assets/_pdfs/chris-froome-bodycompaerophys.pdf (Bell *et al.*, 2017).

| **Physiological Profile** | | |
|---|---|---|
| Age | | 31 |
| Body mass (kg) | | 67.0* |
| Height (cm) | | 185.7 |
| Lactate Threshold (2 mmol·$L^{-1}$) | | |
| | Power Output (W) | 379 |
| | W·$kg^{-1}$ | 5.7 |
| | Heart Rate (b·$min^{-1}$) | 127 |
| Maximal Aerobic Profile | | |
| | $\dot{V}O_2$ peak (ml·$kg^{-1}$·$min^{-1}$) | 88.2 |
| | Peak Power Output (W) | 525 |
| | W·$kg^{-1}$ | 7.5 |

**Denotes known optimal racing weight, all performance/physiological data are expressed relative to the athlete's known race weight.*

### 8.3.3 Overview of the Giro d'Italia 2018

The Giro d'Italia is the first of three Grand Tours within the UCI World Tour professional cycling calendar and the 2018 edition was competed during the month of May over 21 stages (see Figure 8.1) with mean daily environmental temperatures of 22.7 ± 3.7˚C. The race accumulated a total distance of 3572.4 Km and a combined elevation of 50388 m with the winner completing in an overall time of 89 h 02' 39". The 21 stages were made up of two individual time-trials, six flat stages, seven hilly stages (including three summit finishes) and six mountain stages (including five summit finishes). The race also included three rest days

throughout, one after Stage 3, one after Stage 9 and one after Stage 15. An overview of the environmental, physical and physiological demands of the specific stages in conjunction with the period of data collection are presented in Table 8.2.

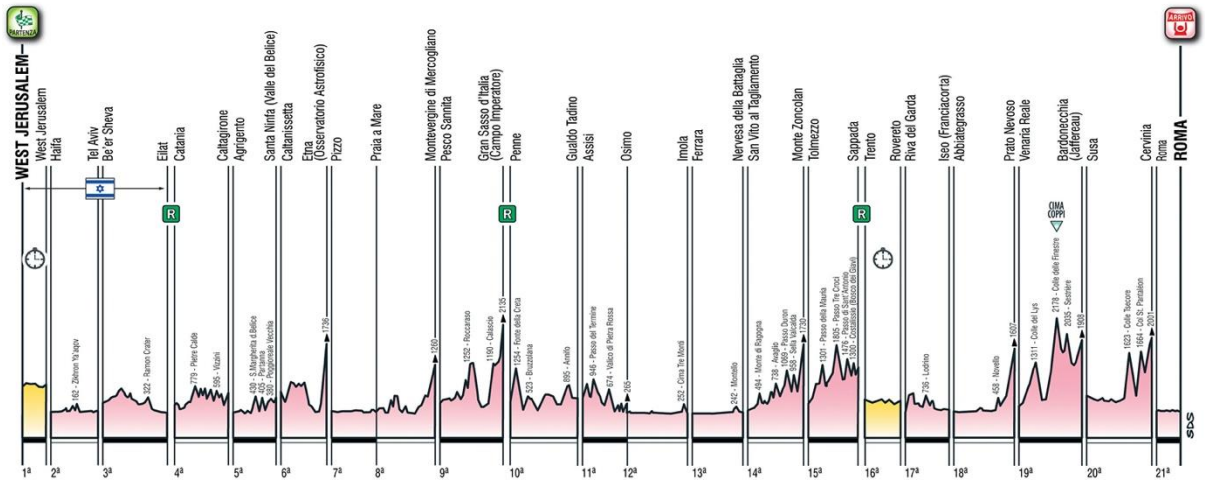


**Figure 8.1** Condensed overview of the 2018 Giro d'italia stages (http://www.giroditalia.it).

### 8.3.4 Theoretical rationale for periodised approach to daily energy and CHO intake

The cyclist intentionally began the race ~2 kg higher than their intended optimal race weight as their primary goal was to achieve peak condition for the Tour de France, occurring 8 weeks later. The athlete also had a heavy crash on the day prior to Stage 1 which meant that the general aim for the rider was to recover from the crash and gradually reduce body mass throughout the course of the race to reach a target weight of 68.5 kg during the last week of the race which contained a key block of mountain stages. To achieve this target weight loss, we opted to attain an energy deficit on specific less intense stages by intentionally under-fuelling but ensure maximal fuelling on more intense and defining stages to help promote performance. Additionally, on those days whereby CHO intake was substantially increased in order to maximise muscle and liver glycogen stores in the days and hours leading up to key stages, dietary fibre intake was significantly reduced with the belief and/or experience that a reduction in bowel contents may contribute to a small but potentially valuable loss of body mass despite still ensuring maximal endogenous CHO stores. Moreover, reduced fat intake was also advised on specific stages in order to reduce overall calorie intake on those days.

### 8.3.5 Dietary assessment

Throughout the duration of the race, breakfast and dinner meals were prepared and served in the mobile kitchen truck by the team chef whilst immediate post-race recovery drinks and food were provided on the team bus. Typically, breakfast options included: eggs, breads, porridge, cereals, rice, pasta, smoothies, fruit juices, fruits and yoghurts and condiments. Immediate post-race recovery foods and drinks included, recovery drinks (protein and carbohydrate), carbohydrate options (e.g. rice and pasta), a protein option (e.g. poultry or fish), smoothie, cakes and additional snacks (e.g. fruit and yoghurts). Dinner had different options that included 1 x meat option (poultry or red meat option), 1 x fish option, 3-4 carbohydrate options (e.g. rice, pasta, potatoes, potatoes, quinoa, risotto, breads), 2 x vegetable and/or salad options, dessert option (e.g. fruit and yoghurt, rice pudding, brownie, banana bread, cake) and snacks and condiments. The daily menus provided were dictated by the team's nutrition staff and chef on race. Nutritional data was collected by two trained investigators during the second half of the race. The investigator who was on the race prescribed and weighed all foods served at breakfast, post-race recovery and dinner for the cyclist before they consumed it. Additionally, the cyclist self-reported the intake of bidons (carbohydrate drinks (Bespoke carbohydrate mix, Science in Sport, Blackburn, UK) and race food (carbohydrate gels (Science in Sport, Blackburn, UK), carbohydrate bars (Science in Sport, Blackburn, UK), protein bars (Science in Sport, Blackburn, UK), paninis, nougat and homemade rice cakes that were consumed during each stage. Throughout each day the cyclist also took a picture of all foods they consumed by the remote food photographic method (RFPM) (Martin *et al.*, 2009) alongside reporting the weight of all weighed foods and a description of each meal in order to enable quantification of all nutritional intake (see Figure 8.2). This information was submitted to a daily mobile app (WhatsApp, California, USA) including the cyclist and the two investigators. The second investigator who was off race then obtained the daily energy and macronutrient composition by analysing the relevant information through the use of a professional dietary analysis software (Nutritics Ltd, Ireland). Information about the nutritional content of foods not available in the software was obtained from the manufacturers. All the food was weighed on a digital scale (Salter Disc Electronic Digital Kitchen Scales, Kent, United Kingdom) with a precision of 1 g increments up to 5 kg, respectively. Sport products such as energy bars, gels and drinks ingested by the cyclist during the race were described from the label of the products.

### 8.3.6 Heart rate, power and energy expenditure

Heart rate was recorded continuously using a portable HR monitor (Garmin Edge, USA) during every stage. Continuous power output data were also collected using a dual-sided power meter (Stages, Colorado, USA). Power output was used to estimate on-bike energy expenditure. After each stage, files were uploaded to Today's Plan online platform (Cranberra, Australia) and later analysed. A power meter determines the power output (watts) using strain gauges that deflect slightly whenever a force is applied. By measuring this torque and combining it with angular velocity, power output can be calculated. Power output is then used to estimate on-bike energy expenditure by assuming an efficiency of 22% and the final kJ value is calculated by the average power of the stage multiplied by the total seconds of riding in the stage divided by one thousand.

### 8.3.7 Body mass measurement

The cyclist completed a daily morning medical and wellness check which included a morning nude body mass recording **(**SECA, Hamburg, Germany). The cyclist also had their nude body mass recorded (SECA, Hamburg, Germany) immediately post-stage before the consumption of any recovery drinks or foods. Weighing scales were calibrated prior to race in line with manufacturing guidelines.

**Table 8.2** Overview of the environmental demands, physiological demands and characteristics for each stage during the period of data collection.

| Stage | Stage type | Avg Temperature (˚C) | Distance (km) | Elevation (m) | On-bike EE (kJ) |
|---|---|---|---|---|---|
| **11** | Hilly | 18 | 163.5 | 1877 | 3635 |
| **12** | Flat | 18 | 221.5 | 964 | 4015 |
| **13**[#] | Flat | - | | | |
| **14** | Mountain (SF) | 20 | 183.9 | 4155 | 5405 |
| **15** | Mid-mountain | 16 | 175.9 | 3776 | 5034 |
| **Rest Day** | - | - | 37.6 | 248 | 853 |
| **16** | Individual TT | 18 | 35.2 | 204 | 1042 |
| **17** | Hilly | 23 | 153.1 | 2020 | 3939 |
| **18** | Hilly (SF) | 27 | 202.2 | 2135 | 4227 |
| **19** | Mountain (SF) | 23 | 185.8 | 4473 | 6180 |

