CARBOHYDRATE FEEDING AND EXERCISE RECOVERY: EFFECTS ON METABOLISM AND PERFORMANCE

By

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Abstract

The importance of sufficient amounts of carbohydrates for optimal performance during endurance exercise is well established. However, it has been demonstrated that a high carbohydrate availability before, during and after training sessions could suppress molecular signalling pathways thought to be important for subsequent training adaptations and/or hinder training adaptations. In addition to that, evidence is inconclusive when it comes to the role of different monosaccharides during the short-term recovery after exhaustive exercise, i.e., how different monosaccharides affect recovery and metabolism during the subsequent exercise bout. The aim of this thesis was to uncover some of the pertaining questions in these areas.

The aim of the first study was to explore a novel approach to sleeping and training with reduced carbohydrate availability whereby carbohydrates are ingested with a delay during a subsequent morning exercise bout. It was found that delayed carbohydrate feeding during exercise following a sleep low approach to training did not supress high fat oxidation rates typically observed under conditions of low carbohydrate availability. However, performance outcomes of the study did not show any significant differences, but there was a trend towards rescuing some of the performance that was lost as a result of avoiding carbohydrates during the recovery.

The aim of the second study was to compare glucose only or fructose-glucose co-ingestion during the post-exercise recovery period on metabolism and performance during a subsequent exercise bout. Study results showed no performance benefits of fructose-glucose co-ingestion as compared to glucose only. However, the fructose-glucose combination led to higher oxidation rates of during recovery ingested carbohydrates during the subsequent exercise bout, hinting at increased whole-body post-exercise glycogen storage.

The final study's aim was to investigate the efficacy of galactose ingestion alone or in combination with glucose during the recovery after glycogen reducing exercise on muscle glycogen synthesis, and metabolism during a subsequent exercise bout. Glucose was shown to be a superior source for post-exercise muscle glycogen repletion over galactose or a combination of galactose and glucose. Interestingly, galactose ingestion resulted in substantial replenishment of muscle glycogen stores similar to those observed with a moderate glucose intake (galactose-glucose combination) without a rise in glucose and insulin levels suggesting that there might be a mechanism for a direct conversion of galactose into glycogen in the muscle.

Collectively, the findings of the work contained within this thesis have successfully advanced the current knowledge in the area of sports nutrition and opened up new interesting questions that require further investigation.

Acknowledgements

“Tell me and I forget, teach me and I may remember, involve me and I learn.”

— Benjamin Franklin

First and foremost, I would like to thank my supervisor Dr Gareth Wallis. I came to Birmingham not knowing what research actually is, how it is done and what it involves. Now at the end of this PhD journey, thanks to your guidance and supervision, I feel like a researcher myself. I cannot express how grateful I am for this guidance, all the help and especially everything you have taught me.

“Great discoveries and improvements invariably involve the cooperation of many minds.” – Alexander Graham Bell

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“Science is a way of life. Science is a perspective. Science is the process that takes us from confusion to understanding in a manner that's precise, predictive and reliable - a transformation, for those lucky enough to experience it, that is empowering and emotional.” – Brian Greene

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“To raise new questions, new possibilities, to regard old problems from a new angle, requires creative imagination and marks real advance in science.” – Albert Einstein

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“Every addition to true knowledge is an addition to human power.” – Horace Mann

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“Call it a clan, call it a network, call it a tribe, call it a family: Whatever you call it, whoever you are, you need one.” – Jane Howard

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List of Publications and Abstracts published during while undertaking PhD studies

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Maunder E., **Podlogar T.**, Wallis G. A. Postexercise Fructose–Maltodextrin Ingestion Enhances Subsequent Endurance Capacity. Med Sci Sport Exerc. 2018 May;50(5):1039–45.

Conference Abstracts:

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Odell O. J., **Podlogar T.**, Wallis G. A. Accurate estimation of exogenous carbohydrate oxidation during exercise requires a low natural abundance 13C-carbohydrate "background" trial.

ACSM Conference 2020:

Odell O. J., **Podlogar T.**, Wallis G. A. Comparable exogenous carbohydrate oxidation with lactose or sucrose feeding during endurance exercise.

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Podlogar T., Free, B., Wallis G. A. Delayed carbohydrate feeding during endurance exercise maintains the high fat oxidation rates observed with training under conditions of low carbohydrate availability.

ECSS Conference 2019:

Podlogar T., Wallis G. A. Exogenous carbohydrate oxidation rates can be accurately determined after 20 minutes of moderate intensity endurance exercise.

ACSM Annual Meeting 2018:

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List of abbreviations

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ATP	Adenosine 5'-triphosphate
CAC	Citric acid cycle
CACT	Carnitine-acetyltranslocase
CaMKII	Calmodulin-dependent protein kinase II
CAT	Carnitine acetyltransferase
CHO	Carbohydrate
CI	Confidence interval
CPT	Carnitine palmitoyltransferase
CREB	cAMP response element-binding protein
CS	Citrate synthase
DM	Dry mass
FA	Fatty acids
GC	Gastrointestinal comfort
GI	Gastrointestinal
GLUT2	Glucose transporter 2
GLUT5	Glucose transporter 5

GP	Glycogen phosphorylase
GS	Glycogen synhtase
HAD	Hydroxylacyl-CoA
HKII	Mitochondrial hexokinase II
HR	Heart rate
IMTG	Intramuscular triacylglycerols
LCFA	Long chain fatty acids
LPL	Lipoprotein lipase
NADH	Nicotinamide adenine dinucleotide
NEFA	Non-esterified fatty acids
p38MAPK	p38 mitogen-activated protein kinase
PCr	Phosphocreatine
PDH	Pyruvate dehydrogenase
PDK	PDH kinase
PDK4	Pyruvate dehydrogenase kinase 4
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
Pi	Phosphate
PPAR	Peroxisome proliferator-activated receptor
RER	Respiratory exchange ratio
RPE	Rate of perceived exertion
SLGT1	Sodium-dependent glucose transporter 1

SS	Steady state
TT	Time trial
UCP3	Mitochondrial uncoupling protein 3
VLDL-TG	Very-low density lipoproteintriacylglycerols
WM	Wet mass

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1. General introduction

1.1. Thesis outline

This thesis explores the effects of carbohydrate feeding of different compositions during recovery after a glycogen reducing exercise bout on metabolism and/or performance during a subsequent exercise bout. It begins with a General Introduction (Chapter 1) in which an outline of the thesis is provided and is followed by a Literature Review (Chapter 2) in which carbohydrate and fat metabolism in relation to exercise are discussed. The chapter finishes by stating the gaps in the literature that this thesis aims to fill. General methods used in the experimental work of this thesis are described in the Chapter 3. Chapter 4 is the first experimental chapter of the thesis. It explores the effects of a delayed carbohydrate feeding strategy incorporated into a popular way of endurance training in which athletes reduce muscle glycogen stores in the afternoon of the first day and perform the next training session the next morning with low carbohydrate availability (also known as sleep low approach to training). Chapter 5, the second experimental chapter, compares ingestion of two different carbohydrate sources (glucose or combined fructose-glucose) after a glycogen reducing exercise bout on metabolism and performance during a second exercise bout. The third and final experimental chapter (Chapter 6) investigated the effectiveness of galactose or a combination of galactose and glucose on repletion of muscle glycogen content after a glycogen reducing exercise bout as compared to glucose only. In addition to that, the metabolic response to a subsequent exercise

bout was assessed. The experimental chapters are followed by a General Discussion and Conclusion (Chapter 7) in which an overview of the findings of the thesis are provided and placed in the wider context of sports nutrition research and practice.

1.2. Introduction

Athletes undertaking endurance exercise expend large amounts of energy. For instance, cyclists at a renowned 3-week long cycling race, the "Giro d'Italia", expend on average ~30 MJ (7000 kcal) of energy per day (Plasqui *et al.*, 2019). It is well documented that a sufficient and well-designed diet is necessary for optimal race performance and in multiple day events also for optimal recovery (Thomas, Erdman and Burke, 2016). Of particular importance is carbohydrate availability and there is a very strong evidence showing that enhancing carbohydrate availability can improve exercise capacity/performance (Bergström *et al.*, 1967; Burke *et al.*, 2011; Stellingwerff and Cox, 2014). Optimisation of carbohydrate intake becomes even more important when the ability to store carbohydrate is taken into account. Namely, storage capacity for carbohydrates in the form of glycogen is ≤ 13MJ (3000 kcal) (Gonzalez *et al.*, 2016) which clearly demonstrates that athletes need to effectively replenish carbohydrate stores if they wish to perform well during a subsequent exercise bout. However, recent evidence indicates that a chronic high carbohydrate availability might hamper some of the desired effects of exercise such as endurance

training adaptation, likely due to carbohydrate and/or overall energy availability being modulators of training response (Philp, Hargreaves and Baar, 2012; Bartlett, Hawley and Morton, 2015).

As a result of this, an approach termed carbohydrate periodisation has emerged (Jeukendrup, 2017; Impey *et al.*, 2018). Its aim is to plan carbohydrate intake so that carbohydrate availability is sufficient for optimal performance and such that it does not impede training adaptations. In practical terms this means a high carbohydrate availability around races and high intensity training sessions and carefully considered intake of carbohydrates (i.e. reduced in certain scenarios) around lower intensity training sessions.

Energy demands are not only high during strenuous competitions, but it is a common observation from analyses of training programmes of elite athletes that they accumulate very large training volumes and expend large amounts of energy (Enoksen, Tjelta and Tjelta, 2011; Solli, Tønnessen and Sandbakk, 2017). One of the fundamentals of endurance training appears to be large overall training volume (Granata, Jamnick and Bishop, 2018a) and thus nutritional support for athletes need to be such that it allows them to achieve this but at the same time such that it does not negatively affect training adaptations. Suboptimal nutrition can not only lead to poor performance, but can result in severe health consequences also known as overtraining syndrome (Schwellnus *et al.*, 2016). Thus, it is very important to optimise training process so that training itself is as efficient as possible, while other aspects

of training, such as nutrition, are planned so that they promote recovery and/or help with adaptations to training.

1.3. Aims and Objectives

The main goal of this thesis and its experimental chapters (4-6) was to investigate the effects of carbohydrate feeding during the recovery period following a glycogen reducing exercise bout on aspects of recovery metabolism and of metabolism and performance during a subsequent exercise bout. More specifically, the aims of the following experimental chapters are:

Chapter 4: It is well demonstrated that carbohydrate ingestion during exercise improves both exercise capacity and performance. There is also plenty of evidence that training with reduced muscle glycogen stores could positively affect molecular signalling thought to be responsible for enhanced adaptations to training and even lead to performance gains. However high carbohydrate availability has been shown to negatively affect signalling and adaptations (Civitarese *et al.*, 2005; Morton *et al.*, 2009). To-date nobody has considered a delayed carbohydrate feeding as a means to improve performance but to sustain the metabolic environment created by preceding low carbohydrate availability. Thus, Chapter 4 aimed to explore the effects of delayed carbohydrate feeding on metabolism and ability to train at high intensities during exercise under a sleep-low approach to training.

Chapter 5: As will be discussed in detail in the next chapter (Literature review), various combinations of carbohydrate sources (i.e., monosaccharides) have been shown to differently affect short-term (i.e., a few hours) replenishment of body carbohydrate stores (i.e., muscle and liver) after a glycogen reducing exercise bout (Wallis and Wittekind, 2013). In addition to that, recent evidence indicates that combining fructose and glucose translates to improved recovery of exercise capacity as compared to ingestion of glucose only (Maunder, Podlogar and Wallis, 2018), but no one has to-date studied whether recovery of exercise performance is also improved. The aim of Chapter 5 was to compare ingestion of combined ingestion of fructose and glucose (maltodextrin) and glucose (glucose and maltodextrin) only in the post-exercise period on metabolism and performance during the subsequent exercise bout.