#No data due to losing Garmin computer during the stage. EE: Energy expenditure

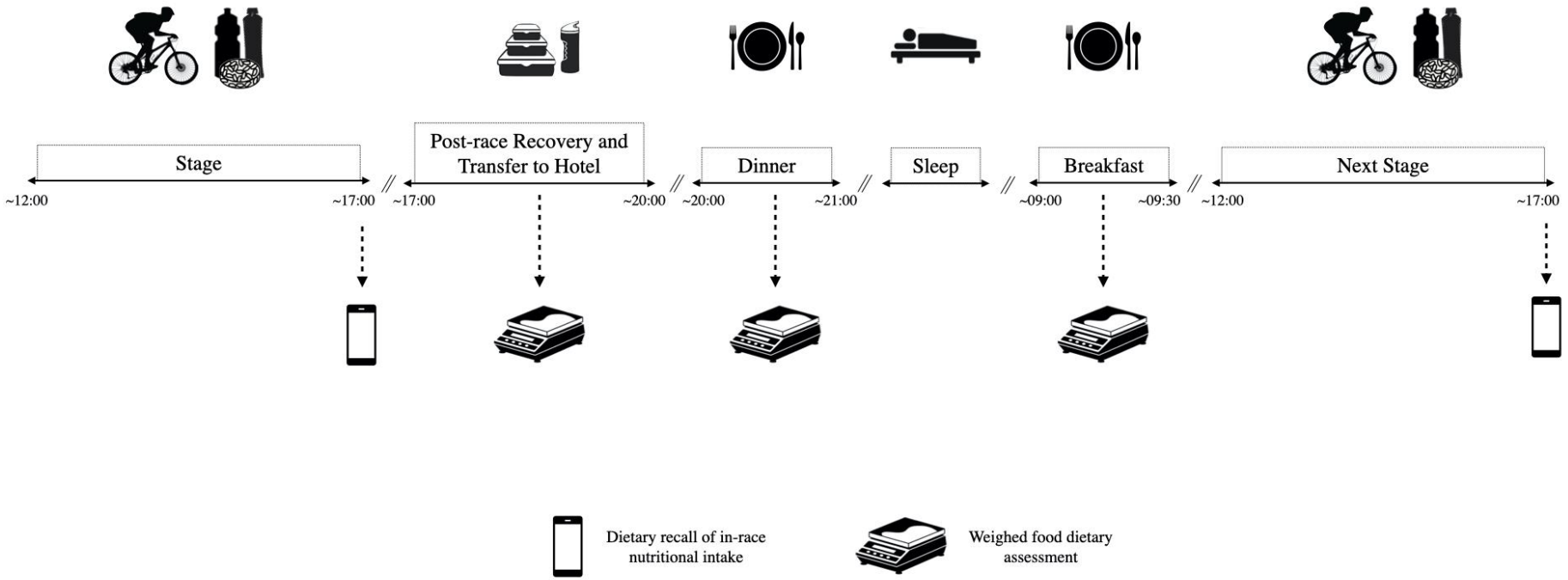


**Figure 8.2** Schematic overview of the typical racing and feeding timings of a typical stage within a Grand Tour.

## 8.4 Results

### 8.4.1 Assessment of daily energy and macronutrient intake

A comparison of the riders' daily energy and macronutrient intake is presented in Figure 8.3. On average the riders' EI and EI relative to their body mass was 3986 ± 1452 kcals (range: 1817 – 6413 kcals) and 58.6 ± 21.4 kcal·kg$^{-1}$ (range: 26.7 – 94.3 kcal·kg$^{-1}$). Specifically, the greatest energy intake can be seen on those stages that were classified as MT stages or in preparation for those stages that produced the higher EEE. In accordance with the large intakes and the vast range of energy intake, CHO was similar with the riders' average CHO and CHO relative to body mass was 739 g (range: 240 – 1305 g) and relative to BM 10.7 ± 4.8 g·kg$^{-1}$ (range: 3.5 – 18.9 g·kg$^{-1}$). Moreover, the riders' average protein intake was 168 ± 36 g (range: 115 – 209 g) and relative to BM was 2.4 ± 0.5 g·kg$^{-1}$ (range: 1.7 – 3.4 g·kg$^{-1}$). Average fat intake was also 47 ± 22 g (range: 20 – 86 g) and relative to BM was 0.7 ± 0.3 g·kg$^{-1}$ (0.3 – 1.2 g·kg$^{-1}$). Both of these macronutrients were maintained within a narrower range as intended, with larger intakes on higher EI days as a consequence of the greater volume of food consumed (see Figure 8.3).

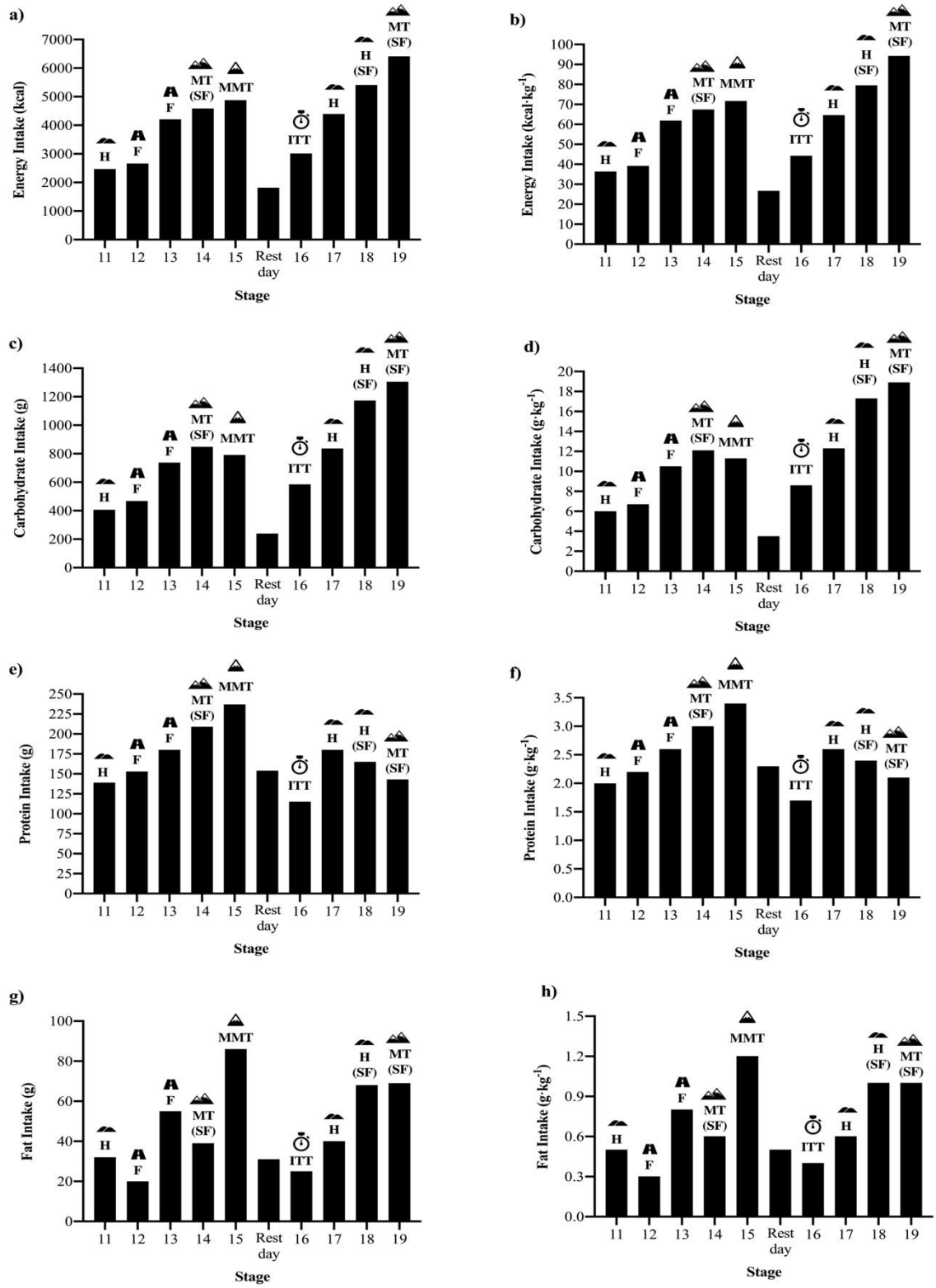


**Figure 8.3** Daily energy and macronutrient intake expressed absolutely and relative to body mass over the testing period. (a) absolute energy intake, (b) energy intake relative to body mass, (c) absolute carbohydrate, (d) relative carbohydrate, (e) absolute protein, (f) relative protein, (g) absolute fat and (h) relative fat. H: Hilly; F: Flat; MT: Mountain; MMT: Mid-mountain; ITT: Individual time-trial; SF: Summit finish.

### 8.4.2 Assessment of within day energy and macronutrient intake

Absolute and relative energy and macronutrient intake distribution across meals is displayed in Table 8.3. CHO intake was greater in the immediate post-race recovery compared with dinner and breakfast which were generally similar. Specifically, both Stage 18 and 19 report substantially higher post-race CHO intake compared to previous stages. Protein intake was also higher in post-race recovery compared with breakfast and dinner, with it also being higher in dinner than at breakfast. Fat intake was similar in breakfast and post-race but higher at dinner. Additionally, on bike CHO intake averaged 327 ± 93 g (range: 229 – 502 g) and relative to the duration of the stage averaged 70 ± 18 $g \cdot h^{-1}$ (range: 48 – 100 $g \cdot h^{-1}$). The highest estimated hourly ingestion rate of 100 $g \cdot h^{-1}$ was observed during Stage 19 (MT stage with summit finish) (see Table 8.3).

**Table 8.3** Daily energy and macronutrient intakes of breakfast, post-race and dinner expressed absolutely and relative to body mass.

| | | Stage | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **11** (Hilly) | **12** (Flat) | **13** (Flat) | **14** (MT SF) | **15** (MMT) | **Rest day** | **16** (ITT) | **17** (Hilly) | **18** (Hilly SF) | **19** (MT SF) | **Mean ± SD** |
| **Energy Intake** | | | | | | | | | | | | |
| | *Breakfast (kcal)* | 523 | 548 | 754 | 1043 | 1319 | 361 | 819 | 879 | 1061 | 997 | 830 ± 292 |
| | *kcal·kg$^{-1}$* | 7.5 | 8.0 | 11.1 | 15.2 | 19.5 | 5.3 | 12.0 | 12.9 | 15.5 | 14.5 | 12.1 ± 4.3 |
| | *Post-stage (kcal)* | 516 | 576 | 1023 | 782 | 1093 | 583 | - | 1007 | 1371 | 2384 | 1037 ± 579 |
| | *kcal·kg$^{-1}$* | 7.7 | 8.4 | 15.3 | 11.6 | 16.1 | 8.5 | - | 14.8 | 20.6 | 35.3 | 13.8 ± 9.5 |
| | *Dinner (kcal)* | 452 | 355 | 1324 | 1241 | 740 | 783 | 686 | 1057 | 1325 | 972 | 894 ± 348 |
| | *kcal·kg$^{-1}$* | 6.6 | 5.1 | 19.5 | 18.3 | 10.9 | 11.5 | 10.1 | 15.5 | 19.5 | 14.3 | 13.1 ± 5.1 |
| **Carbohydrate** | | | | | | | | | | | | |
| | *Breakfast (g)* | 78 | 84 | 133 | 196 | 241 | 33 | 156 | 164 | 203 | 189 | 148 ± 65 |
| | *g·kg$^{-1}$* | 1.1 | 1.2 | 2.0 | 2.9 | 3.5 | 0.5 | 2.3 | 2.4 | 3.0 | 2.7 | 2.2 ± 1.0 |
| | *Post-stage (g)* | 50 | 65 | 154 | 93 | 146 | 90 | - | 162 | 370 | 430 | 173 ± 135 |
| | *g·kg$^{-1}$* | 0.7 | 0.9 | 2.2 | 1.3 | 2.1 | 1.3 | - | 2.4 | 5.4 | 6.3 | 2.5 ± 2.0 |
| | *Dinner (g)* | 50 | 54 | 224 | 215 | 50 | 110 | 110 | 181 | 218 | 184 | 140 ± 73 |
| | *g·kg$^{-1}$* | 0.7 | 0.8 | 3.3 | 3.2 | 0.7 | 1.6 | 1.6 | 2.7 | 3.2 | 2.7 | 2.1 ± 1.1 |
| **Protein** | | | | | | | | | | | | |
| | *Breakfast (g)* | 28 | 28 | 30 | 33 | 40 | 37 | 30 | 28 | 33 | 31 | 32 ± 4 |
| | *g·kg$^{-1}$* | 0.4 | 0.4 | 0.4 | 0.5 | 0.6 | 0.5 | 0.4 | 0.4 | 0.5 | 0.4 | 0.5 ± 0.1 |
| | *Post-stage (g)* | 70 | 73 | 76 | 84 | 95 | 35 | - | 81 | 93 | 76 | 76 ± 18 |
| | *g·kg$^{-1}$* | 1.0 | 1.0 | 1.1 | 1.2 | 1.4 | 0.5 | - | 1.2 | 1.4 | 1.1 | 1.1 ± 0.3 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Dinner* | *(g)* | 36 | 32 | 49 | 70 | 53 | 78 | 51 | 42 | 30 | 32 | 47 ± 16 |
| | | *g·kg$^{-1}$* | 0.5 | 0.5 | 0.7 | 1.0 | 0.8 | 1.1 | 0.8 | 0.6 | 0.4 | 0.5 | 0.7 ± 0.2 |
| **Fat** | | | | | | | | | | | | | |
| | *Breakfast* | *(g)* | 11 | 11 | 11 | 14 | 22 | 9 | 9 | 12 | 13 | 13 | 13 ± 4 |
| | | *g·kg$^{-1}$* | 0.2 | 0.2 | 0.2 | 0.2 | 0.3 | 0.1 | 0.1 | 0.2 | 0.2 | 0.2 | 0.2 ± 0.1 |
| | *Post-stage* | *(g)* | 4 | 3 | 8 | 8 | 14 | 14 | - | 4 | 8 | 40 | 11 ± 11 |
| | | *g·kg$^{-1}$* | 0.1 | 0.1 | 0.1 | 0.1 | 0.2 | 0.2 | - | 0.1 | 0.1 | 0.6 | 0.2 ± 0.2 |
| | *Dinner* | *(g)* | 12 | 1 | 25 | 11 | 38 | 3 | 5 | 18 | 37 | 12 | 16 ± 13 |
| | | *g·kg$^{-1}$* | 0.2 | 0.1 | 0.4 | 0.2 | 0.6 | 0.1 | 0.1 | 0.3 | 0.5 | 0.2 | 0.3 ± 0.2 |

**Note.** No “Post-race” data for Stage 16 as it was an individual time trial with dinner being the next meal post time trial. MT: Mountain; MMT: Mid-mountain; SF: Summit finish; ITT: Individual time trial.

**Table 8.4** Overview of daily in race nutritional intake, relative carbohydrate intake to duration of stage and distribution of carbohydrate form.

| | | Stage | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **11** (Hilly) | **12** (Flat) | **13** (Flat) | **14** (MT SF) | **15** (MMT) | **Rest day** | **16** (ITT) | **17** (Hilly) | **18** (Hilly SF) | **19** (MT SF) | **Mean ± SD** |
| **Carbohydrate** | | | | | | | | | | | | |
| | *Solids (g)* | 105 | 128 | 167 | 130 | 126 | - | - | 126 | 188 | 84 | 132 ± 33 |
| | *Gels (g)* | 110 | 132 | 66 | 154 | 176 | - | - | 132 | 176 | 308 | 157 ± 71 |
| | *Fluids (g)* | 20 | 20 | 0 | 40 | 20 | - | - | 60 | 20 | 160 | 43 ± 51 |
| | *(g)* | 235 | 280 | 233 | 324 | 322 | - | - | 318 | 384 | 522 | 327 ± 93 |
| | *($g \cdot h^{-1}$)* | 59 | 52 | 58 | 59 | 64 | - | - | 95 | 69 | 100 | 70 ± 18 |
| **Protein** | | | | | | | | | | | | |
| | *(g)* | 5 | 20 | 25 | 22 | 29 | - | - | 29 | 9 | 4 | 18 ± 10 |
| **Fat** | | | | | | | | | | | | |
| | *(g)* | 5 | 5 | 11 | 6 | 6 | - | - | 6 | 10 | 4 | 7 ± 3 |
| **Energy Intake** | | | | | | | | | | | | |
| | *kcal* | 981 | 1185 | 1107 | 1422 | 1502 | - | - | 1450 | 1654 | 2060 | 1420 ± 342 |

**Note.** Rest day and Stage 16 (individual time-trial) report no carbohydrate intake during in-race. MT: Mountain; MMT: Mid-mountain; SF: Summit finish; ITT: Individual time trial.

### 8.4.3 Assessment of daily energy availability

The riders' daily morning and post-stage body mass is presented in Figure 8.4. It is difficult to ascertain the true changes in body mass due to the daily fluctuations. However, by the critical Stage 19 the rider weighed 68.9 kg after an extremely aggressive CHO load and in a fully replenished and rehydrated state. The daily EI and on-bike EE are presented in Figure 8.4. On average the rider was consuming 3986 ± 1452 kcals daily and expending 3814 ± 1814 kJ (range: 853 – 6180 kJ) during the stage, with the highest expenditure being reported on the MT stages as expected. Daily average energy availability (EA) was observed to be 2.3 ± 17 kcal·kg$^{-1}$ FFM with large variations between days. Indeed, on Stage 11 and 12, EA was estimated as -18.3 and – 21.2 kcal·kg$^{-1}$ FFM, respectively. In contrast, higher daily EA of 31.0 and 18.6 kcal·kg$^{-1}$ FFM was reported on Stage 16 and 18, respectively, in accordance with the higher EI stages.

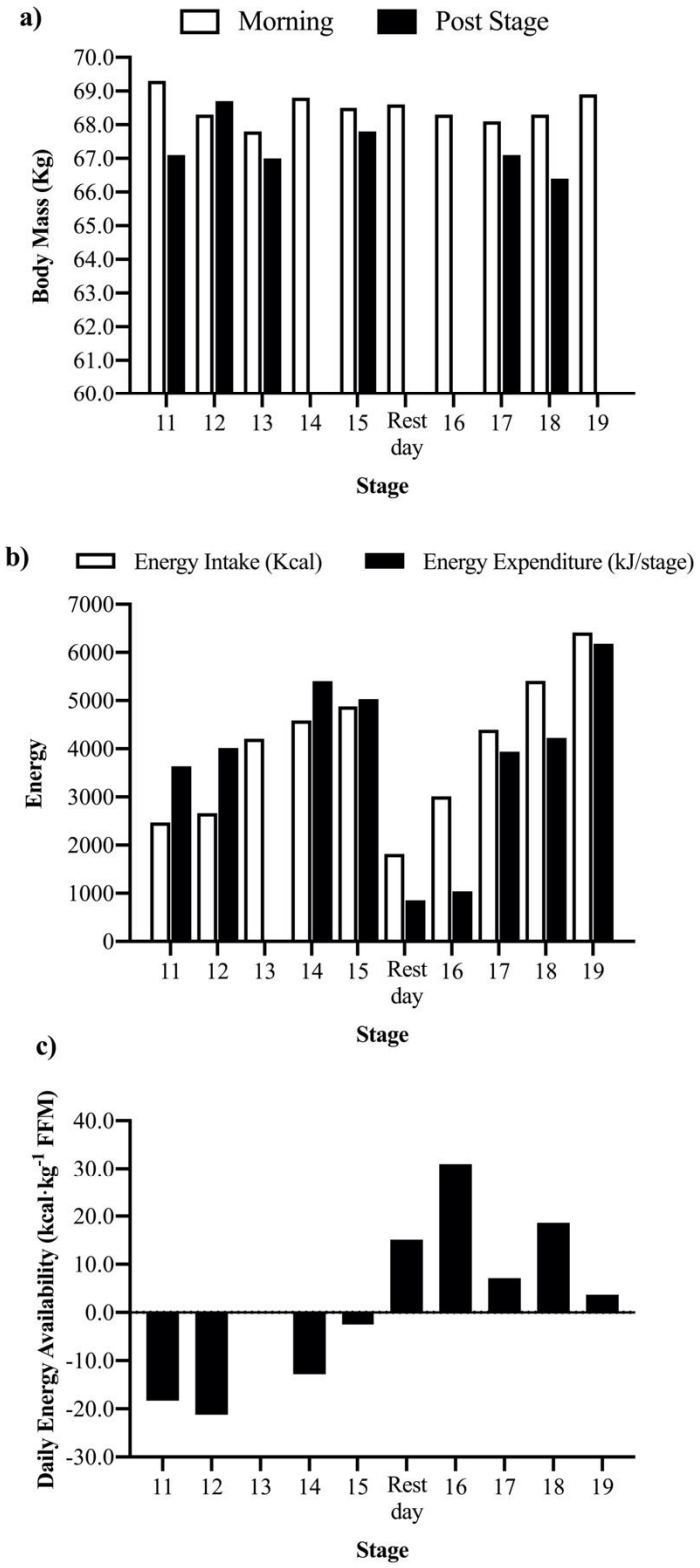


**Figure 8.4** (a) Body mass morning and post stage, (b) total energy intake and on bike energy expenditure and (c) calculated energy availability for stage during the period of data collection. No "Post Stage" body mass recorded on Stage 14 and 16. No exercise energy expenditure for calculating energy availability on Stage 13 due to losing Garmin computer during the stage.

**Table 8.5** An overview of the food consumption in preparation for and during Stage 19, 'Mission Critical'. For full nutritional breakdown see Table 8.3 and Figure 8.3.

| Meal/Time | Item and Description | Amount (g) | Carbohydrate (g) |
|---|---|---|---|
| **Stage 18** | | | |
| *Post-Race Recovery* | CHO + protein recovery drink | 70 | 40 |
| | Basmati rice | 400 | 125 |
| | Maple syrup | 20 | 15 |
| | Tuna steak | 80 | 0 |
| | 2 x Cherry juice | 500 (ml) | 40 |
| | Fruit based smoothie | 300 (ml) | 70 |
| | Sweet potato rice pudding | 600 | 75 |
| | Potato and leek soup | 100 (ml) | 5 |
| | **Total (g)** | | **370** |
| | **Total ($g \cdot kg^{-1}$)** | | **5.4** |
| *Dinner* | Basmati Rice | 400 | 125 |
| | Prawns | 70 | 0 |
| | Potato Bake | 200 | 25 |
| | Orange Polenta Cake | 100 | 30 |
| | Rhubarb and date fool | 150 | 30 |
| | Orange juice | 200 (ml) | 20 |
| | **Total (g)** | | **230** |
| | **Total ($g \cdot kg^{-1}$)** | | **3.3** |
| **Stage 19** | | | |
| *Breakfast* | Basmati rice | 400 | 125 |
| | Omelette | 180 | 0 |
| | 4 x Pancakes with jam | 120 | 40 |
| | Honey | 7 | 5 |
| | Orange juice | 200 (ml) | 20 |
| | **Total (g)** | | **190** |
| | **Total ($g \cdot kg^{-1}$)** | | **2.7** |
| *In Race* | 2 x SiS Beta Fuel drinks | 84 | 160 |
| | 4 x Rice cake | - | 84 |
| | 14 x SiS Isotonic carb gels | 60 (ml) | 308 |
| | **Total (g)** | | **552** |
| | **Total ($g \cdot h^{-1}$)** | | **100** |
| *Recovery* | CHO + protein recovery drink | 70 | 40 |
| | Basmati rice | 400 | 125 |
| | Maple syrup | 20 | 15 |
| | Salmon | 80 | 0 |
| | Cherry juice | 500 (ml) | 20 |
| | Fruit based smoothie | 300 (ml) | 70 |
| | Haribo | 25 | 20 |
| | 4 x Banana Slices | 300 | 150 |

| | | | |
|---|---|---|---|
| | **Total (g)** | | **440** |
| | **Total (g·kg$^{-1}$)** | | **6.4** |
| *Dinner* | Basmati rice | 400 | 125 |
| | Salmon Sashimi | 100 | 0 |
| | Sweet Potato Rice Pudding | 125 | 60 |
| | **Total (g)** | | **185** |
| | **Total (g·kg$^{-1}$)** | | **2.7** |

**Note** CHO: carbohydrate.

## 8.5 Discussion

The aim of the present case-study was to practically deliver a periodised daily CHO feeding strategy for a male professional World Tour road cyclist during the 2018 Giro d'Italia Grand Tour. To the author's knowledge, this is the first report to document a General Classification winners' daily EI and EA during that winning Grand Tour.