Chapter 6: While glucose and fructose have been relatively well investigated in relation to the effectiveness in replenishment of muscle glycogen stores in the post-exercise period, this is not the case for galactose. Thus, the third aim of this thesis was to investigate the efficacy of galactose ingestion alone or in combination with glucose during recovery after glycogen reducing exercise on muscle glycogen synthesis, and metabolism during a subsequent exercise bout.

2. Literature review

2.1. Introduction to endurance exercise

Exercise refers to structured physical activities that people perform in order to stay healthy, get fitter or achieve certain sporting goals (i.e., athletes). Exercise is usually classified into two broad categories: aerobic (endurance), and resistance exercise (Cartee *et al.*, 2016). This doctoral thesis focuses on nutrition and metabolism during endurance exercise (e.g., cycling, marathon running) during which most of the energy is derived by complete oxidation of macronutrients (oxidative phosphorylation) and throughout the thesis, the term endurance exercise will be used to represent sustained work efforts lasting longer than 1 minute (Chamari and Padulo, 2015), as approximately at this exercise duration the predominant source of energy becomes oxidative phosphorylation (Spencer and Gastin, 2001).

2.1.1. Fatigue

There are several different definitions of fatigue, however, this thesis is interested in fatigue related to endurance exercise and therefore the definition will be focused on this. For the purposes of this thesis, the definition from Enoka and Stuart will be adopted: "*fatigue is a general concept intended to denote an acute impairment of performance that includes both an increase in the perceived effort necessary to exert*

a desired force and an eventual inability to produce this force" (Enoka and Stuart, 1992).

Already in the early 20th century a famous exercise physiologist and Nobel Prize winner Archibald Vivian Hill made scientific contributions on the topic of fatigue during exercise by analysing world records in various different sports disciplines (Hill, 1925). Based on the results of this study and his previous work, he concluded that there are different factors causing fatigue and, when it comes to sports disciplines, there is a major difference in the origin of fatigue between short (e.g., 1-min) or long duration (e.g., several hours) exercise bouts. He predicted that fatigue is mainly caused by perturbations in cellular homeostasis during short and intense exercise, while longer efforts are most likely to be limited by fuel availability. Although the underlying causes of fatigue have been extensively investigated over the following decades, the debate regarding the theories on what really causes fatigue during endurance exercise is not yet concluded (Gladden, 2016; Joyner, 2016; Reid, 2016; St Clair Gibson, Swart and Tucker, 2018).

2.1.2. Energy stores

Energy required to sustain prolonged endurance exercise primarily comes from the oxidative metabolism of carbohydrates and fats, as amino acids are used in very small quantities. While it is almost impossible to run out of fat (an average 75-kg

human has >100,000 kcal or >400 MJ of energy stored as fat), carbohydrate availability can become a limiting factor due to its finite storage (<3000 kcal or <13MJ) (Gonzalez *et al.*, 2016).

Carbohydrates in the body are predominantly stored in the form of glycogen in two storage sites – liver and muscle. Approximately ~100-120 g of it is stored in the liver and ~400 g (300-700 g, depending on muscle characteristics) is stored in skeletal muscle in the post-absorptive state in an average 75 kg male, while there are also ~2.5 grams of glucose in a free form in the bloodstream (Wasserman, 2009; Burke, van Loon and Hawley, 2017). With increasing exercise intensity and thus energy requirements, the proportion of energy derived from carbohydrates increases both absolutely and relatively, while absolute fat oxidation rates either remain unchanged or decrease (Romijn *et al.*, 1993; van Loon *et al.*, 2001). In regard to exercise duration, during steady state exercise reliance on carbohydrates decreases over time, while utilization of fats increases (Coggan and Coyle, 1989). It is important to note that there is a large inter-individual variability in substrate utilisation during exercise (Venables, Achten and Jeukendrup, 2005; Fletcher *et al.*, 2017; Dandanell *et al.*, 2018). For example, time of day, composition of the diet, menstrual cycle in females, adiposity and training status can all influence fuel selection during exercise (Friedlander *et al.*, 1997, 1998; van Loon *et al.*, 1999).

2.2. Basics of exercise metabolism

One of the more important aspects of metabolism is the provision of energy that is required to maintain normal functions of the body and to meet requirements of performing body movements. Energy balance is the difference between energy input and energy expenditure and needs to equal zero or changes in body weight will occur. Energy expenditure is usually divided into three subcategories: resting metabolic rate, the thermic effect of feeding and the thermic effect of activity (Poehlman, 1989). During endurance exercise, the latter represents by far the highest contribution to energy expenditure.

2.2.1. Muscle function is dependent on ATP supply

Adenosine 5'-triphosphate (ATP) is a molecule that is crucial for energy turnover, as most of the metabolic reactions rely on energy that becomes available when ATP is hydrolysed to adenosine 5'-diphosphate (ADP) and inorganic phosphate (Pi) (Hara and Kondo, 2015). This reaction is reversible and requires energy that is produced using different metabolic pathways that will be briefly described in the following paragraphs.

ATP stores are very limited and can only supply energy for ~1 second of high intensity exercise, however during exercise ATP is resynthesized using other pathways so that

during exercise its concentration remains relatively stable (Söderlund, Greenhaf and Hultman, 1992). ATP is synthesised from adenosine diphosphate (ADP) and a phosphate (Pi) under the influence of ATP synthase. Energy required for ATP synthesis comes from chemical reactions that are broadly divided into anaerobic and aerobic. As the name implies, for the latter oxygen is required, while the former can function in its absence.

The first energy system that is activated once energy requirements increase is the phosphocreatine (PCr) system. In the breakdown process of PCr, creatine, Pi and ATP are formed. This process can supply energy only for a few seconds of high intensity work (e.g., sprinting) and very quickly provides large quantities of ATP. This chemical reaction is reversible and creatine and Pi can again form PCr with the energy derived from the oxidative phosphorylation (Guimarães-Ferreira, 2014).

Glycolysis is the next pathway in line for energy production, and similar to the PCr system, oxygen is not involved in the chemical reactions. Glycolysis sees glucose converted into pyruvate, while ATP molecules and nicotinamide adenine dinucleotide (NADH) are produced. Pyruvate can subsequently be used in the mitochondrion for the generation of ATP in the citric acid cycle (CAC), where it is further broken down to CO_2 and H_2O or can be converted to lactate (Brooks, 2018). Lactate can then be used for oxidative energy production in the same cell or transferred to other cells/tissues for energy production or glycogen storage (Brooks, 2018). There has been a lot of speculation whether lactate or lactic acid is produced in the process of

glycolysis (Robergs, 2004; Brooks, 2018) and whether it causes production of H^+ that in turn acidifies the environment. Whichever theory holds true, it is a fact that lactate is far from a waste product and that it can be an important energy substrate.

For fatty acids (FA) to be utilized as an energy source, they first need to enter fatty acid ß-oxidation. Before entering the mitochondria and ß-oxidation, FA are converted to acyl-CoA. ß-oxidation is a cyclic reaction in which acyl-CoA is shortened to acetyl-CoA and NADH and flavin adenine dinucleotide ($FADH_2$) are formed (Houten and Wanders, 2010).

Citric acid cycle (CAC), also known as the Krebs cycle, sees acetyl-CoA broken down to H_2O and CO_2 molecules as well as NADH and $FADH_2$ that are subsequently used in the oxidative phosphorylation pathway. Most of the acetyl-CoA comes from ß-oxidation and from glycolysis. As previously mentioned, the end product of glycolysis is pyruvate that is converted to acetyl-CoA by an enzyme pyruvate dehydrogenase (PDH).

During prolonged steady state exercise most of the ATP is produced by oxidative phosphorylation. The latter process is formed by four mitochondrial complexes. In these complexes, NADH and $FADH_2$ produced in the CAC are oxidised with protons and electrons being transported into the mitochondrial intermembrane space, generating potential energy gradient. This proton gradient is then used to generate

ATP from ADP and Pi via ATP synthase. Molecules reduced during oxidative phosphorylation are then used again in aforementioned reactions.

Although described in a linear order, it is important to note that these processes usually occur simultaneously as is depicted in Figure 2.1. The biggest determinants on which energy system provides most energy is dependent on the exercise intensity and duration of the activity. Common classification of efforts as “aerobic” and “anaerobic” is thus erroneous given that energy is always derived from both aerobic (complete oxidation of fats and carbohydrates) as well as anaerobic (PCr system and glycolysis) sources (Chamari and Padulo, 2015).

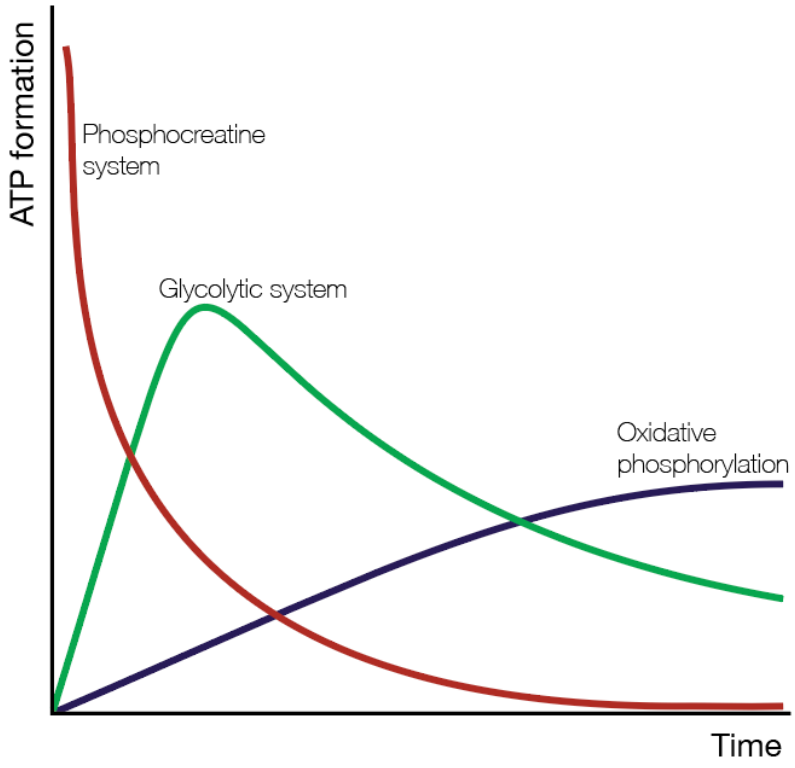

Figure 2.1 Energy continuum.

2.3. Carbohydrates

Monosaccharides (glucose, galactose and fructose) are the simplest form of dietary carbohydrates. Bonded with covalent bonds, monosaccharides form larger molecules. Two monosaccharides form a disaccharide (e.g., lactose, sucrose, maltose). A chain of 3-10 monosaccharides is called oligosaccharide, while carbohydrates with a chain of more than 10 monosaccharides is termed a polysaccharide.

Western diets mostly consist of glucose-based saccharides, while fructose is mainly present in certain fruits and as a part of sucrose and high-fructose corn syrup that are common in certain foods/beverages. Galactose on the other hand is almost exclusively present in milk products (i.e., in the disaccharide lactose, consisting of galactose and glucose).

2.3.1. Carbohydrate digestion

Starch is the most common type of carbohydrate in the human diet. It is a polymeric carbohydrate consisting of glucose units bound together by glycosidic bonds. Starch can be found in two forms differentiated by their structure – branched amylopectin and linear or helical amylose. Before being absorbed into the bloodstream it has to be broken down to glucose molecules. Breakdown of carbohydrates starts in the

mouth where the α-amylase enzyme hydrolyses α-1,4 links. Although the acidic environment of the stomach stops activity of α-amylase, it appears that in the upper parts of the stomach it is still active and that up to 50% of starch is broken down before leaving the stomach (Frayn, 2010). Food (chyme) then enters the small intestine where further digestion occurs, and most of the absorption takes place. The hormone secretin triggers the exocrine pancreas to produce a highly alkaline fluid causing the pH to be neutralized in the small intestine. This again enables α-amylases to further hydrolyse carbohydrates. Not all bonds are broken down by α-amylases but are hydrolysed by other more specific enzymes at the brush border membrane. For example sucrase digests sucrose to glucose and fructose, and lactase decomposes lactose into galactose and glucose (Gray, 1975).

Hydrolysis of polysaccharides could in theory present a limiting factor for the rate of carbohydrate absorption, as more time is required for the breakdown of bonds as compared to when monosaccharides are ingested. However, it appears that glucose polymers (i.e., maltodextrins) and amylopectin starch do not significantly impact digestion, while amylose starch delays digestion (Rehrer *et al.*, 1992; Wagenmakers *et al.*, 1993; Jeukendrup and Jentjens, 2000).

2.3.2. Carbohydrate transport from the small intestine into the portal vein

Carbohydrates are absorbed in the small intestine into the portal vein that subsequently goes to the liver and leaves the liver through a number of hepatic veins. The small intestine has a distinct folded structure which makes the surface area very large. For absorption of monosaccharides to take place, special transporters are required. While glucose and galactose are mainly absorbed using the sodium-dependent glucose transporter 1 (SLGT1) and glucose transporter 2 (GLUT2), fructose uses the glucose transporter 5 (GLUT 5) (Figure 2.2) (Jones, Butler and Brooks, 2011; Daniel and Zietek, 2015).

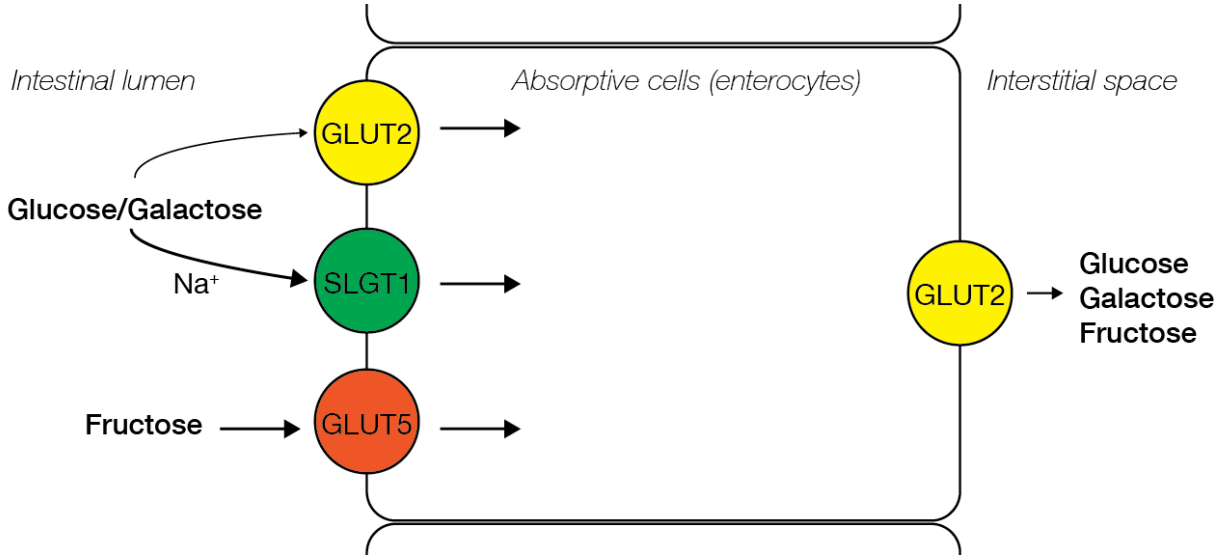

Figure 2.2. Carbohydrate transport. Galactose and glucose are transported into enterocytes via SLGT1 and GLUT 2 transporters, while fructose is transported using GLUT5 transporter. All three sugar types subsequently use GLUT2 to pass into the interstitial space. GLUT2 - glucose transporter 2; SLGT1 - sodium glucose transporter 1, GLUT5 - glucose transporter 5.

2.3.3. Carbohydrate metabolism

Metabolism of glucose, fructose and galactose differ significantly even though the chemical formula is the same for all three of them ($C_6H_{12}O_6$). Of all three monosaccharides, only the structure of glucose enables muscle cells to directly oxidise it (Frayn, 2010). Once in the blood stream, glucose can be up-taken by the majority of cells (e.g., muscle and liver), where it can be used to replenish glycogen stores or be immediately oxidised via the glycolytic pathway. For this to occur, glucose has to be first converted to glucose-6-phosphate by an enzyme hexokinase. Conversely, the majority of fructose enters the liver via a GLUT2 transporter where it is further metabolized by the enzyme hepatic fructokinase (also known as ketohexokinase) (Tappy and Le, 2010). Only a small fraction of fructose stays in the blood and plasma concentrations are usually below 0.5 mmol · L^{-1} (Rosset *et al.*, 2017). Apart from the liver, other cells do not metabolise circulating fructose under normal physiological conditions, as they are lacking fructokinase (Tappy and Le, 2010). Hexokinase, present in muscle cells can metabolize fructose as well (Rikmenspoel and Caputo, 1966). However, in order for this pathway to be active, fructose concentrations have to be very high (e.g., ~5.5 mmol · L^{-1}). This is unlikely to happen under the physiological conditions, unless fructose is infused (Ahlborg and Björkman, 1990). Once in the liver, the majority of fructose is metabolised into the glucose or lactate, which subsequently appear in the blood stream, or fructose is used to replenish liver glycogen stores (Sun and Empie, 2012). A small amount of

fructose can be converted into fat via the process called de novo lipogenesis, however this appears to account for less than 1% of ingested fructose (Chong, Fielding and Frayn, 2007; Sun and Empie, 2012). Knowledge of galactose metabolism is relatively scarce, however the current view is that similarly to fructose, most of galactose metabolism occurs in the liver. Galactose is converted via the Leloir pathway to glucose-6-phosphate which can then be directly oxidised, directed towards glycogen storage or released into the circulation as glucose (Hansen and Gitzelmann, 1975).

2.3.4. Carbohydrate availability limits endurance capacity and performance

The first to test how diet influences exercise capacity were Danish scientists (Christensen and Hansen, 1939a) who showed that when study participants were fed a carbohydrate rich diet as compared to low carbohydrate diet each for 3 days, their exercise capacity at a fixed intensity of 175 W was substantially improved. Unfortunately, at the time it was not possible to determine the cause of this observation and thus scientifically prove or disprove aforementioned Hill's hypothesis that energy availability (i.e., low glycogen stores) is a limiting factor during moderate intensity exercise (Hill, 1925). Hill's proposition was subsequently confirmed in a study investigating the effects of different diets on muscle glycogen content and exercise capacity (Bergström *et al.*, 1967). Bergström and colleagues initially fed study participants a "mixed diet" comprised of carbohydrates, fats and protein.

Exercise capacity was assessed by cycling at ~75% $\dot{V}O_2$max after overnight fast until reaching volitional exhaustion. For the subsequent 3 days, they were fed a diet consisting of fat and protein and on the 4th day again had their muscle glycogen concentration determined and time to exhaustion at 75% $\dot{V}O_2$max assessed. Over the next 3 days they consumed a high carbohydrate diet and had all the study outcomes assessed again. It was found that muscle glycogen concentration and exercise capacity were highly correlated. Exercise capacity was much higher with higher muscle glycogen stores. Also, it was shown that varying the quantities of carbohydrates in the diet affected muscle glycogen stores with a higher amount of carbohydrates in the diet leading to a larger muscle glycogen concentrations and thus higher exercise capacity.

Over the last few decades a substantial amount of evidence accumulated on how carbohydrate availability most likely influences exercise capacity. Coyle and colleagues had participants cycling at a ~70% $\dot{V}O_2$max in two conditions, water ingestion only or glucose ingestion at 2 g · min^{-1} (Coyle *et al.*, 1986). Their data demonstrated that fatigue was preceded by a decline in the carbohydrate oxidation rate, whereas carbohydrate intake during exercise prevented this decline. However, they did not observe sparing of muscle glycogen with glucose feeding but detected maintenance of blood glucose levels when fed carbohydrates. Given that time to exhaustion was 33% longer when fed carbohydrates and that no sparing of muscle glycogen occurred at the same time points (i.e., point of fatigue when only given

water), exogenous provision of glucose enabled subjects to maintain high rates of carbohydrate oxidation and by doing so, prolong time to fatigue.

2.3.5. Glycogen storage

Glycogen is a multibranched polysaccharide of glucose which serves as a reservoir for glucose. It is stored in both skeletal muscle and liver. During fasting, blood glucose concentration is maintained by the release of glucose from the liver via a process called glycogenolysis. On the other hand, in muscle it can directly be used for production of ATP via glycolysis (Jensen and Richter, 2012). Liver glycogen thus plays a crucial role in regulating blood glucose and preventing hypoglycaemia, as it is the only endogenous store of glucose that regulates blood glucose levels. The reason for this is that in the muscle glucose-6-phosphate cannot be converted back to glucose and be transported out of the cell due to lack of enzyme glucose-6-phosphatase (Burke, van Loon and Hawley, 2017). More recently, it has been shown that glycogen plays other important roles as well, such as being a fuel sensor, a regulator of intracellular signalling pathways thought to be responsible for the promotion of exercise training adaptations and a mediator of osmotic characteristics of the muscle cell (Philp, Hargreaves and Baar, 2012).

2.3.5.1. Muscle glycogen

Glycogen distribution in skeletal muscle is compartmentalized and heterogeneous – there are 3 distinct subcellular locations of glycogen, namely: 1) intermyofibrillar glycogen (glycogen particles are located between myofibrils in proximity to I-band, mitochondria and sarcoplasmic reticulum), 2) intramyofibrillar glycogen (glycogen particles located between contractile filaments) and 3) subsarcolemmal glycogen (glycogen particles located just beneath the surface membrane). The largest glycogen reservoir is located in the intermyofibrillar space (Marchand *et al.*, 2002). Over the past few years, the role of each compartment has been further investigated (Kent *et al.*, 2016; Kasper D. Gejl *et al.*, 2017). This is, however, beyond the scope of this literature review.

Due to high energy demands during exercise, muscle glycogen is utilised, while after exercise when carbohydrate availability is high, it is replenished. Muscle glycogen and overall carbohydrate metabolism are mainly regulated by two enzymes. Glycogen phosphorylase (GP) is a prerequisite for muscle glycogen breakdown and the flux through it is regulated by energy demand, whereas PDH subsequently regulates pyruvate entry into mitochondria (Howlett *et al.*, 1998). Muscle glycogen utilization during exercise is mainly determined by exercise intensity (i.e., energy demand – higher the exercise intensity, higher the rate of glycogen utilisation) and its availability. It has been demonstrated that higher muscle glycogen content at the

onset of exercise increases its utilisation, whereas when the glycogen stores are reduced, more fat is utilised (Hargreaves, McConell and Proietto, 1995; Arkinstall *et al.*, 2004).