The mean daily CHO (10.7 g·kg$^{-1}$) and energy intake (3986 kcal·day$^{-1}$) consumed by the rider was lower than the ~12.6 g·kg$^{-1}$ and 5415 – 7815 kcal·day$^{-1}$ reported previously within Grand Tours (García-Rovés *et al.*, 1998; Muros *et al.*, 2019; Saris *et al.*, 1989). However, in accordance with the periodised approach, daily CHO and energy intake ranged considerably during the 10-day period with values of 3.5-18.9 g·kg$^{-1}$ and 1817-6413 kcal·day$^{-1}$, respectively. Indeed, the periodised approach was intended to adopt a gradual reduction in body mass through intentional under-fuelling on flat and less energetically demanding stages (see Table 8.2) before ensuring sufficient CHO availability prior to mountain stages and summit finishes. Such a strategy was an attempt to promote climbing performance by maximising power to weight ratio (Mujika & Padilla, 2001; Olds *et al.*, 1995; Van Erp *et al.*, 2020). For example, the rider consumed a low CHO and low residue diet (Reale *et al.*, 2017) during Stage 11, 12 and 13 (6.0, 6.7 and 10.5 g·kg$^{-1}$) during which time morning body mass decreased from 69.3 to 67.8 kg. In recovery from Stage 13 and in preparation for Stage 14, the rider then consumed a higher CHO intake (8.4 g·kg$^{-1}$) in order to promote glycogen storage prior to the stage. As such, body mass increased on the morning of Stage 14 to 68.8 kg and the rider was considered well fuelled. Additionally, the rider also consumed 59 g·h$^{-1}$ of CHO during Stage 14 and produced a stage winning performance winning by 6 seconds.

In keeping with the periodised approach, the lowest daily CHO and energy intakes typically occurred on rest days (3.5 g·kg$^{-1}$ and 1817 kcal) and on flat and hilly days such as Stage 11

(6.0 g·kg$^{-1}$ and 2472 kcal), 12 (6.7 g·kg$^{-1}$ and 2664 kcal) and 13 (10.5 g·kg$^{-1}$ and 4208 kcal). In contrast, the highest daily energy and CHO intakes were reported on Stage 18 and 19 with Stage 19 corresponding to the most physically demanding (6180 kJ) stage of the race. In preparation for Stage 19, the rider consumed 5.4 g·kg$^{-1}$ CHO and 1371 kcal in the early recovery period (i.e., 3-4 hour post-stage) from Stage 18 and 3.2 g·kg$^{-1}$ CHO and 1325 kcal in the evening dinner in recovery from Stage 18. Additionally, the rider consumed 2.7 g·kg$^{-1}$ CHO at breakfast on the morning of Stage 19. In this way, the rider prepared for Stage 19 after consuming a total of 11.3 g·kg$^{-1}$ of high glycaemic CHO in an 18-h period, a rate of CHO feeding that is considered conducive to maximal glycogen storage (Burke *et al.*, 1993; Thomas *et al.*, 2016). Throughout Stage 19, the rider also consumed 522 g of CHO equating to 100 g·h$^{-1}$, therefore in agreement with a dosing strategy considered optimal for performance (Chapter 5). During this stage, the rider attacked solo with 80 km to the finish which required him to ride on the front on his own for over 3 hours. To ensure a fuelling pattern consistent with feeding every 15-20 minutes (Mears *et al.*, 2020), the rider consumed a mix of solids (4 × rice cakes: 84 g CHO), gels (14 × CHO gels: 308 g CHO) and liquids (2 × CHO drinks: 160 g) whereby team support staff was strategically positioned at set distances on the course to physically hand over the required fuel source. Importantly, this was the stage when the rider successfully took the overall lead of the race by regaining 03 minutes and 23 seconds in the final 80 km. Whilst no objective measurement of GI discomfort or symptoms was measured, the rider did mention upon race completion that at times he felt high levels of gut fullness and minor feelings to vomit during the stage, likely a reflection of the high dose of CHO consumed and gut ischemia associated with the high exercise intensity (De Oliveira & Burini, 2011; Ter Steege *et al.*, 2008; Ter Steege *et al.*, 2011). Nonetheless, the rider was still able to consume 9.0 g·kg$^{-1}$ CHO and 3356 kcal in the early recovery from Stage 19 and in the evening dinner so as to prepare for the final racing stage on Stage 20. As such, the total CHO consumed on Stage 19 equated to 18.9 g·kg$^{-1}$ (see Table 8.4) which to the authors' knowledge, is the highest reported in the literature.

In relation to the within day distribution of energy and macronutrient intake (excluding in-race macronutrient and energy intake), we observed that the greatest CHO and energy intakes were typically observed during the post-stage recovery period (2.5 g·kg$^{-1}$ and 1037 kcal) (which consisted of a 3-4 h period between the stage finishing and having dinner, see Figure 8.2) as compared with both breakfast (2.2 g·kg$^{-1}$ and 830 kcal) and dinner (2.1 g·kg$^{-1}$ and 894 kcal). This is in contrast to previous observations in GT whereby riders consumed most of their daily

CHO and energy distribution at breakfast (Muros *et al.*, 2019) or dinner (García-Rovés *et al.*, 1998). However, the distribution of CHO and energy between feeding time points within the 10-day assessment period in the current study varied in accordance with the aforementioned daily CHO periodisation approach, which was implemented on a meal-by-meal basis. As such, CHO intake rates at breakfast, post-stage and dinner ranged from 0.5-3.5, 0.7-6.3 and 0.7-3.3 $g \cdot kg^{-1}$, respectively. Moreover, similar to the previously noted trend of lower overall daily intakes on flat stages and rest days, CHO and energy intake was lowest at breakfast (0.5-2.0 $g \cdot kg^{-1}$ and 361-754 kcal), post-stage (0.7-2.2 $g \cdot kg^{-1}$ and 516-1023 kcal) and diner (0.7-1.6 $g \cdot kg^{-1}$ and 452-783 kcal) during such days, whilst during and in preparation for mountain and summit finishes, CHO intake was highest at breakfast (2.4-3.5 $g \cdot kg^{-1}$ and 879-1319 kcal), post-stage (2.1-6.3 $g \cdot kg^{-1}$ and 1093-2384 kcal) and dinner (1.6-3.2 $g \cdot kg^{-1}$ and 686-1325 kcal). The higher CHO and energy distribution in the early 3-4 h period post stage was especially evident in Stage 18 and 19, in accordance with the most physically demanding Stage 19. Additionally, such high intakes were purposely advised within this early 3-4 hour period in order to rapidly stimulate glycogen resynthesis rates (Ivy *et al.*, 1988; Sjøberg *et al.*, 2017; Zachwieja *et al.*, 1991) immediately after the stage, with CHO intake rates equating to approximately 1.5 $g \cdot kg^{-1} \cdot h^{-1}$ for the 4-hour period (Burke *et al.*, 2017). This subsequently led to considerably higher intakes compared to dinner (~6 vs ~3 $g \cdot kg^{-1}$ CHO). In contrast, the distribution of CHO and energy intake was more evenly matched during less demanding flat and hilly stages such as Stage 11 and 12 whereby lower intakes were consumed at breakfast (1.1 and 1.2 $g \cdot kg^{-1}$ CHO, and 523 and 548 kcal), post-stage (0.7 and 0.9 $g \cdot kg^{-1}$ CHO, and 516 and 517 kcal) and dinner (0.7 and 0.8 $g \cdot kg^{-1}$ CHO, and 452 and 355 kcal).

In addition to the distribution of CHO intake, the mean CHO intake during each stage reported values of 70 $g \cdot h^{-1}$ with a range of 52-100 $g \cdot h^{-1}$. Such values were lower than the ~90 $g \cdot h^{-1}$ previously reported during the Tour de France and Vuelta a España (García-Rovés *et al.*, 1998; Muros *et al.*, 2019; Saris *et al.*, 1989) but higher than the intakes rates reported during 1-day professional Spring Classic races (51 $g \cdot h^{-1}$) (Heikura *et al.*, 2019) and shorter stage races (~60 $g \cdot h^{-1}$) (Pfeiffer *et al.*, 2012; Ross *et al.*, 2014). Nonetheless, the large variation in CHO intake was strategically devised dependent on the physical demands and performance aims of each stage. For instance, during Stage 11-15, which included one important stage in regard to the overall GC (Stage 14), CHO intake was ~60 $g \cdot h^{-1}$ compared to Stage 17-19 whereby CHO intake was 95, 69 and 100 $g \cdot h^{-1}$ with each of these stages being highly contested between GC rivals. As such, intakes of ~60 $g \cdot h^{-1}$ would have been sufficient to maintain elevated rates of

CHO availability during those less demanding stages whilst higher intakes of 70-100 g·h$^{-1}$ would have been considered more conducive to maintaining optimal CHO availability during those CHO dependent and race-defining stages. The in-race feeding strategies were devised of a combination of CHO forms such as solids (homemade rice cakes, CHO bars and paninis), gels (isotonic CHO gels) and fluids (single and multi-source CHO drinks) as based on the premise that there is little difference in exogenous CHO oxidation rates (albeit in fluid matched conditions) between the aforementioned forms (Pfeiffer *et al.*, 2010a; Pfeiffer *et al.*, 2010b). These in-race CHO forms also consisted of both single (maltodextrin) and multi-source (sucrose and fructose) CHO to promote exogenous CHO oxidation with higher intakes (Jeukendrup, 2011). Typically, the greatest contribution to overall in-race CHO intake came from both solid and gel forms with reduced CHO intake from fluids. Additionally, in-race fuelling strategies typically comprised of solid choices during the first half of the race when intensity is considered low-to-moderate to aid digestion and absorption, before switching to CHO gels for the latter part of the race when exercise intensity increases, and feeding may become more difficult. The rider was also advised to adopt a 'drink to thirst' approach to hydration, where absolute fluid intake can vary between 350 to 800 ml·h$^{-1}$ but unfortunately, overall on-bike fluid intake was not measured within the current study due to the difficulties of self-reporting bidon consumption and measuring the consumed content of bidons passed to cyclists during stages.

As a consequence of variations in daily energy intake and EEE, we also observed significant variations in daily EA, ranging from -21 kcal·kg$^{-1}$ to 31 kcal·kg$^{-1}$ FFM. This significant variation was most notably the consequence of the intended reduction in body mass in preparation for Stage 18, 19 and 20 combined with the high EEE associated with mountain stages and summit finishes (Stage 14: 5405 kJ, Stage 15: 5034 kJ, Stage 18: 4227 kJ and Stage 19: 6180 kJ). Similarly, recent insights from Heikura *et al.* (2019) also reported professional cyclists to demonstrate alternate day low-EA during professional 1-day Spring Classic races whereby values of 14.4 kcal·kg FFM$^{-1}$ were reported on race days followed by 56.9 kcal·kg FFM$^{-1}$ during the rest-days, which were interspersed between the 1-day races. The distinct difference between the studies is that the Spring Classics require riders to compete a single day race followed by 1-3 recovery days before then competing in another 1-day race. This enabled riders to restore higher EA values between races given the high EI (5050 kcal) and low EEE during these training recovery days (954 kJ). Conversely, the current study involved continuous daily racing which consistently demonstrated daily EA values of $\leq$ 30 kcal·kg FFM$^{-1}$. Despite

the daily low EA demonstrated by the rider, it seemed to have no negative implications upon the riders' performance or health though we acknowledge that no specific biochemical markers were obtained in the present study.