In addition to being an important energy store, recent evidence indicates that glycogen depletion can also negatively impact muscle contraction (reduced sarcoplasmic Ca^{2+} release) and thus contribute to the onset of fatigue (Ørtenblad, Westerblad and Nielsen, 2013).

In the post-exercise period, muscle glycogen synthesis is mainly regulated by glycogen synthase (GS) which is in turn under the influence of insulin as well as muscle contraction (Nielsen and Richter, 2003). In addition to that, there is a relationship between GS activity and glycogen stores, so that the higher the glycogen storage, the lower the GS activity (Nielsen *et al.*, 2001).

2.3.6. Muscle glucose uptake

Glucose uptake into the muscle is a tightly regulated process influenced by numerous factors (for comprehensive reviews see (Richter and Hargreaves, 2013; Mul *et al.*, 2015)). Crucial for contracting skeletal muscle to obtain glucose is the presence of insulin-sensitive GLUT4 transporter located on the membrane surface (Ryder *et al.*, 1999) and a diffusion gradient, as glucose uptake via GLUT4 transporter relies on

facilitated diffusion. After a meal high in glucose-based carbohydrates, blood glucose concentrations increase and so does insulin enabling muscle tissue to take up glucose. During exercise, however, blood insulin concentration declines, while glucose uptake increases (Sanders *et al.*, 1964; Wahren *et al.*, 1971; Ahlborg and Felig, 1982; Richter and Hargreaves, 2013). Reasons for this apparent contradiction could be twofold and are explained below.

Firstly, muscle contraction itself has an additive effect on glucose uptake in muscle tissue as a result of exercise induced GLUT4 translocation (DeFronzo *et al.*, 1981; Richter and Hargreaves, 2013). Indeed, studies have shown that during exercise, plasma glucose transport into the muscle is mainly augmented by contractile activity (Ploug, Galbo and Richter, 1984; Goodyear *et al.*, 1990; Lund *et al.*, 1995). In the post-absorptive phase the only plasma glucose source is glucose released from the liver (Ahlborg and Felig, 1982). If glucose uptake was only regulated by contractile activity, then liver glycogen depletion would occur rapidly and be followed by fatigue (Ahlborg *et al.*, 1974; Ahlborg and Felig, 1982). To prevent that, glucose uptake by muscle tissue is also dependant on glucose concentration in the bloodstream, with the lower the concentration, the lower the uptake (Ahlborg *et al.*, 1974).

Secondly, as blood flow to exercising muscles increases, so does the insulin delivery, meaning that insulin flux could actually increase or be maintained concomitant with decreased concentrations (Richter and Hargreaves, 2013). It is uncertain how much of an effect this has on the glucose uptake in humans, however increased insulin

delivery in dogs accounts for ~30% increase in glucose uptake (Zinker *et al.*, 1993). Once glucose is in the cell, it is phosphorylated by hexokinase to glucose-6-phosphate which subsequently enters the glycolytic pathway.

It is well documented that GLUT4 content and thus maximal glucose uptake capacity increase as a result of endurance training (Dela *et al.*, 1993; Ebeling *et al.*, 1993). However, exercise at the same absolute intensity before and after training results in decreased reliance on carbohydrates partly because of decreased glucose uptake at least partly as a result of blunted exercise-induced translocation of GLUT4 transporter to the cell membrane (Coggan *et al.*, 1990, 1995; Friedlander *et al.*, 1997; Richter *et al.*, 1998). In line with this, GLUT4 content and glucose uptake are inversely related (McConell, McCoy, *et al.*, 1994) which further supports the notion that GLUT4 content per se is not defining glucose uptake and that intrinsic activity of GLUT4 or its translocation determines glucose uptake. Although this might seem unexpected, at higher intensities increased GLUT4 content could enable higher glucose uptake rates. This has been shown in low muscle glycogen states where reliance on extracellular carbohydrates was increased (Kristiansen *et al.*, 2000). In addition to that, increased GLUT4 content appears to be at least partly linked to faster replenishment of glycogen content post-exercise and higher overall content (McCoy, Proietto and Hargreaves, 1996; Greiwe *et al.*, 1999).

2.4. Lipids

While the terms lipids and fats are terms often used interchangeably, they are not synonyms. Lipids are an umbrella term for molecules soluble in nonpolar solvents whereas fat is a synonym for triacylglycerols (also triglycerides). The human diet is comprised of fats, which can subsequently be converted into other forms of lipids (e.g., FA). Because they hardly dissolve in the water, FA are mostly carried in plasma loosely bound to the plasma protein albumin, while triacylglycerols form complex structures lipoproteins.

The majority of lipids in human bodies are stored in subcutaneous and visceral adipose tissue. In addition to that, muscles store lipids in the form of intramuscular triacylglycerols (IMTG), while some lipids are freely circulating in the bloodstream bound to albumin or as a part of lipoproteins (Frayn, 2010). As a source of fuel during exercise, lipids provide energy in three distinct forms: albumin-bound long-chain fatty acids (LCFA) very-low density lipoprotein-triacylglycerols (VLDL-TG) and IMTG (Kiens, 2006).

The rate of fat utilization during exercise depends on factors such as exercise intensity, time from the last meal, composition of the last meal, daily diet and nutritional intake during exercise nutrition (Montain *et al.*, 1991; McConell, Fabris, *et al.*, 1994; Febbraio and Stewart, 1996). For instance, fat oxidation during exercise is

highly influenced by plasma insulin levels which could be elevated due to a high carbohydrate content in the previous meal ingested in close proximity to the exercise session (McConell, Fabris, *et al.*, 1994). Elevated insulin levels supress lipolysis and consequently fat oxidation (Horowitz *et al.*, 1997). In addition to lipids derived from the blood stream, IMTG could also be utilized during exercise (van Loon, 2004). Under certain conditions, IMTG utilization can in part replace diminished adipose tissue lipolysis (Watt, Holmes, *et al.*, 2004; van Loon, Thomason-Hughes, *et al.*, 2005).

2.5. Proteins

The building blocks of proteins are amino acids. Dietary protein source varies in the quantity and the type of amino acids. The majority of amino acids are water soluble enabling them to move freely in the bloodstream.

Amino acids are oxidised during exercise in a relatively small amount as compared with carbohydrates and fats (Tarnopolsky, 2004). The proportion of energy derived from amino acids has been reported to account for up to 10% of energy expenditure in conditions of low muscle glycogen availability (Lemon and Mullin, 1980). The contribution to the energy expenditure is mostly dependent on carbohydrate availability as protein degradation and amino acid oxidation rates increase when carbohydrate availability is limited (Lemon and Mullin, 1980; Wagenmakers *et al.*, 1991). Although common equations for calculation of substrates oxidation rates

during exercise allow the determination of amino acid oxidation (Jeukendrup and Wallis, 2005), most studies assume protein oxidation to be negligible, because in order to quantify oxidation rates, nitrogen excretion rates need to be known. While in the field of sports nutrition carbohydrate and fats are usually discussed when it comes to energy, protein is more commonly regarded as an important factor for recovery and training adaptations (Close *et al.*, 2016; Knuiman *et al.*, 2018).

2.6. Regulation of fatty acid and carbohydrate metabolism

As previously alluded to, one of the most important determinants of the balance between fat and carbohydrate oxidation during exercise is exercise intensity. Maximal fat oxidation rate occurs at the intensity corresponding to approximately 50% $\dot{V}O_2$max, and can vary between individuals from as low as 22% and all the way up to 89% $\dot{V}O_2$max (Randell *et al.*, 2017). As the exercise passes this intensity, proportion of energy derived from fat oxidation declines both relatively and absolutely, while carbohydrate oxidation rates increase (van Loon *et al.*, 2001).

To better understand the relationship between carbohydrate and FA metabolism it is important to understand the metabolism of each. As previously mentioned, fat and carbohydrate metabolism share the same final pathway; CAC. Acetyl-CoA used in the CAC can come both from either glycolysis (carbohydrates) or β-oxidation (fats). As CAC has a limited capacity, a mechanism needs to exist that regulates which

source of acetyl-CoA is being used in CAC. Given that absolute rates of fat oxidation decline as an exercise intensity increases, there has to be a mechanism causing a reduction in fat oxidation rates.

One of the possible mechanisms behind a reduction of FA oxidation with increasing exercise intensity could be a reduction in FA delivery to the skeletal muscle due to reduced lipolysis caused by a reduction in adipose tissue blood flow. It is indeed a common finding that NEFA concentrations decrease as an exercise intensity increases (van Loon *et al.*, 2001). However, it is important to understand that since muscle blood flow increases, reduced NEFA concentrations do not necessarily mean a reduced delivery of NEFA to skeletal muscle. A study that measured concentrations of LCFA in muscles actually observed increases of LCFA within the cells at higher exercise intensities, indicating that reduced lipolysis does not completely explain suppressed fat oxidation at higher exercise intensities (Kiens, Roemen and van der Vusse, 1999). In line with this, another study investigated what happens when NEFA levels are increased during exercise at higher intensities and has found that fat oxidation increases, however it is still lower than at lower intensities (Romijn *et al.*, 1995).

Skeletal muscle FA uptake is a tightly regulated process and involves different steps that are well controlled: transmembrane, cytosolic, mitochondrial membrane transport and intramitochondrial FA oxidation (Lundsgaard, Fritzen and Kiens, 2017). An overview can be seen in Figure 2.3.

Uptake of FAs across cell membranes of skeletal muscles is mainly dependant on the CD36 binding protein that does not appear to be a limiting step in fat oxidation (Bezaire *et al.*, 2006; Lundsgaard, Fritzen and Kiens, 2017).

Before being oxidised, FAs need to enter the mitochondria, where ß-oxidation, a final step in muscle FA oxidation takes place. Entry of FAs into mitochondria is dependent on mitochondrial membrane protein carnitine palmitoyltransferase (CPT) (Bonnefont *et al.*, 2004). Before entering, FAs are first converted to fatty acyl-CoAs under the influence of long chain fatty acyl-CoA ligase (or synthetase). Under the influence of CPT1, acyl-CoAs are bound with carnitine to form fatty acylcarnitine which can then be first transferred across the outer mitochondrial membrane and subsequently across the inner membrane via carnitine-acetyltranslocase (CACT) (Pande, 1975; Lundsgaard, Fritzen and Kiens, 2017). For this step to be operational, carnitine needs to be available in sufficient quantities and its availability could be a limiting factor (Bonnefont *et al.*, 2004). Once acylcarnitine is transferred across both membranes, CPT2 converts acylcarnitine back to fatty acyl-CoA and free carnitine. That mitochondrial fat translocation is likely a limiting step was nicely demonstrated by Coyle and colleagues in a study in which oxidation of long chain FAs (dependent on CPT1 transport) and medium chain FAs (not dependant on CPT1 transport) was investigated. In conditions of high carbohydrate availability, oxidation of long chain FAs was suppressed, whereas this did not happen for medium chain FAs (Coyle *et al.*, 1997).

Carnitine plays a role within a mitochondria as well, where it buffers excess acetyl-CoA to form acetylcarnitine and free CoA under the influence of carnitine acetyltransferase (CAT) (Friedman and Fraenkel, 1955). This reaction ensures that CoA availability is maintained for normal operation of PDH (Constantin-Teodosiu *et al.*, 1991). Increased acetyl-CoA coming from glycolysis could overwhelm mitochondrial capacity to metabolise it, which could result in accumulation of acetylcarnitine and consequently in reduction of the availability of free carnitine and suppression of FA transport into mitochondria (Lundsgaard, Fritzen and Kiens, 2017). Decreased free carnitine and increased acetylcarnitine levels have been observed together with increased exercise intensity and decreased fat oxidation (Sahlin, 1990; van Loon *et al.*, 2001). Inversely, increasing total carnitine content in the muscle increases fat oxidation and decreases PDH activity at moderate intensity and increases PDH activity at higher intensities while reducing lactate accumulation (Wall *et al.*, 2011).

A key enzyme in aerobic carbohydrate metabolism is PDH complex that orchestrates pyruvate transformation into acetyl-CoA (Harris *et al.*, 2002). PDH is therefore a key for oxidative metabolism of carbohydrates, as pyruvate needs to be decarboxylated to acetyl-CoA to be used in the mitochondria in the CAC cycle (Pilegaard and Darrell Neufer, 2004). PDH exists in two forms, active PDHa and inactive PDHb, with PDH kinase (PDK) transforming PDHa to PDHb, while PDH phosphatase is responsible for the reverse reaction (Spriet and Heigenhauser, 2002).

There are numerous mechanisms that regulate PDH activity, of which exercise intensity and duration play an important part. Increasing exercise intensity increases its activity (as a result of Ca^{2+} release and later increased ADP:ATP ratio), while increasing exercise duration decreases its activity (increased PDK activity) (Howlett *et al.*, 1998; Harris *et al.*, 2002; Spriet and Heigenhauser, 2002; Watt *et al.*, 2002; Watt, Heigenhauser, *et al.*, 2004). In turn, PDK activity is reduced by increased levels of glycolysis and a high production rates of pyruvate and is activated by a high ratio of acetyl-CoA:CoA ratio as well as $NADH:H^{+}$, as happens when the respiratory chain is working at full capacity (Pilegaard and Darrell Neufer, 2004). When pyruvate production exceeds its conversion into acetyl-CoA, lactate is produced (Robergs, 2004; Brooks, 2018).

Once the rate of glycolysis is increased, as happens during higher intensity exercise, acetyl-CoA production is increased. Given that acetyl-CoA deactivates PDH (Holness and Sugden, 2003), formation of acetylcarnitine enables normal functioning of glycolysis and thus ATP production (Lundsgaard, Fritzen and Kiens, 2017). On the other hand, during lower intensity exercise, reduced glycolytic activity would mean increased availability of carnitine and thus increased FA transport into mitochondrial and subsequently its oxidation. Collectively, it appears that with increasing exercise intensity, carbohydrate oxidation rates increase and in turn downregulate FA oxidation rates.

In the long term, dietary carbohydrate/fat content play an important role in PDH activity during exercise, as a low carbohydrate diet reduces its activity (Putman *et al.*, 1993) and this reduction persists even after loading with carbohydrates (Stellingwerff *et al.*, 2006). Together with an upregulation of certain enzymes (e.g., CPT) this likely explains higher fat oxidation rates after an exposure to carbohydrate restricted diet (Yeo *et al.*, 2011). However, although higher fat oxidation rates might be desired in order to spare glycogen stores, reduction in maximal PDH activity could in turn result in reduced power production (LeBlanc *et al.*, 2004).

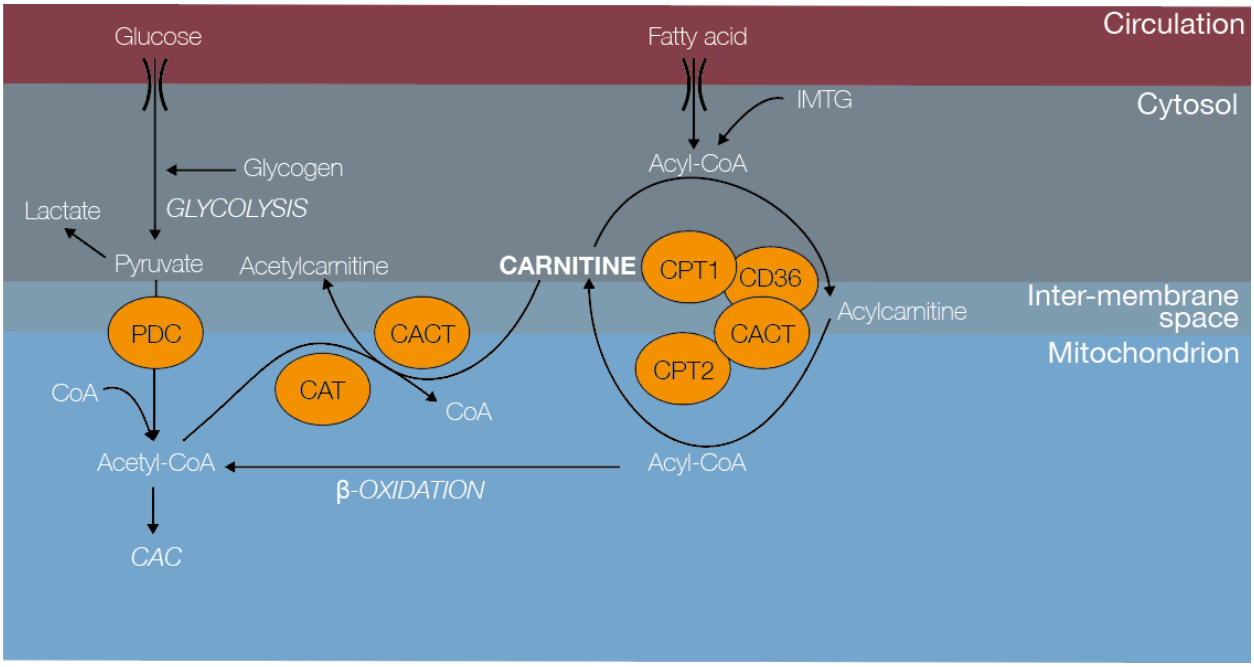

Figure 2.3. Regulation of fat and carbohydrate metabolism showing importance of carnitine. Adapted from (Stephens, Constantin-Teodosiu and Greenhaff, 2007) PDC - pyruvate dehydrogenase complex, CPT - carnitine palmitoyltransferase, CAT - carnitine acetyltransferase, CACT - carnitine-acylcarnitine translocase, CAC - citric acid cycle

2.7. Enhancing post-exercise glycogen synthesis by nutritional manipulations

As it appears that glycogen availability is an important limiting factor for exercise capacity and performance, repletion of glycogen stores post-exercise is an important feature of the recovery period. In line with this, studies have shown that increasing carbohydrate content of the diet in the post-exercise period positively influences subsequent endurance exercise capacity (Fallowfield and Williams, 1993; Fallowfield, Williams and Singh, 1995).

Glycogen synthesis is regulated by enzyme GS (Jensen and Richter, 2012; Roach *et al.*, 2012). Synthesis of muscle glycogen is mainly triggered by muscle glycogen utilization and the greater the utilization, the greater its subsequent synthesis (Zachwieja *et al.*, 1991). Synthesis is initiated even in the absence of dietary CHO provision, as shown in a study where participants depleted muscle glycogen stores and received no food for the next 12 hrs. Even under these conditions, muscle glycogen stores were increased at a rate 1-2 mmol · kg^{-1} wet muscle mass (WM) per hour (4-9 mmol · kg^{-1} dry muscle weight (DM) per hour), most likely via the process of gluconeogenesis taking place in the liver (Maehlum and Hermansen, 1978). However, rates of synthesis can be much higher when optimal carbohydrate intake is provided and can reach up to 5-10 mmol · kg^{-1} WM · h^{-1} (22-44 mmol · kg^{-1} DM · h^{-1}) (Burke,

van Loon and Hawley, 2017). In order to fully replenish muscle glycogen stores, 20-24 hrs are usually needed (Coyle, 1991).

It is common for athletes to train or compete multiple times a day and thus have a limited time between each exercise bout (i.e., less than 20 hrs). In order to be able to train/compete at the highest intensity and highest efficacy in the subsequent exercise bout, they have to start replenishing muscle glycogen stores as quickly as possible and as efficiently as possible. Over the past few decades methods for elevating muscle glycogen stores after exercise, with the highest efficiency possible, have been extensively investigated (reviewed in (Burke, van Loon and Hawley, 2017)).

2.7.1. Quantity

Probably the most important determinant of glycogen restoration is the quantity of carbohydrates provided following an exercise bout. Numerous studies have investigated this topic by providing varying amounts of carbohydrates during the recovery. It has been found that providing carbohydrates at a rate of 1.2 $g \cdot kg^{-1} \cdot h^{-1}$ yields the highest rates of muscle glycogen synthesis, as lower doses were not as efficient, while larger doses did not provide any further benefit (Blom *et al.*, 1987; Ivy, Lee, *et al.*, 1988; Howarth *et al.*, 2009).

2.7.2. Protein co-ingestion

As amino acids are also insulinogenic, it has been speculated that addition of protein could augment synthesis of muscle glycogen. In a number of studies it has been shown that protein could help with muscle glycogen synthesis but only when carbohydrate dose is suboptimal (i.e., <1.2 $g \cdot kg^{-1} \cdot h^{-1}$) (Burke *et al.*, 1995; van Hall, Shirreffs and Calbet, 2000; van Loon *et al.*, 2000; Jentjens *et al.*, 2001)

Furthermore, addition of proteins in a post-exercise meal increases protein synthesis as well as enhances activation of certain molecular signalling pathways (Breen *et al.*, 2011; Cogan *et al.*, 2018) which could, at least in the long term, aid to improvements in body composition, recovery and/or training adaptations (Knuiman *et al.*, 2018). Therefore, a combination of proteins and carbohydrates at a combined dose of 1.2 $g \cdot kg^{-1} \cdot h^{-1}$ (i.e., 0.8 $g \cdot kg^{-1} \cdot h^{-1}$ of carbohydrates and 0.4 $g \cdot kg^{-1} \cdot h^{-1}$ of protein) could be recommended (Alghannam, Gonzalez and Betts, 2018). Recently, it has been shown that immediate post-exercise protein supplementation aids subsequent exercise performance independent of caloric intake (Sollie *et al.*, 2018). Since no additional muscle glycogen synthesis is expected from additional protein, it appears that protein has, at least in trained individuals, other short-term important roles in recovery.

2.7.3. Timing

Following an exhaustive exercise bout that results in a significant decrease in muscle glycogen content, there are two distinct phases of glycogen repletion, a rapid and a slow phase. The initial rapid phase lasting for 30-60 minutes appears to be mainly triggered by low muscle glycogen concentrations and high synthesis rates are achieved by an increased uptake of glucose as a result of upregulation of GLUT4 transporters on plasma membrane as well as increased glycogen synthase activity. The subsequent slow phase that could last for more than 48 hrs is a result of increased insulin sensitivity that is thought to be regulated via various factors, such as AMP-activated protein kinase (AMPK), insulin signalling molecules, muscle glycogen concertation and serum factors (Jentjens and Jeukendrup, 2003).

The timing of ingestion appears to be important up to a certain point. It has been shown that by delaying ingestion of carbohydrates by 2 hrs, muscle glycogen repletion in the first few hours (2 and 4 hrs) is lower as compared to ingesting carbohydrates immediately after exercise (Ivy, Katz, *et al.*, 1988). However, when researchers investigated the effects of 2 hrs delayed feeding by taking muscle biopsies 8 and 24 hrs post-exercise, there was no difference in muscle glycogen content (Parkin *et al.*, 1997).

Collectively, it appears that the timing of ingestion of carbohydrates does not have a major effect on the rates of glycogen synthesis. However, when there is a limited time available between each exercise bout, it is indeed advised to consume carbohydrates as soon as possible to achieve the highest glycogen concentrations before the onset of the subsequent exercise bout.