Despite the novelty and practical aspects of the current study, our data are not without limitations, largely a reflection of the practical demands of data collection within the elite cycling environment given the number of logistical obstacles associated with the ever-changing demands of multi-stage racing. Indeed, to minimise the over- or underestimation of quantifying EI, both a weighed food approach and self-reporting with RFPM was used to collectively estimate energy and macronutrient intake and distribution. However, it is possible that in some cases the quantity of CHO consumed on the bike during a stage may have been over- or underestimated as a consequence of the rider self-reporting and recalling what they consumed during the stage immediately after it and the associated stress levels of the racing environment potentially effecting such recollections. Similarly, the impossibility of measuring the consumed content of bidons passed to the rider during stages is also acknowledged as a limitation associated with dietary assessment. Data were only collected from Stage 11 – 19 and in order to have an understanding of the complete nutritional intake during the full Grand Tour race, this study would have to be replicated across the whole duration of the race. Indeed, future studies may also wish to perform more comprehensive testing of body composition and also assess total daily EE as opposed to on-bike EE per se. Above all, unlike traditional randomised controlled trials incorporating large sample cohorts, this study provided a 'real world' example of one rider only and hence data are limited to the specific context of this rider and this race.

In summary, we simultaneously quantified for the first time, the daily energy and macronutrient intakes of an elite professional cyclist during a race winning Grand Tour. Our data confirm the role of high CHO intake in supporting winning performances whilst also documenting use of in-race CHO and energy periodisation strategy intended to simultaneously manipulate body mass and promote performance during the most physically demanding stages.

# Chapter Nine:

# Synthesis of findings

The aim of this Chapter is to critically appraise the experimental findings in relation to the original aims and objectives set out in Chapter 1. The Chapter begins by briefly revisiting the aims and objectives of the thesis and reviewing if each, in turn, have been achieved. A critical discussion of the findings is then presented whereby specific attention is given to how the present data have advanced our understanding of the effects of CHO feeding on muscle fuel selection and exercise capacity. A discussion of the practical implications is subsequently presented alongside an overview of important experimental and methodological limitations. Finally, the thesis closes by outlining directions and recommendations for future research.

## 9.1 Achievement of aims and objectives

The primary aim of this thesis was to determine the dose-response relationship of CHO feeding during endurance exercise on muscle fuel selection and exercise capacity. This aim was intended to be realised by completion of a scheme of work comprising five integrated studies.

**Objective 1: To develop a prolonged submaximal endurance-based cycling protocol and a subsequent exercise capacity test that is reflective of endurance road cycling competition, which will provide an exercise protocol that can be used in subsequent chapters to determine the dose-response relationship of carbohydrate feeding during exercise on muscle fuel selection and endurance cycling capacity (Study 1, Chapter 4).**

Data presented in Study 1 demonstrated that under conditions of high pre-exercise CHO availability, trained male cyclists are capable of completing 180-min of submaximal exercise at lactate threshold and that fatigue during the subsequent exercise capacity typically occurred within <10 minutes. However, the significant reductions in capillary lactate, RPE and HR during the submaximal protocol between Trial 1 and 3 considered in combination with the significant extension in exercise capacity between Trial 1 and Trial 3 suggested that at least one full familiarisation session was required prior to studying the dose-response relationship of CHO feeding on metabolism and performance. As such, completion of this study provided important methodological rigour for Study 2.

**Aim 2: To determine the dose-response relationship of carbohydrate feeding during endurance exercise on endurance cycling capacity (Study 2, Chapter 5).**

Having developed a suitable prolonged endurance cycling based exercise protocol in Study 1, Study 2 utilised this protocol to subsequently assess the dose-response relationship of CHO feeding on exercise capacity. The data presented in this study demonstrated that in conditions of high pre-exercise CHO availability (i.e., after CHO loading and consumption of a pre-exercise CHO meal), CHO feeding improves cycling exercise capacity in a dose dependent manner whereby 90 $g \cdot h^{-1}$ of CHO extended exercise capacity over both 45 and 0 $g \cdot h^{-1}$. Such ergogenic effects were also independent of muscle glycogen sparing (at least when assessed in whole muscle homogenate) and were suggested to be due to a combination of liver glycogen sparing, increased plasma glucose availability and maintenance of CHO oxidation. Given that Grand Tours in professional cycling can be won or lost with a matter of seconds, these findings are of practical significance in that the chosen CHO strategy may improve the capacity to "hold a wheel" or "mount attacks" during the closing periods of race defining mountain stages.

**Aim 3: To determine the dose-response relationship of carbohydrate feeding during endurance exercise on muscle fuel selection (Study 3, Chapter 6).**

Whilst Study 2 demonstrated an ergogenic effect in the absence of muscle glycogen, assessment of glycogen utilisation was limited by the lack of fibre specific measurements. As such, Study 3 assessed the effects of CHO feeding on both muscle glycogen and IMTG utilisation in type I and II fibres. Consistent with Study 2, the data presented here also demonstrated that CHO feeding did not induce a glycogen sparing effect in either type I or II muscle fibres. Additionally, our data also demonstrate that CHO feeding does not affect IMTG utilisation in either fibre type. It was suggested that the lack of effects of CHO feeding on muscle fuel selection may be due to the fact that high pre-exercise muscle glycogen availability may offset the regulatory effects of consuming CHO "during" exercise. Additionally, it was also suggested that biopsies should be obtained throughout exercise so as to provide more insight on the time-course of muscle fuel selection and the potential effects of any CHO feeding.

**Aim 4: To determine the dose-response relationship of carbohydrate feeding during endurance exercise on subcellular substrate utilisation (Study 4, Chapter 7).**

Before definitively ascertaining that CHO feeding during exercise does not alter muscle fuel selection, we deemed it appropriate to study muscle glycogen utilisation in specific subcellular

storage pools, as measured using transmission electron microscopy. However, due to the associated laboratory restrictions of the COVID-19 world pandemic, it was not possible to complete this aim. Nonetheless, the development of this technique within the LJMU laboratories now provides opportunity for further study in this area.

**Aim 5: To practically deliver a periodised daily carbohydrate feeding strategy for a male professional cyclist during a Grand Tour (Study 5, Chapter 8).**

In using a detailed case-study design, we delivered a periodised approach to CHO feeding during a Grand Tour with the aim of optimising performance during critical stages of the race. To this end, we were able to quantify daily energy and macronutrient intake, exercise energy expenditure and energy availability during the final 10 stages of the 2018 Giro d'Italia. This approach was successful in supporting a winning performance and provides the first report in the literature to document the practical application of a periodised approach of CHO feeding in professional cycling. Importantly, the winning performance was supported by application of high CHO availability during critical stages and was informed by the laboratory data collected during this thesis.

## 9.2 General discussion of findings

The provision of CHO during prolonged endurance exercise has been extensively researched with initial reports beginning as far back as the 1920s during the Boston Marathon (Gordon *et al.*, 1925). From the plethora of evidence over the years demonstrating the beneficial effects of CHO ingestion on endurance performance and capacity (Cermak & Van Loon, 2013; Stellingwerff & Cox, 2014; Vandenbogaerde & Hopkins, 2011) (see Table 2.2, 2.3, 2.4 and 2.6), it is now a readily practiced strategy by athletes during competition. Current sport nutrition guidelines for endurance exercise advocate CHO ingestion at a rate of $\leq$30 g or CHO mouth rinse during exercise <60 mins, 30-60 $g \cdot h^{-1}$ of single source CHO during 1-2.5 h of exercise and 90 $g \cdot h^{-1}$ of multi-transportable CHOs during >2.5-h of exercise (Jeukendrup, 2014; Thomas *et al.*, 2016). The mechanisms underpinning these performance enhancements are dependent on exercise modality, duration, intensity and CHO feeding rate and availability and likely encompass sparing of muscle and liver glycogen utilisation (Björkman, Shalin, Hagenfeldt & Wahren, 1984; Gonzalez *et al.*, 2015; Stellingwerff *et al.*, 2007a), maintenance

of plasma glucose and elevated CHO oxidation rates (Coyle *et al.,* 1986) and direct effects upon the central nervous system (CNS) (Carter, Jeukendrup & Jones, 2004).

### 9.2.1 What is the optimal dose of carbohydrate feeding to improve endurance capacity?

Although the effects of CHO feeding on promoting endurance performance and extending exercise duration are well documented, the dose-response relationship of CHO ingestion and performance has been less well characterised (see Table 2.5). Indeed, comparisons between studies are often limited owing to the fact that pre-exercise CHO availability has not been tightly controlled and that the form of CHO consumed during exercise is often confined to beverages only. Accordingly, the present thesis attempted to utilise a dietary protocol that was considered best practice whereby experimental trials were commenced after a CHO loading protocol combined with a high CHO pre-exercise meal. Additionally, our chosen CHO feeding strategy comprised of a mixture of fluids, solids and gels so as to replicate the typical feeding schedules adopted by professional cyclists. In this way, the pre-exercise dietary regime ensured that participants commenced each trial with high muscle glycogen concentrations (as reported in Chapter 5 and 6) and likely, high liver glycogen stores (Casey *et al.,* 2000). Such dietary strategies and feeding protocols were collectively utilised to promote the practical relevance of experimental findings given that elite endurance cyclists will typically consume high CHO diets in the day(s) and hours prior to and during competition (García-Rovés *et al.,* 1998; Heikura *et al.,* 2019; Muros *et al.,* 2019; Morton, unpublished observations; Sánchez-Muñoz *et al.,* 2016).

Using this design, we were able to demonstrate that CHO feeding improves exercise capacity in a dose dependent manner whereby 90 $g \cdot h^{-1}$ > 45 $g \cdot h^{-1}$ > 0 $g \cdot h^{-1}$. Additionally, the magnitude of improvement between doses (i.e., 108 to 233 seconds) was considered of practical significance considering that such a range encompasses the winning margin of professional cycling Grand Tours. In relation to previous experimental data, our data agree favourably with previous observations that identified 90 $g \cdot h^{-1}$ of CHO in the form of a 2:1 glucose and fructose blend to be superior for cycling TT performance after a 2- (King *et al.,* 2018) and 3-h (King *et al.,* 2019) preload when CHO doses between 60-112.5 $g \cdot h^{-1}$ (King *et al.,* 2018) and 80-100 $g \cdot h^{-1}$ (King *et al.,* 2019) were examined. Similarly, Smith *et al.* (2013) also estimated an optimal upper ingestion rate of 88 $g \cdot h^{-1}$ of CHO during prolonged endurance cycling (>2-h) to maximise TT performance when graded quantities of 10–120 $g \cdot h^{-1}$ were assessed, but they did

however conclude the dose-response relationship of CHO ingestion to be curvilinear in nature. Taken together, our findings highlight important practical implications that suggest consuming 90 $g \cdot h^{-1}$ of CHO when riding in the peloton may subsequently improve the capacity to "hold a wheel" or "mount attacks" during the closing periods of mountain stages which can be characterised as important determinants between winning or losing during professional road cycling.

### 9.2.2 Does carbohydrate feeding improve exercise capacity by altering muscle fuel selection?

A longstanding hypothesis underpinning the ergogenic effect of CHO feeding during exercise is the concept that muscle glycogen sparing occurs, thus ensuring more glycogen is available to support energy production later in exercise. However, the data presented in this thesis clearly demonstrate that CHO feeding does not spare muscle glycogen, as assessed in whole muscle homogenate (see Figure 5.3) or from a fibre-specific level within type I or type II muscle fibres (see Figure 6.2). This lack of a glycogen sparing effect when compared with previous studies (Bergstrom and Hultman, 1967; Bosch et al., 1996; Björkman *et al.*, 1984; De Bock *et al.*, 2007; Erickson *et al.*, 1987; Hargreaves *et al.*, 1984; Stellingwerff *et al.*, 2007; Tsintzas *et al.*, 1995; Tsintzas *et al.*, 1996) is most likely related to distinct methodological differences between studies related to muscle glycogen availability, pre-exercise CHO feeding, exercise duration and timing of assessment of substrate metabolism. Most notably, initial pre-exercise glycogen status is known to be a potent regulator of glycogen metabolism (Arkinstall *et al.*, 2004; Hargreaves *et al.*, 1995), therefore any potential regulatory effects of consuming CHO "during" exercise may have been offset by the effects of the high pre-exercise muscle glycogen concentrations on absolute muscle glycogen utilisation as well as the metabolic conditions created by consumption of a pre-exercise meal.