2.7.4. Combined ingestion of glucose and fructose or glucose and galactose

Currently widely adopted guidelines for post-exercise recovery advise athletes to consume carbohydrate-rich foods with a moderate-to-high glycaemic index in the first few hours after an exercise bout when limited time is available between two exercise sessions (Burke *et al.*, 2011). However, such recommendations might be too simplistic given the evidence that different monosaccharides can differently affect glycogen storage as described below.

As discussed earlier, monosaccharides differ in metabolism, their glycaemic index and this could collectively potentially lead into differences in glycogen repletion rates. Nilson and Hultman were the first to compare the effects of glucose and fructose on replenishment of muscle and liver glycogen (Nilsoon and Hultman, 1974). They infused either of the monosaccharides to healthy individuals after an overnight fast with unrestricted diet the day before and showed that fructose infusion results in a higher rate of liver glycogen formation and no differences in muscle glycogen content

as compared to infusion of glucose (Nilsoon and Hultman, 1974). While liver glycogen concentrations were likely reduced after an overnight fast, this was not the case for muscle glycogen given a lack of exercise preceding the experiment and this likely influenced the results. The first study to assess effects of ingestion of different monosaccharides on replenishment of muscle glycogen immediately after exercise was a study Norwegian scientists (Blom *et al.*, 1987). Blom and colleagues found that when a carbohydrate dose of 0.7 $g \cdot kg^{-1}$ was given immediately following an exercise bout, 2 hrs and 4 hrs later, there was no difference in the rate of muscle glycogen replenishment between sucrose (i.e., glucose and fructose) and glucose, whereas fructose alone resulted in ~50% lower rates of muscle glycogen replenishment.

Subsequently, it was found that ingestion of glucose or glucose and fructose in 2:1 ratio at a rate of 90 $g \cdot h^{-1}$ does not differentially affect muscle glycogen storage over 4 hrs of recovery as compared to glucose only (Wallis *et al.*, 2008). Unfortunately, neither Blom and colleagues nor Wallis and colleagues measured liver glycogen concentrations or a subsequent exercise performance or capacity.

The first study to investigate the effects of different sugar types on liver glycogen was the study by Casey and colleagues. They investigated effects of a single bolus consisting of 1 $g \cdot kg^{-1} \cdot h^{-1}$ of glucose or sucrose or a placebo on exercise capacity after a 4 hrs recovery at 70% $\dot{V}O_2max$ and assessed muscle and liver glycogen content (Casey *et al.*, 2000). They found no significant differences in exercise capacity between all three conditions. However, there was a trend towards carbohydrate

supplementation increasing time to exhaustion. Neither were there any differences in muscle or liver glycogen content among three experimental conditions. However, given how small amount of carbohydrates was given, results are not surprising.

Another study has found that a combination of glucose and fructose or glucose and galactose at an ingestion rate of ~70 $g \cdot h^{-1}$ results in a higher restoration of liver glycogen during 6.5 hrs recovery period as compared to glucose alone (Décombaz *et al.*, 2011). This indicates that addition of fructose and galactose is beneficial for liver glycogen replenishment.

Hypothesising that Wallis and colleagues could have increased the dose of carbohydrates as it was below *optimal,* a Dutch research group conducted another study in which they provided participants 1.2 g of glucose and 0.3 g of fructose or 1.5 g of glucose or 0.9 g glucose and 0.6 g sucrose per kg of body weight per hour. Again, it was shown that co-ingestion of fructose did not accelerate post-exercise muscle glycogen content. However, they did find a combination to be beneficial for those suffering from gastrointestinal (GI) issues as ingesting multiple transportable carbohydrates decreases GI issues (Trommelen *et al.*, 2016).

Recently it was further investigated whether there are any beneficial effects of fructose and glucose co-ingestion on liver glycogen replenishment. Following a glycogen depleting exercise, participants were provided with 1.5 $g \cdot kg^{-1} \cdot h^{-1}$ hour of sucrose or glucose. After 5 hrs, muscle and liver glycogen contents were measured.

This study's data confirmed previous findings that there are no differences in muscle glycogen content, however, liver glycogen content increased to a greater degree when sucrose was ingested (Fuchs *et al.*, 2016).

Based on the findings of improved storage when fructose and glucose were co-ingested, Maunder and colleagues tested whether co-ingestion of fructose-glucose as compared to glucose alone at a rate of 90 g per hour during 4 hrs recovery after exhaustive exercise translates into improved running exercise capacity. They found a 33% improvement in running time to exhaustion when fructose was co-ingested with glucose (Maunder, Podlogar and Wallis, 2018). The results of this study were the foundation for the study described in Chapter 5, which aimed to investigate whether improvements could also be observed when performance rather than exercise capacity was assessed.

When fructose is given in isolation and without glucose, positive effects are not apparent and actually exercise capacity is impaired (Rosset *et al.*, 2017). In this study participants exercised to reduce muscle glycogen stores after which they were provided with a mixed diet in which most of carbohydrates were either in the form of glucose (5.0 g · kg^{-1}) or fructose (4.4 g · kg^{-1} of fructose and 0.6 g · kg^{-1} of glucose) for 24 hrs. There was a similar repletion of muscle glycogen stores for fructose and glucose conditions, although plasma glucose and insulin levels were higher when participants were fed with glucose. Researchers observed an increased postprandial energy expenditure and net carbohydrate oxidation in fructose condition. This

indicates that carbohydrate storage was less efficient with fructose and given that muscle glycogen concentrations have recovered to the similar degree, repletion of liver glycogen is likely the storage site where fructose was less efficient. This study also assessed exercise metabolism during the subsequent exercise bout. Participants were asked to cycle for 3 hrs at exercise corresponding to 50% maximal power output or until exhaustion. Many participants in the fructose condition could not finish this exercise bout. At the time of the exhaustion, blood plasma glucose levels were below 4 mmol · L^{-1} indicating liver glycogen stores depletion and somehow confirming the hypothesis that liver glycogen levels were lower in fructose condition. Collectively, this study challenges previous results showing that fructose infusion results in an increased liver glycogen content (Nilsoon and Hultman, 1974). However, the nature of both experiments (i.e., infusion vs. ingestion and preceding exercise) could explain the divergent findings.

While ingestion of fructose and glucose or a combination of the two was relatively extensively investigated, at least when it comes to metabolism, galactose on the other hand has not been. Co-ingestion of fructose and glucose appears to be beneficial not only from the perspective that such an approach decreases GI discomfort but could also improve subsequent exercise capacity. On the other hand, there is no data available on how effective galactose alone or in combination with glucose is on replenishment of muscle glycogen stores. Chapter 6 tries to fill this gap by investigating the effectiveness of galactose or a combination of galactose and glucose on replenishment of muscle glycogen in the post-exercise period.

2.7.5. Effects of exercise mode

Eccentric exercise as opposed to concentric results in higher rates of muscle injury (Proske and Morgan, 2001). It has been shown that damage induced by a heavy eccentric exercise negatively impacts rates of muscle glycogen synthesis, as more than 10 days could be needed to replenish muscle glycogen stores in both type I and type II muscle fibres (O'Reilly *et al.*, 1987). Another study tried to differentiate the effects of concentric and eccentric exercise on muscle storage and has found that only eccentric exercise results in impaired glycogen synthesis rates (Costill *et al.*, 1990). However, it appears that impaired glycogen synthesis rates are only observed after more than 6 hrs of recovery as shown in a study assessing muscle glycogen content 6, 24, 48 and 72 hrs into recovery while receiving 7 $g \cdot kg^{-1} \cdot day^{-1}$ (Widrick *et al.*, 1992). However, another study has found that glycogen replenishment is reduced only after 48 hrs post-exercise (Doyle, Sherman and Strauss, 1993).

Mechanisms that could underlie these observations are still to be completely understood, however some report that they are associated with a decreased concentrations of GLUT4 protein (Asp, Daugaard and Richter, 1995), while a later study did not support this theory (Asp, Rohde and Richter, 1997). Another proposed explanation was a reduced glucose uptake as a result of insulin resistance (Asp *et al.*, 1996).

2.8. Carbohydrate supplementation during exercise

Beneficial effects of carbohydrate ingestion during exercise have been observed already in the 1930s (Christensen and Hansen, 1939b). They had participants cycling at a fixed intensity of 175 W, while measuring substrate oxidation and blood glucose levels. At the point of fatigue participants were hypoglycaemic, indicating that blood glucose availability could have been limiting their exercise capacity. At the point of fatigue, participants were given 200 g of glucose and after 15 minutes signs of hypoglycaemia disappeared (blood glucose rose as well) and participants were able to continue exercising.

Over the recent decades evidence has accumulated and is clearly showing beneficial effects of carbohydrate supplementation on endurance capacity and performance (Stellingwerff and Cox, 2014). Current nutritional guidelines endorsed by American College of Sports Medicine (Thomas, Erdman and Burke, 2016) recommend athletes to consume carbohydrates during exercise sessions that are longer than 45-min.

There are two proposed mechanisms by which carbohydrate supplementation during exercise could help athletes to perform better. Firstly, by stimulation of central nervous system and secondly by sparing endogenous glycogen stores and maintaining glycaemia.

2.8.1. Carbohydrate intake in shorter events (<1h)

Athletes commencing an exercise bout with non-depleted (i.e., full) muscle and liver glycogen stores are unlikely to deplete glycogen stores in events lasting less than 1 hour (Areta and Hopkins, 2018). However, a systematic literature review (Stellingwerff and Cox, 2014) has shown that most studies show that carbohydrate supplementation improves performance/capacity in events shorter of 1 hour as well. Seminal work in this area showed that mouth rinsing with a carbohydrate solution had a positive effect on 1-h time trial cycling performance (Carter, Jeukendrup and Jones, 2004). The authors hypothesised that this had to be unrelated to effects on metabolism since no carbohydrates were ingested and proposed that performance improvement might be due to stimulation of certain receptors in the oral cavity that could have a downstream impact on motivation. The same research group also investigated whether infusing participants with glucose solution would improve performance (Carter *et al.*, 2004). As compared to ingestion of glucose containing beverage/food, infusion bypasses oral cavity and other parts of the digestive tract. Infusion of glucose did not improve performance when compared to a placebo indicating that there has to be other mechanism than adding additional glucose source during exercise.

Subsequent studies have confirmed these findings both in cycling and running events and even showed that performance can be improved using both sweet (e.g., sucrose

or glucose) and non-sweet (i.e., maltodextrin) carbohydrate sources as compared to an artificial noncaloric sweet-taste placebo (Stellingwerff and Cox, 2014). In addition to that, carbohydrate mouth rinsing coupled with caffeine was shown to improve capacity to perform high intensity intervals even in the condition of low muscle glycogen content (Kasper *et al.*, 2016).

A mechanistic study (Chambers, Bridge and Jones, 2009) employing magnetic resonance brain imaging has shown that mouth rinsing with carbohydrates (glucose and maltodextrin) activates brain regions involved in the regulation of motor activity. This work therefore further implies that there are receptors in the oral cavity that sense the presence of carbohydrates.

2.8.2. Carbohydrate intake in longer events (>1h)

As hypothesised by Hill and confirmed a few decades later, limited availability of carbohydrates can limit exercise capacity (Hill, 1925; Christensen and Hansen, 1939b; Bergström *et al.*, 1967). Exogenous provision of carbohydrates could therefore be a way to reduce utilization of endogenous carbohydrate (glycogen) stores and increase exercise capacity. There are two potential mechanisms by which carbohydrate intake during exercise could theoretically improve exercise capacity. Firstly, exogenous carbohydrate intake could reduce or completely suppress glucose output from the liver and thus prevent the onset of hypoglycaemia. Secondly,

provision of exogenous carbohydrates could reduce utilization of muscle glycogen and thus prolong time needed to deplete muscle glycogen stores.

That exogenous carbohydrate intake can reduce or even prevent utilization of hepatic glycogen has been nicely documented by (Jeukendrup *et al.*, 1999). They have shown that provision of carbohydrates at ~36 g · h^{-1} reduces hepatic glucose output and the ingestion of ~177 g · h^{-1} completely prevents liver glycogen utilization. However, this study did not observe sparing of muscle glycogen. Collectively these data suggest that when carbohydrates are provided at a currently recommended dose (60-90 g · h^{-1}), glycaemia can be maintained for a long period of time, and that liver glycogen is not a limiting factor in multiple hour endurance events.

Many studies indicate that exogenous carbohydrate provision decreases muscle glycogen utilization (Bjorkman *et al.*, 1984; Hargreaves *et al.*, 1984; Tsintzas *et al.*, 1995, 1996; Stellingwerff *et al.*, 2007). However these findings are not uniform across all studies (Coyle *et al.*, 1986; Flynn *et al.*, 1987; Mitchell *et al.*, 1989), and a recent meta-analysis has shown that carbohydrate feeding during exercise does not spare muscle glycogen (Areta and Hopkins, 2018). However, these contradictory findings could be down to methodological differences between studies (e.g., sparing has been commonly found in type I muscle fibres during running exercise (Tsintzas *et al.*, 1995, 1996)) or simply changes are so small that studies were underpowered to detect differences.

2.8.3. Exogenous carbohydrate oxidation rates

Although it has become apparent already in first half of 20th century that carbohydrate intake during exercise has a positive effect on exercise capacity and maintenance of glycaemia, early studies were unable to show how much exogenous carbohydrate is actually used during exercise bouts. Over the last few decades numerous studies have been conducted using isotope tracer methodology to determine the effects of various different combinations of carbohydrate intake on exercise metabolism (Hawley, Dennis and Noakes, 1992; Jeukendrup and Jentjens, 2000).

Studies have shown that when glucose is ingested during exercise, a peak oxidation rate is ~1 g · min^{-1} and does not increase if glucose is ingested at rates higher than 1 g · min^{-1} (Wagenmakers *et al.*, 1993; Jeukendrup and Jentjens, 2000; Jentjens *et al.*, 2004). The currently most accepted view is the most important limiting factor is the rate of absorption of carbohydrates from the small intestine (Jeukendrup, 2004). There is no direct evidence for this conclusion, however indirect evidence strongly suggests this is the case. As discussed previously, glucose and fructose use different transporters from the intestine. Observed exogenous carbohydrate oxidation rates are elevated when multiple transporter carbohydrates are utilized as compared to a single transporter (Wallis *et al.*, 2005; Rowlands *et al.*, 2015). In addition to that, when glucose is infused and thus intestinal transport is not a limiting factor, exogenous oxidation rates of glucose are elevated well above 1 g · min^{-1} (Hawley *et al.*, 1994).

2.9. Enhancement of training adaptations with altering substrate availability

So far, this introduction focused on strategies to acutely improve exercise performance. However, it is now well documented that this approach might not be optimal for achieving training adaptations, as high carbohydrate availability at all times and nutrients per-se can modulate training responses (Bartlett, Hawley and Morton, 2015; Hawley, Maughan and Hargreaves, 2015; Jeukendrup, 2017).

Endurance athletes undertake large volumes of training to promote exercise adaptations. For instance, world class cyclists train 5-8 times a week, each training session lasts between 1 and 6 hours, they undertake 100-200 races per year and accumulate over 30,000 km on the bike in a single year (Jeukendrup, Craig and Hawley, 2000). Similar volumes are observed in other endurance sports as well (Solli, Tønnessen and Sandbakk, 2017). A recent laboratory assessment of a multiple Tour de France winning cyclist (Bell *et al.*, 2017) has revealed exceptional physiological values, such as VO2peak value of 84 ml · kg^{-1} · min^{-1} and a lactate turn-point above 5 W · kg^{-1}. Numerous models of endurance performance exist trying to explain determinants of endurance performance (Coyle, 1995; Hawley and Stepto, 2001; Joyner and Coyle, 2008) and these have shown that in terms of physiology, absolute and relative values of both aerobic and anaerobic metabolism play an important role (e.g., $\dot{V}O_2max$, lactate turnpoint).

To improve performance athletes periodise their training programmes by manipulating training variables such as intensity, duration and frequency (Bishop, Granata and Eynon, 2014; Tnønessen *et al.*, 2014; Granata, Jamnick and Bishop, 2018a). The concept of training periodisation has evolved over the past few decades, yet the fundamental principle stayed the same – manipulating training variables with an aim to achieve specific goals for each training session in mind (Loturco and Nakamura, 2016).

Athletes and their coaches are mainly interested in performance benefits of training, as this is what counts in the world of elite sport. On the other hand, scientists are also interested in mechanisms underlying changes in performance. For example, over the past few decades they have determined that increases in mitochondrial volume and function increase oxidative capacity (Irrcher *et al.*, 2003), angiogenesis improves muscle blood flow (Kiens *et al.*, 1993), and a content or/and function of certain metabolic enzymes changes and affects metabolism (Holloszy, 1967; Gollnick *et al.*, 1985; Kiens *et al.*, 1993), which could in turn lead into improved performance.

Based on observations that carbohydrate availability is an important factor in endurance performance (see above), it is currently recommended that athletes compete with a high carbohydrate availability, as this has been established to maximise performance (Thomas, Erdman and Burke, 2016; Helge, 2017). However, during the training phase more and more athletes employ the concept of nutritional

periodisation that has received a lot of scientific interest over the last decade (Baar and McGee, 2008; Jeukendrup, 2017; Impey *et al.*, 2018; Stellingwerff, Morton and Burke, 2019). The principle is that nutrient intake is manipulated in order to achieve specific goals, such as 'training the gut' to improve tolerability to a high carbohydrate intake during competitions; practicing 'low-carb lifestyle' in order to increase fat oxidation; or strategically manipulate carbohydrate availability on a daily basis to achieve certain positive metabolic adaptations.

The relatively new research field molecular exercise physiology offers us insights into cellular processes that in the long-term most likely underpin adaptations to exercise. Researchers have to-date mostly been interested in mitochondrial function and aerobic metabolism and how this is influenced by training (Granata, Jamnick and Bishop, 2018b). Using molecular biology techniques enables us to get acute snapshots of molecular signalling activated after a single training session as well us see long term cellular adaptations (Egan and Zierath, 2013; Burke and Hawley, 2018). These include changes in protein content and enzyme activities which are preceded by activation and/or repression of signalling pathways known to regulate transcription and translation of gene expressions (Egan and Zierath, 2013).

It is now well documented that altering substrate availability alters acute signalling responses to exercise which could in the long term lead to desired metabolic adaptations and/or performance improvements (Impey *et al.*, 2018). Most of the research has focused on how can carbohydrate availability influences molecular

signalling and long term adaptations both from health (Wallis and Gonzalez, 2018) as well as performance improvement standpoint (Impey *et al.*, 2018).

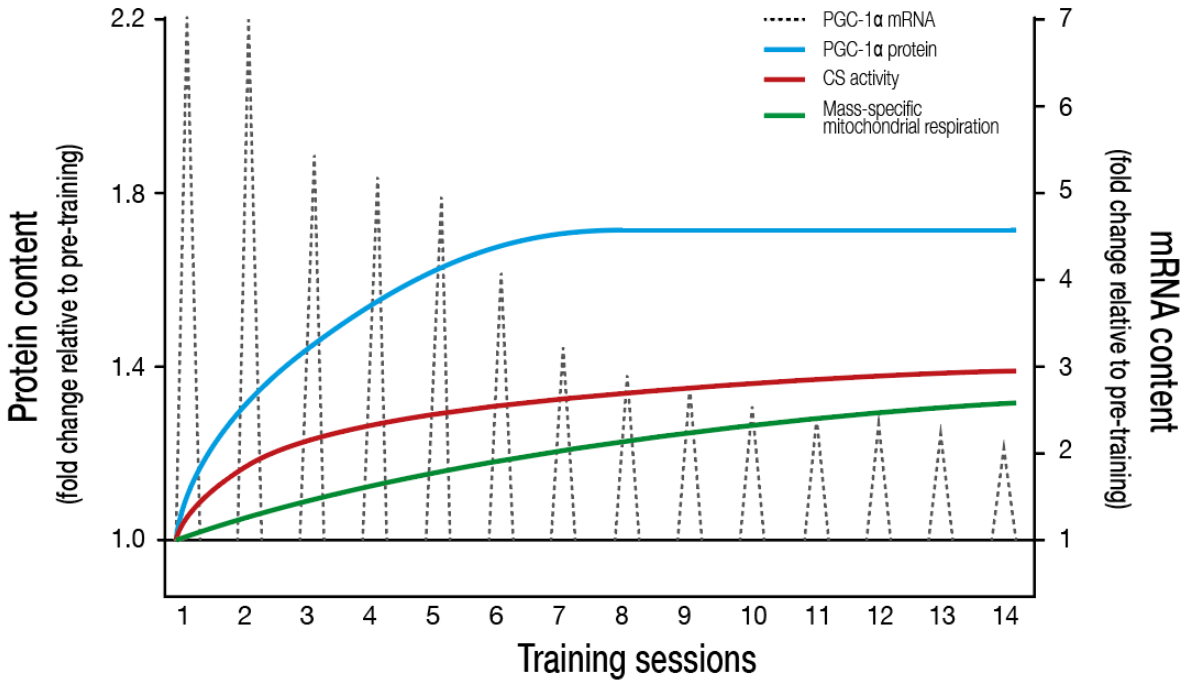

Figure 2.4. Acute exercise bout increases expression of certain mRNA content that in turn over time translate to increased protein content (e.g., PGC-1α) and consequently translates to altered function of an organelle (e.g., mitochondrial respiration). Adapted from (Granata, Jamnick and Bishop, 2018b).

Changes in mitochondrial content and function observed after a training/diet manipulation are thought to be caused firstly by transient changes in gene expression (e.g., peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) mRNA) and secondly, if repeated, accumulative changes in gene expression (e.g., lower PGC-1α mRNA response after the same exercise bout) and protein content (e.g., increased PGC-1α protein content) (Figure 2.4) and ultimately an increase in

functional parameters (e.g., mass specific mitochondrial respiration) (Perry *et al.*, 2010).

This chain of events is thought to be initiated mostly by muscle contraction in addition to nutrient availability. This then causes perturbations within cell environment (e.g., Ca^{2+} flux, altered AMP/ATP ratio, formation of reactive oxygen species, production of lactate) that in turn affect post-translation status of key signalling kinases (e.g., calmodulin-dependent protein kinase II (CaMKII), p38 mitogen-activated protein kinase (p38MAPK) and AMPK) (Bartlett, Hawley and Morton, 2015). A downstream consequence is activation of transcription factors and co-activators that co-ordinate the expression of both nuclear and mitochondrial encoded proteins.

2.9.1. Training with low muscle glycogen availability

One of the most well studied approaches to carbohydrate periodisation is training with low carbohydrate availability and especially reduced muscle glycogen stores. This can be achieved by restricting carbohydrate intake after the first training session which is designed to deplete glycogen stores. When exercise is commenced with low muscle glycogen content, drastic shifts in metabolism are observed as compared to when exercise is undertaken with normal muscle glycogen stores. These include lower reliance on muscle glycogen, increased fat metabolism (from IMTG stores and adipose tissue), higher protein breakdown as well as change in hormonal status (e.g.,

increased catecholamine levels) (Coyle *et al.*, 1985; Hargreaves, McConell and Proietto, 1995; Blomstrand and Saltin, 1999; Steensberg *et al.*, 2002). It is now well established that this causes increased cellular stress and altered molecular signalling (Philp, Hargreaves and Baar, 2012).