In addition to glycogen, we also observed that CHO feeding did not alter IMTG utilisation in type I and IIa fibres consistent with previous observations (Stellingwerff *et al.*, 2007a) but in contrast to others (De Bock *et al.*, 2005). In the same way as glycogen, the absence of any apparent effect of CHO feeding upon IMTG use seemed to be potentially offset by the elevated glycogen concentrations and CHO feeding prior to exercise. As such, it seemed the metabolic conditions during the initial 60-90 minutes of exercise may not have been distinct enough between trials to alter the pattern of IMTG use (see Figure 5.4 and 5.5), in combination with

previous observations suggesting that IMTG utilisation appears to predominate during the first two hours of exercise with an attenuated use thereafter (Romijn *et al.*, 1993; Watt *et al.*, 2002). Although a potential suppression of IMTG mobilisation and/or oxidation may have occurred during the third hour of exercise as a result of the progressive difference in plasma NEFA availability and delivery (see Figure 5.4) (Van Loon *et al.*, 2003a; Watt *et al.*, 2002; Van Loon *et al.*, 2005a; Van Loon *et al.*, 2005b; Watt *et al.*, 2004), it is possible that the predominant IMTG utilisation had already occurred during the first 1-2 h of exercise given that whole body rates of fat oxidation did not differ between trials until the second and third hour of exercise.

When taken together, these data therefore suggest that the ergogenic effects of CHO feeding do not arise from alterations to muscle fuel metabolism but are more likely underpinned by liver glycogen sparing and maintenance of plasma glucose and CHO oxidation (see Figure 5.4 and 5.5). In this regard, Gonzalez *et al.* (2015) demonstrated that CHO feeding at a rate of 102 $g \cdot h^{-1}$ of either glucose or sucrose during 3-h of endurance cycling prevented liver but not muscle glycogen depletion. The CHO feeding protocol utilised in Study 2 and 3 of the current thesis consisted of CHO from a range of single and multi-transportable sources with the inclusion of glucose, maltodextrin and sucrose which were derived from the 'real foods' aspects of the feeding protocol. This may have elucidated a potential liver glycogen sparing effect in accordance with these previous findings. Additionally, Newell *et al.* (2018) further supported these metabolic explanations in that the performance gains associated with the ingestion of 39 and 64 $g \cdot h^{-1}$ of single source CHO during prolonged endurance exercise (>2-h) was found to be related to a preservation of endogenous glycogen stores, most likely hepatic stores, and maintaining high rates of CHO oxidation through suppression of circulating NEFA concentration. Moreover, further correlation and regression analyses from the same study suggested that the rate of exogenous CHO oxidation and suppression of NEFA were the two most closely linked metabolic parameters associated to a significant prediction of the subsequent performance task outcome. Nonetheless, we acknowledge the necessity to further examine the effects of endurance exercise and CHO feeding on subcellular glycogen utilisation before definitively concluding that CHO feeding does not alter muscle fuel selection.

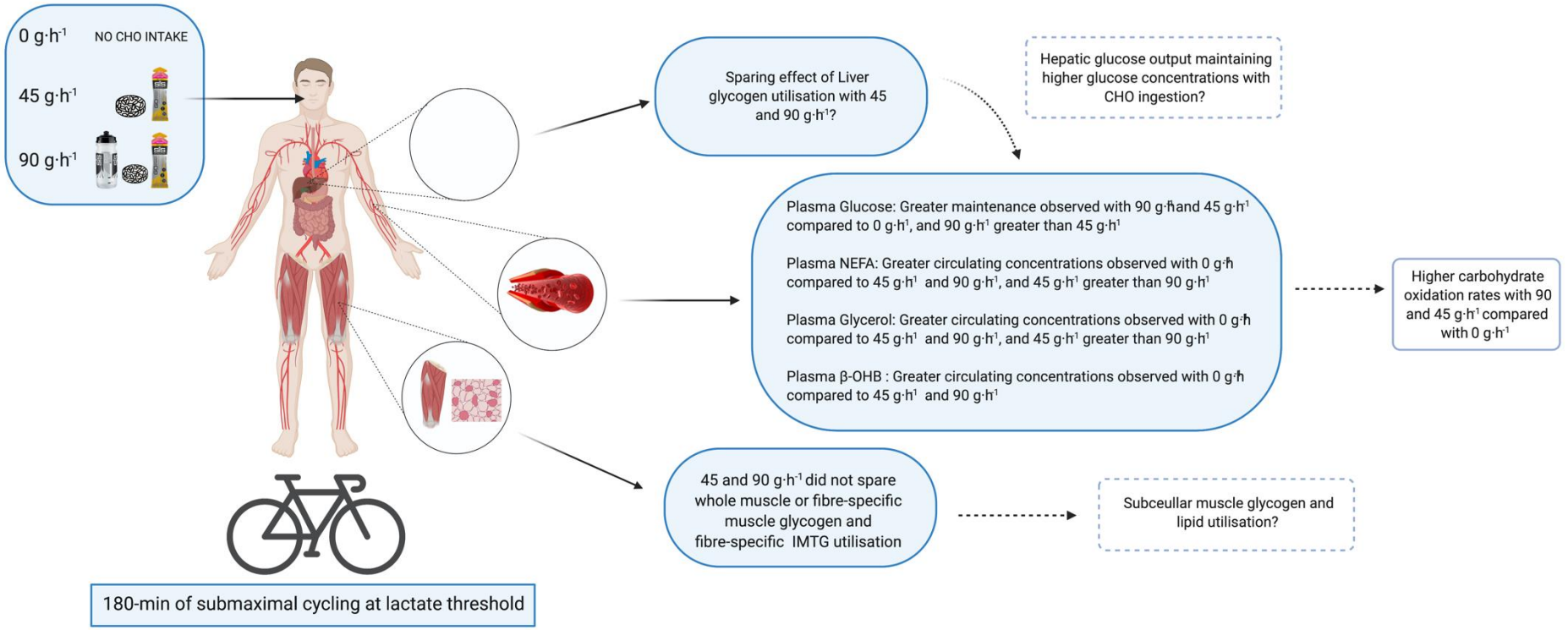


**Figure 9.1** Illustration of the metabolic effects outlined in Study 2 and 3 of 0, 45 and 90 g·h$^{-1}$ of carbohydrate (CHO) feeding during and after 180-min of endurance-based cycling. CHO feeding had no effect upon whole muscle and fibre-specific glycogen utilisation but resulted in a greater maintenance of plasma glucose and higher CHO oxidation rates as a result of a potential liver glycogen sparing mechanism.

### 9.2.3 The application of carbohydrate feeding strategies in real world settings

On the basis of the observed laboratory data reported within Study 2 and 3 (Chapter 5 and 6) coupled with the interpretation from the paper to podium matrix (P-2-P) (Close *et al.*, 2019) critically evaluated in Table 9.1, we felt confident that the findings within this thesis could be readily applied to the elite sporting environment and more specifically, professional road cycling. We felt the application of these findings would be especially relevant given the fine margins between winning and losing within professional road cycling as demonstrated in the most recent edition of the Tour de France (2020) whereby the winning rider was only 59 seconds ahead of 2$^{nd}$ place who in turn was only 3 minutes and 30 seconds ahead of the rider finishing in 3$^{rd}$ position. As such, the direct application of our findings was demonstrated within Study 5 (Chapter 8) whereby it was used to help inform the daily CHO requirements for the GC winner during the 2018 Giro d'Italia. Whilst a small number of previous studies have outlined the energy and macronutrient intake of professional cyclists during Grand Tours (García-Rovés *et al.*, 1998; Muros *et al.*, 2019; Saris *et al.*, 1989), none have done so in combination with outlining the daily periodisation of CHO intake throughout a GT and the subsequent variation in daily EA. In this regard, Study 5 (Chapter 8) therefore demonstrated the first report to the author's knowledge that documented the practical delivery of a periodised daily CHO feeding strategy for a GT winning cyclist.

The periodised CHO strategy was devised in order to adopt a gradual reduction in body mass through intentional under-fuelling on less energetically demanding stages whilst ensuring sufficient CHO availability prior to highly demanding and race defining mountain stages and summit finishes. This was evident in the daily variation in CHO and energy intake which ranged considerably during the 10-day assessment period with values of 3.5-18.9 g·kg$^{-1}$ and 1817-6413 kcal·day$^{-1}$, respectively. In line with this approach, the rider's morning body mass varied throughout the race (Stage 11, 12, 13, 17, 18 and 19 was 69.3, 68.3, 67.8, 68.1, 68.3 and 68.9, respectively).

The lowest CHO intakes were evident on the less energetically demanding stages such as Stage 11 (6.0 g·kg$^{-1}$; Hilly stage), 12 (6.7 g·kg$^{-1}$; Flat stage) and 16 (8.6 g·kg$^{-1}$; Individual time trial) as well as on the rest day (3.5 g·kg$^{-1}$), whilst the highest CHO intakes occurred on Stage 18 (17.3 g·kg$^{-1}$) and 19 (18.9 g·kg$^{-1}$), with Stage 19 corresponding to the most physically demanding stage of the race. Specifically, the rider prepared for Stage 19 after consuming a

total of 11.3 g·kg$^{-1}$ of CHO in an 18-h period followed by the intake of 522 g of CHO during the stage itself which equated to 100 g·h$^{-1}$, in agreement with the dosing strategy considered optimal for performance as reported in Study 2 (Chapter 5) with a schematic representation of the intended feeding strategy provided to the rider in Figure 9.2. The in-race strategy was also comprised of a feeding protocol reflected from Study 2 whereby CHO feeds were aimed to be consumed every 20-min during the stage with more solid based foods (rice-cakes) during the earlier stages of the race followed by CHO gels towards the end of the race with CHO fluid consumed throughout. Importantly, this was the stage when the rider successfully took the overall GC lead of the race by regaining 03 minutes and 23 seconds in the final 80 km. Additionally, Stage 14 also represented a stage winning performance by 6 seconds whereby the rider consumed a high CHO diet (8.4 g·kg$^{-1}$) between finishing Stage 13 and beginning Stage 14. Whilst during the stage, the rider consumed an estimated 59 g·h$^{-1}$ of CHO throughout. The quantity of CHO consumed before and during Stage 14 and the time difference between first and second place (6 seconds) also appeared reflective of what was observed in laboratory data from Chapter 5. Despite the in-depth analysis of nutritional intake, the observations within Chapter 8 are not without their limitations from a translation perspective given the case study is only representative of one athlete in this specific context and may not be truly applicable to different settings and contexts. Nonetheless, it does provide important insights into the management and logistics of nutritional intake within the turbulent sporting arena and provide a platform for which to formulate specific nutritional guidelines for this unique population.

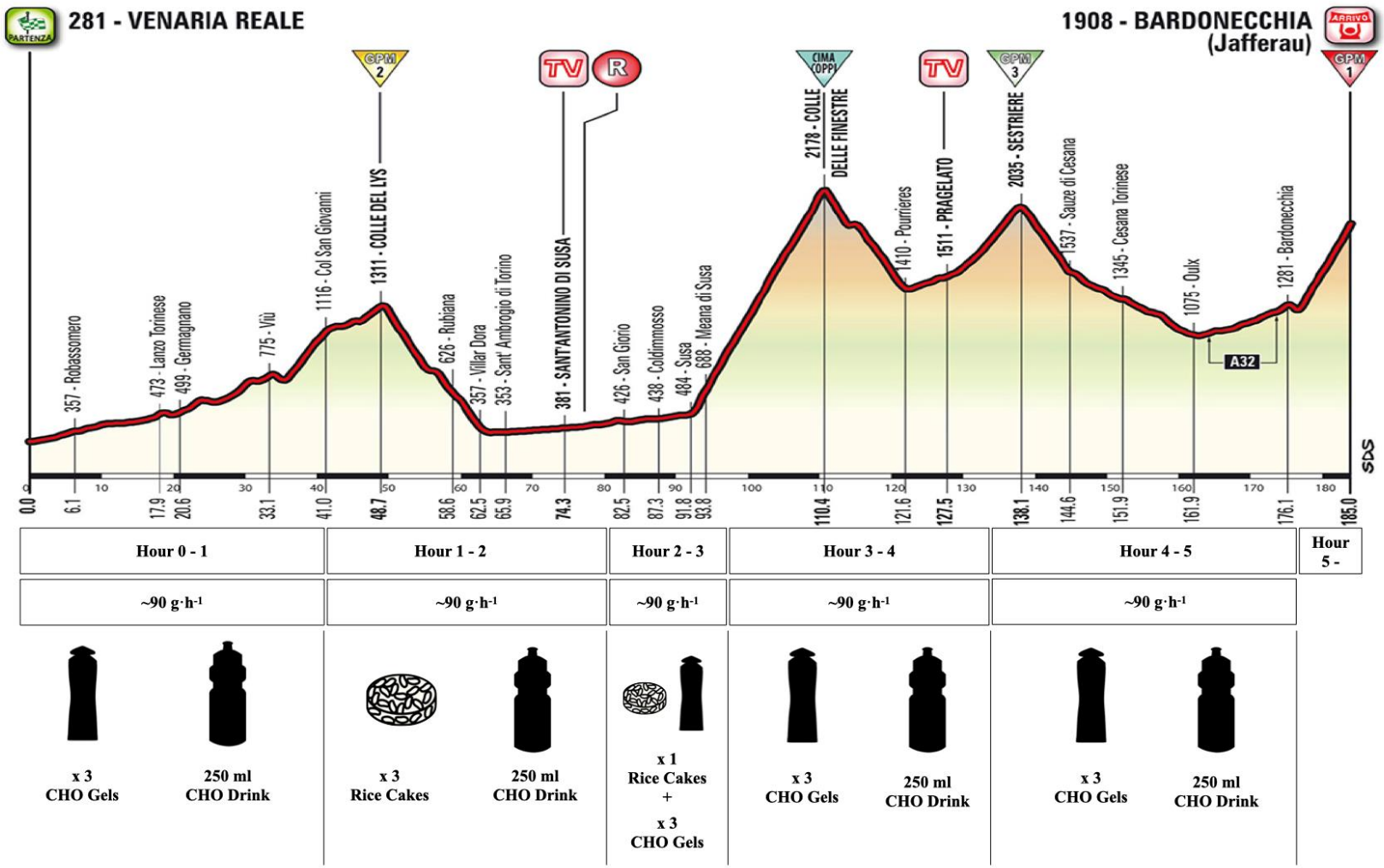


**Figure 9.2** Schematic representation of the in-race carbohydrate feeding strategy for Stage 19 during the 2018 Giro d'Italia that was presented to the rider and comprised of a mixture of carbohydrate (CHO) forms (solids, gels and fluids) at an intended carbohydrate rate of ~90 g·h$^{-1}$, in accordance with the findings of Study 2 and 3. CHO: carbohydrate.

**Table 9.1** Evaluation of the translation potential of the research of Study 2 and 3 combined, to professional road cycling using the "Paper-2-Podium" (P-2-P) matrix (Close, Kasper & Morton, 2019).

| **Criteria** | **Study 2 and 3** |
|---|---|
| | Effects of CHO feeding upon endurance cycling capacity and the associated mechanisms. |
| Research Context | +2<br>Human participants, exercise performance measures and evaluation of mechanisms (applied and mechanistic study) |
| Research Participants | +1<br>Close to appropriate training status for the context required, e.g. trained level participants when wanting to translate to elite athletes (with defined criteria), and in the required age group |
| Research Design | 0<br>Randomised cross-over trial with repeated measures or matched groups design, inclusion of control group but no blinding of intervention. Sample size commensurate with previous research in the area but no sample size calculations provided |
| Dietary and Exercise Controls | +2<br>Dietary provision provided by researchers, exercise control cited, supported by relevant objective data and representative of real-world context |
| Validity and reliability | +2<br>Inclusion of familiarisation trial and citation of reliability data and measurement tool error. Exercise protocol is representative of the relevant exercise modality and includes both laboratory- and field- based protocols that are applicable to real-world context |
| Data Analytics | +2<br>Analytics reported, appropriate significance or MBI tests provided, effect sizes included and presentation of individual responses to treatment intervention if appropriate |

| | |
|---|---|
| Feasibility of Application | +2<br>Cheap to implement, extremely simple and minimal risk of non-compliance |
| Risk / Reward | +2<br>Minimal risk in terms of anti-doping violation and safety data available. Solid evidence of no side effects and optimal dose clear. |
| Timing of Intervention | +2<br>Age-appropriate for the athlete. Time available for dosing is considered optimal to be effective. Time from the major competition is also sufficient to warrant testing the new strategy. |
| Total Score / Interpretation | +15<br>An appropriate study to guide practice |

### 9.3 Experimental limitations

*Protocol design* – As discussed previously, professional endurance road cycling stages or 1-day races typically last from 3–6 h in duration. Although the current protocol utilised within Study 2 and 3 lasted approximately just over 180-min, this is not truly reflective of the longer duration events within professional road cycling and additionally the effects of CHO provision during these substantially longer stages or races are not well characterised within the literature. Additionally, the protocol utilised a prolonged submaximal steady state component set at LT followed by a short high intensity exercise capacity test. The submaximal steady state intensity was devised to be reflective of riders riding conservatively within the peloton at submaximal intensities, whereby mountain stages have been observed to mostly require submaximal, constant power outputs for longer periods (Vogt *et al.*, 2007; Sanders *et al.*, 2018). Nonetheless, there will always be an intermittent element of professional road cycling whereby there will be variations in power output as result of the ever-changing terrain, general parcours of the race and race tactical situation. Therefore, the protocol is short coming in mimicking the true metabolic demands reflective of the intensities of professional cycling. The exercise performance assessment used within the protocol in Study 2 was an endurance capacity test in the nature of TTE at 150% LT in order to replicate an uphill attack or ‘holding a wheel’. The capacity times typically lasted 5-10-min as seen in Chapter 5. This could also be considered a

potential limitation as often within professional road cycling these types of attacks or 'holding a wheel' may extend between 10 – 20-min (Van Erp *et al.*, 2020) and as such the true magnitude of performance gain observed in Chapter 5 may therefore be limited. However, the ability to truly mimic the chaotic nature of professional sport within the laboratory setting is extremely challenging and the controlled, calm and temperature-controlled laboratory environment is never representative of the elite sporting arena. Based upon this, future studies are warranted to examine specific nutritional interventions using exercise protocols and performance assessments that are even more applicable to the chosen sporting arena.

*Participants* – The participants used for the studies in this thesis were defined as trained cyclists in accordance with the criteria outlined by Jeukendrup, Craig and Hawley (2000). Nevertheless, as can be seen in Table 3.1 elite professional cyclists complete substantially greater amounts of weekly training compared with trained cyclists coupled with an extremely impressive physiological capability. Because of this factor, the metabolic elements of subjects may markedly differ in line with the induced adaptations of increased training status thereby altering the effects of CHO feeding on specific metabolic factors. As such, it is unclear whether the metabolic alterations and performance increases in Study 2 and 3 would be of the same magnitude in elite endurance populations given the physiological and metabolic difference that would exist. Future studies should pay particular attention to the inclusion criteria for subject recruitment so as to minimise the effect of training status upon exercise metabolism with better trained subjects in peak training condition having more practical application.

*Dietary interventions* – Due to the nature of Study 2 and 3 incorporating 'real foods', it was not possible to truly blind each experimental trial. Whilst examination of physiological data suggest that the performance improvements were due to maintenance of plasma glucose and rates of CHO oxidation, it is of course possible that improved performance were to the conscious effect of eating more food. Future studies may wish to blind conditions through the use of CHO versus sugar free placebo jelly as opposed to the rice cake feeding strategy adopted here.

*Muscle biopsies and muscle analysis* – The timing of muscle biopsy sampling within Study 2 corresponded to immediately before and after 180-min of exercise. Two time points were chosen to reduce the discomfort associated with repeated biopsy sampling for participants, particularly given the conchotone needle biopsy technique was used which is perhaps

considered somewhat more traumatic than other biopsy methods. These sampling points were perhaps limited in regard to lack of time-dependent insights into substrate utilisation "during" the exercise protocol and if there was any associated sparing during hour 1, 2 or 3. In this way, future studies should aim to assess the time-dependent utilisation of substrate during exercise through sampling of muscle biopsies during exercise.

*Assessment of dietary intake* – Currently there is no gold standard measure for assessing energy intake. Therefore, the assessment of dietary intake within Study 5 is not without its limitations coupled with the challenges of having to record such aspects in the stressful sporting arena. Although the athlete was provided with weighed CHO portions at each main meal along with cross referencing what the athlete consumed using the RFPM method (Martin *et al.*, 2009), the athlete had to recollect from memory what they consumed and drank during each stage immediately. Such inferences may be associated with some level of error with self-reporting and recollection in conjunction with the stress levels of the racing environment potentially effecting such recollections. Additionally, there is also the associated impossibility of measuring the consumed content of bidons passed to the rider during stages.

*Case study* – The data collected within Study 5 was only gathered from Stage 11 – 19 of that Grand Tour and as such is limited in that it does not provide the overall dietary intake of the athlete across all 21 stages. Furthermore, energy availability was estimated from a previous dual energy X-ray absorptiometry (DEXA) measurement of the athlete that was not undertaken in close proximity to the race, so it was unknown what the current fat free mass (FFM) of the athlete was during the quantified stages. Additionally, the exercise EE component of the energy availability equation was quantified from the on-bike power meter which may also have associated error when determining EE from this. These estimations of energy availability will therefore have obvious limitations and future studies should be conducted in order to better understand the complete nutritional intake of cyclists during a full Grand Tour race in combination with more precise estimates of energy availability.

## 9.5 Recommendations for future research

There are several potential areas of future research which have emerged from the data presented in this thesis, namely relating to further understanding the mechanisms associated with the ergogenic effects of CHO provision as well as utilising exercise protocols that closely resemble

real world practice and competition. The continual understanding of the dietary habits of elite professional endurance athletes during multi-day competition with more precise estimations of energy availability during such events also warrant further study. In the following section, the reader is provided with a brief outline of potential studies addressing these issues.

*Recommendation 1* – Based upon the data obtained from Chapter 4, it appears that future studies should continue to develop more reliable and valid exercise protocols examining the repeatability of the associated metabolic and physiological variables whilst further assessing the performance variations related to any novel more specific performance tests. Such protocols should further replicate the demands of professional road cycling competition by consisting of 4 – 6-h in duration and including more intermittent aspects throughout. Figure 9.3 outlines a potential future exercise protocol which may be more applicable, however this proposed experimental design is hypothetical in nature given the vast number of muscle biopsies prescribed which may in turn be considered unethical.

*Recommendation 2* – The results of Study 2 highlighted the dose-response relationship of CHO ingestion during prolonged endurance exercise and performance. However, the study was limited to the investigation of only two CHO doses only, with a maximum dose of 90 $g \cdot h^{-1}$. Future studies should aim to further extrapolate the optimal dose of CHO required to maximise the performance response of such interventions. This could be achieved by replicating the current research design of Study 2 but examining more CHO doses. Multiple CHO doses should also include doses beyond 90 $g \cdot h^{-1}$ to assess any further benefit or detriments to performance and indicating if there is a potential ceiling effect of CHO feeding during prolonged exercise. This is especially apparent given the recent evidence suggesting that CHO intakes of 120 $g \cdot h^{-1}$ during ultra-endurance events can limit metabolic fatigue, exercise induced muscle damage and internal load during a mountain marathon (Viribay *et al.*, 2020). Figure 9.3 proposes a potential hypothetical experimental design to examine the metabolic and performance effects of higher CHO doses.

*Recommendation 3* – The research design employed within Study 2 and 3 should be closely replicated within the field to determine if similar performance gains would be observed. Although certain metabolic and physiological measurements may not be realistic in the field setting (e.g. using a metabolic cart for gas analysis or repeated blood measurements throughout the protocol) the field design could include a similar muscle biopsy and venous blood sample

before and after the set duration as many studies have now performed such measurements in the field setting. The exercise protocol would aim to maintain similar prescribed intensities between trials through the use of on-bike power meters along with physiological feedback through the use of heart rate monitors and subjective information through from RPE scales. The field setting would enable a reflective performance assessment through the incorporation of a specific uphill section or 'climb' lasting ~10 – 20-min and also provide real meaningful performance outcomes. This performance test would have the added benefit of incorporating true effects of outdoor competition, for instance the added gravity aspect of hill climbing which is absent within laboratory performance tests. These types of studies would also perhaps be more familiar for participants as they would regularly undertake cycling on the road as opposed to in the controlled laboratory environment.

*Recommendation 4* – The data obtained from Study 3 (Chapter 6), appear to suggest that fibre-specific muscle glycogen and IMTG utilisation is unaltered with CHO feeding. However, before definitively ascertaining that CHO feeding during exercise does not alter muscle fuel selection, we had intended to study muscle glycogen utilisation in specific subcellular storage pools, as measured using transmission electron microscopy. However, the failure to complete this aim suggests the necessity to complete future work in this area and is especially relevant given recent findings underpinning the importance of intramyofibrillar glycogen within type I fibres for endurance capacity and the supercompensation of subsarcolemmal glycogen from high pre-exercise CHO intake reducing the utilisation of intramyofibrillar glycogen during exercise (Jensen *et al*., 2020). Furthermore, given that the degradation of glycogen and IMTG during exercise is under the control of many key regulating enzymes, the assessment of these enzymes should be considered to provide further insights into the underpinning mechanisms of CHO ingestion. Such enzymes as glycogen phosphorylase would provide useful links to glycogen utilisation whilst HSL, ATGL and carnitine palmitoyl transferase 1 (CPT-1) would provide additional insights into IMTG and lipid utilisation which can be examined through specific western blots analysis. Additionally, important transport proteins like GLUT-4 can provide further insights into glucose metabolism aspects and GLUT-4 translocation could be assessed through immunofluorescence microscopy.

*Recommendation 6* – Previously stated within the limitations section was the lack of muscle biopsy sampling during exercise to assess the time-dependent utilisation of muscle glycogen and IMTG utilisation. Indeed, previous findings by Stellingwerff *et al.* (2007a) reported a

sparing early in exercise with CHO provision when assessed through tracer analysis. Accordingly, future studies should aim to evaluate whole muscle homogenate, fibre-specific and subcellular muscle glycogen and IMTG utilisation during exercise in a time-dependent manner whereby muscle biopsy sampling is conducted at set time points during the exercise bout itself.

*Recommendation 7* – Study 5 highlighted valuable insights into the dietary intake of a multiple Grand Tour winner during a specific Grand Tour which the athlete went on to win. However, given the paucity of dietary intake information detailed within the literature from the elite endurance population during competition, there is a requirement to better understand the nutritional habits of such athletes during competition, specifically during multi-day events like those in professional road cycling. Such information will help to further elucidate how athletes and support teams formulate nutrition strategies to combat the large energy expenditures associated with such races and in turn balance energy availability with fuelling, recovery and body composition. Success in many endurance cycling races is determined by a cyclist's ability to maintain their 'optimal physique' during a race whilst consuming substantial amounts of CHO and energy during the event (Jeukendrup, 2011). Therefore, further insights into how cyclists switch between low and high energy intakes relative to their on-bike EE depending on the desire for fat mass loss and the performance benefits of being well fuelled would be unique. Future studies in this area would also benefit from using DEXA as an accurate indication of FFM, and doubly labelled water as an accurate indication of total energy expenditure across a study period in order to accurately determine energy availability. Further measurements of specific blood markers such as testosterone, IGF-1, and hepcidin should also be incorporated to give an overall picture of energy availability and any acute RED-s syndrome responses.

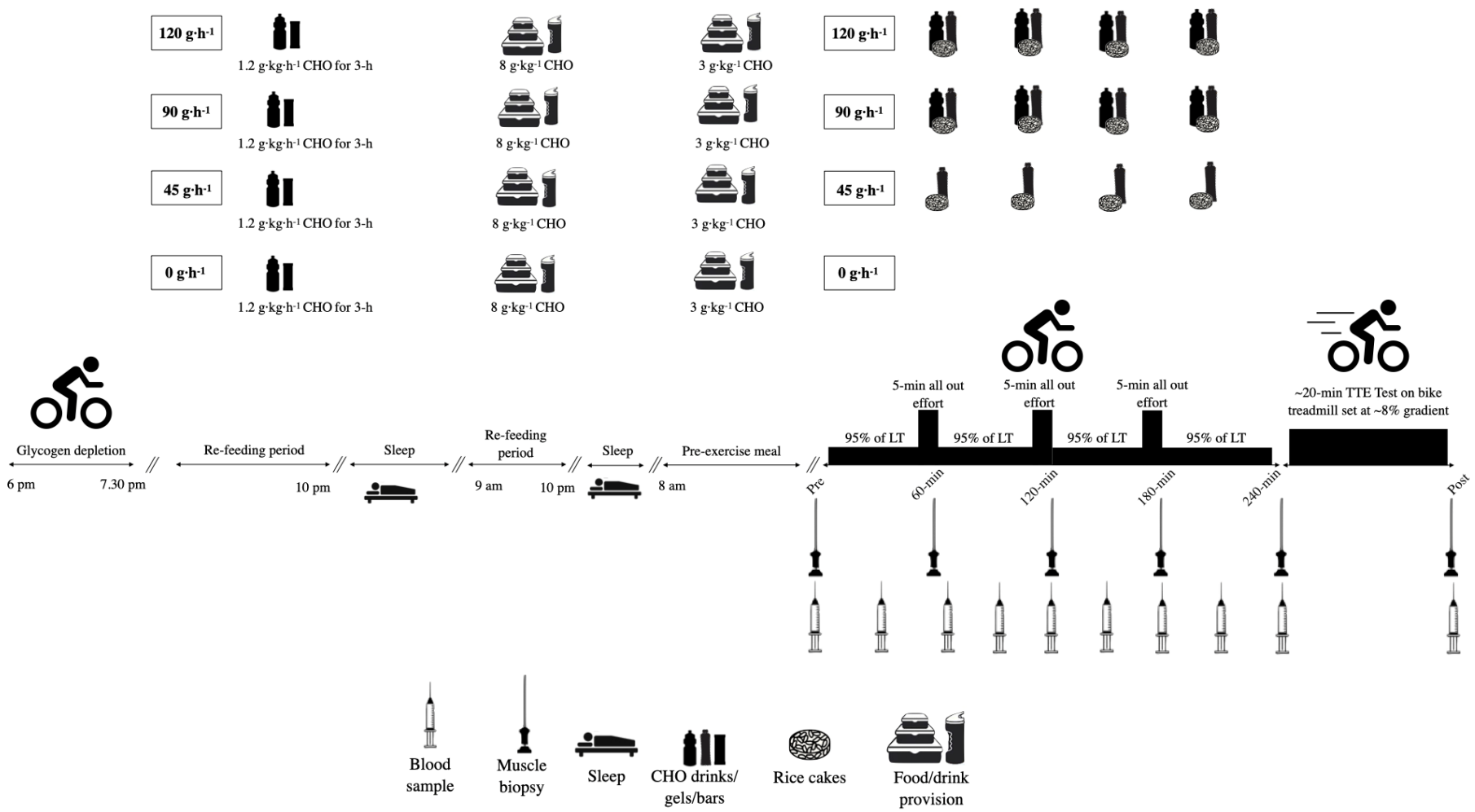


**Figure 9.3** Proposed hypothetical experimental design for future research. The precise intensities and characteristics of the exercise protocol remain to be determined, especially for the time to exhaustion test. It is recommended that additional biopsies obtained during exercise and immediately post time to exhaustion will provide further insights into substrate utilisation during endurance exercise. CHO: carbohydrate; TTE: time to exhaustion.

## 9.6 Closing thoughts

In summary, the data presented within this thesis demonstrate that endurance exercise capacity is increased in a dose-dependent manner when CHO is ingested during prolonged endurance exercise under conditions of best nutritional practice. This enhancement in performance is independent of a muscle glycogen sparing effect (as measured in whole muscle and type I and II fibres) and most likely due to a potential liver glycogen sparing mechanism, a greater maintenance of plasma glucose concentrations and subsequent higher whole-body CHO oxidation rates. When taken together, these data have practical implications by suggesting that during key endurance cycling events or stages, i.e., mountain stages within a specified race, cyclists should aim to maintain high CHO availability through consuming 90 $g \cdot h^{-1}$ of CHO when in the peloton in attempts to increase performance when it comes to key parts of that given stage, i.e., mountain attacks and “holding a wheel”. From a research perspective, it is hoped that the present thesis will stimulate further laboratory and field-based studies that aim to formulate optimal CHO feeding strategies.

# Chapter Ten:

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# Appendix 1 – Ethical approval Study 1-4

Dear John

With reference to your application for Ethical Approval:

**16/SPS/030 - John Fell, PGR - The effects of CHO feeding on fibre type specific and intracellular muscle glycogen utilisation during endurance cycling (James Morton)**

The University Research Ethics Committee (UREC) has considered the above application by proportionate review and I am pleased to inform you that ethical approval has been granted and the study can now commence.

UREC would like to thank the applicant for submitting an excellent application

**Notes:** Please confirm the time period between pre and post biopsies. Ensure the risk assessment matches B5A. Is it 6 or 4?

Please clarify the status of the destruction of sample and deletion from database. G9 contradicts other information.

On the PI sheet please confirm whether the visits are on 5 separate occasions.

Approval is given on the understanding that:

- any adverse reactions/events which take place during the course of the project are reported to the Committee immediately;
- any unforeseen ethical issues arising during the course of the project will be reported to the Committee immediately;
- the LJMU logo is used for all documentation relating to participant recruitment and participation e.g. poster, information sheets, consent forms, questionnaires. The LJMU logo can be accessed at http://www2.ljmu.ac.uk/corporatecommunications/60486.htm

Where any substantive amendments are proposed to the protocol or study procedures further ethical approval must be sought.

Applicants should note that where relevant appropriate gatekeeper / management permission must be obtained prior to the study commencing at the study site concerned.

For details on how to report adverse events or request ethical approval of major amendments please refer to the information provided at http://www2.ljmu.ac.uk/RGSO/93205.htm