For instance, AMPK, an energy sensing kinase, is activated directly by increases in intracellular ratios of AMP and ATP as well as ADP and ATP, upstream kinases (e.g., CaMKII) and by glycogen stores (Towler and Hardie, 2007; McBride *et al.*, 2009). In line with this it was demonstrated that when exercise is commenced with reduced muscle glycogen stores, AMPK activation is augmented (Wojtaszewski *et al.*, 2003; Yeo *et al.*, 2010). Furthermore, achieving glycogen sparing by exogenous carbohydrate provision appears to attenuate AMPK activation (Akerstrom *et al.*, 2006). However, when glycogen sparing does not occur, AMPK activation is not different between high and low glucose availability (Towler and Hardie, 2007). Collectively this indicates that AMPK is capable of sensing glycogen stores.

One of most important targets of AMPK is transcription coactivator PGC-1α often known as a master regulator of mitochondrial synthesis (Jager *et al.*, 2007) as it is thought to be a major factor regulating muscle mitochondrial biogenesis. Indeed, PGC-1α mRNA expression and protein content increase after acute exercise bout and an increase is augmented by low muscle glycogen (Mathai *et al.*, 2008; Psilander *et al.*, 2013).

Another proposed mechanism of how low glycogen availability modulates mitochondrial biogenesis is via p38 MAPK that also upregulates transcription of PGC-1α (Akimoto *et al.*, 2005). It is well documented that glycogen binds water and changes in glycogen content within the cell could influence osmotic pressure (Philp, Hargreaves and Baar, 2012). In line with this, p38 MAPK activity is increased concomitantly with increases in hyperosmotic cellular stress as happens when glycogen stores get depleted (Gustin and Sheikh-Hamad, 2004). Unsurprisingly, activity of p38 MAPK is increased in a glycogen-depleted muscle (Chan *et al.*, 2004).

In addition the rise in catecholamines as observed during exercise in glycogen depleted state have also been linked to the perturbations needed to influence molecular signalling (Wojtaszewski *et al.*, 2003). Catecholamines have been linked to targeting PGC-1α via cAMP response element-binding protein (CREB) (Philp, Hargreaves and Baar, 2012). However, this is still debatable as some evidence suggests that PGC-1α expression and mitochondrial biogenesis are not induced in skeletal muscle but only in brown adipose tissue (Kim *et al.*, 2013).

So far only signalling concerning mitochondrial biogenesis was mentioned, but there are also other signalling pathways activated including expression of GLUT4 mRNA (Steinberg *et al.*, 2006), expression of pyruvate dehydrogenase kinase 4 (PDK4),

mitochondrial uncoupling protein 3 (UCP3), mitochondrial hexokinase II (HKII) and lipoprotein lipase (LPL) (Pilegaard *et al.*, 2002).

More recently, the major tumour suppressor protein p53 was implicated to affect mitochondrial biogenesis (Saleem, Adhihetty and Hood, 2009; Bartlett *et al.*, 2013) and training with reduced muscle glycogen has been shown to augment p53 signalling (Bartlett *et al.*, 2014).

Readers interested in more detailed effects of low glycogen availability and acute training responses on a molecular level are recommended to consult other recent reviews on the topic (Philp, Hargreaves and Baar, 2012; Bartlett, Hawley and Morton, 2015).

Hansen and colleagues conducted the first investigation of long-term training with reduced muscle glycogen stores (Hansen *et al.*, 2005). This study employed a leg-kicking exercise performed in a group of untrained individuals in a 10-week training study. Each leg was a subject to a different treatment. Half of training sessions in one leg were performed in a state of reduced muscle glycogen content, which was achieved by commencing the second training session only 2 hrs after the finish of the first session, while the other leg's second training session took place on the following day after muscle glycogen stores repletion. There was a substantial improvement in time to exhaustion at 90% of maximal workload in leg that trained with reduced glycogen stores (+294%), while the increase in the other leg was much smaller

(+125%). Muscle biopsy analysis also showed more positive adaptations (hydroxyacyl-CoA dehydrogenase (HAD) and citrate synthase (CS) activity) in the leg training with reduced muscle glycogen stores. Although results are compelling, this study has numerous limitations for applied practice, such as employment of unusual exercise type, training with only one leg at a time, different time window between exercise bouts and the fact that subjects were untrained, to name a few.

Since then, numerous other long term studies have been conducted looking into effectiveness of undertaking certain training sessions with low muscle glycogen content (reviewed in (Impey *et al.*, 2018)). After the seminal work published in 2005 by Hansen and colleagues, only a few other studies found functional benefits of training with reduced muscle glycogen concentrations (Cochran *et al.*, 2015; Marquet, Brisswalter, *et al.*, 2016; Marquet, Hausswirth, *et al.*, 2016). Conversely, observations of positive adaptations on the molecular level have been more commonly observed. Namely, some studies have shown an increase in activity of CS (Hansen *et al.*, 2005; Yeo *et al.*, 2008), succinate dehydrogenase (Morton *et al.*, 2009) and β-HAD (Yeo *et al.*, 2008; Hulston *et al.*, 2010) among others. It is important to note that none of the studies showed any negative effects of training with reduced muscle glycogen stores (Impey *et al.*, 2018).

Discrepancies between studies could very well be a result of different methodological approaches and thus different effects as well as type II statistical error, especially as laboratory tests are often not representative of real-world situations (e.g., cycling road

races last 4-6 hrs, whereas laboratory assessments are rarely longer than 3 hrs). Furthermore, a glycogen threshold hypothesis has been proposed (Philp, Hargreaves and Baar, 2012; Impey *et al.*, 2018) that proposes that certain level of glycogen depletion needs to be achieved in order to observe adaptations. In line with this, some of the studies not observing enhanced adaptations as a result of training with reduced muscle glycogen content had their muscle glycogen content outside the range thought to stimulate enhanced adaptations (Kasper Degn Gejl *et al.*, 2017; Impey *et al.*, 2018). Although the glycogen threshold hypothesis is sound, it still warrants further investigations.

The downside of training with low carbohydrate availability and especially commencing training sessions with reduced muscle glycogen content is a reduction in training volume as a result of a reduction of either exercise duration and/or intensity (Bergström *et al.*, 1967; Yeo *et al.*, 2008; Hulston *et al.*, 2010; Impey *et al.*, 2016a). Given that carbohydrate provision during exercise can increase exercise duration and/or intensity, it remains to be seen what impact delayed carbohydrate ingestion has on training commenced with reduced muscle glycogen content. Especially, as previous studies showed that delaying carbohydrate feeding does not supress fat oxidation rates (Horowitz *et al.*, 1999). The study described in Chapter 4 tried to uncover some of the questions in this regard.

3. General methods

This chapter's aim is to describe the general methods that were used in this thesis and discuss their principles.

3.1. Assessment of VO_2peak and Wmax

Research study participants are commonly classified according to the maximal oxygen uptake and maximal power output during incremental exercise test to exhaustion (Jeukendrup, Craig and Hawley, 2000; De Pauw *et al.*, 2013). Due to strict criteria for determination of VO_2max (Spurway *et al.*, 2012), it was decided that reporting VO_2peak rather than VO_2max values was more appropriate.

Participants performed an incremental test to exhaustion to determine VO_2peak and Wmax on a cycle ergometer (Lode, Groningen, Netherlands). The test started at an intensity of 100 W and the workload increased by 30 W every 2 minutes. During the test, gas exchange measurements were made using an automated online gas analysis system (Vyntus, Vyaire Medical, IL, US). The gas analysers were calibrated with a known gas mixture (15.04 % O_2, 5.06 % CO_2; BOC Gases, Surrey, UK) and the volume transducer was calibrated with a 3-litre calibration syringe (Jaeger, Wurzburg, Germany) prior to each experiment. The highest 30-s average of O_2 uptake was considered to represent VO_2peak. Wmax was calculated as the power output from

the last completed stage plus the fraction of the time spent in the next stage multiplied by 30 W.

3.2. Indirect calorimetry

Assessment of carbohydrate and fat oxidation during exercise in all three experimental chapters of this thesis adopted the principles of indirect calorimetry. This technique has been extensively used for years and has become a widely used method for assessment of energy expenditure and substrate oxidation rates.

The foundation of indirect calorimetry is the measurement of oxygen (O_2) uptake from the inspired air and excretion of carbon dioxide (CO_2) from the body (Frayn, 1983; Jeukendrup and Wallis, 2005). This technique is based on two assumptions. Firstly, completely oxidising carbohydrates and fats results in production of carbon dioxide (CO_2) and water (H_2O). Secondly, carbohydrates and fats differ in their chemical structure (i.e., different number of carbon, hydrogen and oxygen atoms) and in the amount of O_2 needed for a complete oxidation to CO_2 and water (H_2O).

For instance, the stoichiometry for glucose is as follows:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$$

And stoichiometry for a common fatty acid (palmitic acid) is:

$$C_{16}H_{32}O_2 + 23O_2 \rightarrow 16CO_2 + 16H_2O$$

Knowing the uptake of O_2 and excretion of CO_2 thus enables calculation of not only energy expenditure but also determination of exact carbohydrate and fat oxidation rates.

However, as discussed in the Literature review chapter, during exercise proteins can be utilised as well (Lemon and Mullin, 1980). They first need to be broken down to amino acids and be deaminated. Keto-acids will be oxidised to H_2O and CO_2 similarly as carbohydrates and fats whereas the nitrogen and sulphur will be secreted in urine, sweat and faeces (Jeukendrup and Wallis, 2005). For determination of the proportion that proteins contribute to energy expenditure, nitrogen excretion in the urine is commonly used. However, due to previous observations that only a small proportion of energy is coming from proteins during exercise (as discussed in the Literature review chapter), an assumption has been made that proteins present a negligible part in substrate oxidation and was assumed that all the energy was produced from carbohydrates and fats.

The ratio between the production of CO_2 and uptake of O_2 is known as respiratory exchange ratio (RER). The RER for carbohydrate oxidation is 1, whereas for oxidation of fats it is 0.7. Based on this data, carbohydrate and fat oxidation rates can be calculated. Throughout the experimental work of this thesis calculations by Jeukendrup and Wallis (2005) for moderate-to high intensity exercise were used.

$$\text{CHO Oxidation} = 4.210\ VCO_2 - 2.962\ VO_2$$

$$\text{Fat Oxidation)} = 1.695\ VO_2 - 1.701\ VCO_2$$

Where VCO_2 and VO_2 are expressed in $L \cdot min^{-1}$ and oxidation rates (CHO and fat) are calculated in $g \cdot min^{-1}$.

3.3. Determination of during recovery ingested carbohydrate oxidation rates

In Chapters 5 and 6 oxidation rates of during recovery period ingested carbohydrates were determined using the principles of stable isotope methodology. Carbon atoms form a backbone of carbohydrates. In nature, carbon has 15 isotopes (i.e., a chemical element that differs in the number of neutrons and thus mass), of which 2 are stable (^{12}C and ^{13}C). Assessment of carbohydrate oxidation rates using stable isotope methodology is based on knowing the total carbohydrate oxidation rates and the premise that by knowing the ratio between ^{12}C and ^{13}C in the pools of stored (glycogen), ingested carbohydrates and the ratio between ^{12}C and ^{13}C in expired CO_2, it is possible to calculate the amount of carbohydrates coming from each of the pools (stored or ingested) (Coggan, 1999; Jeukendrup and Jentjens, 2000).

Thus, oxidation of during recovery ingested carbohydrates (g · min^{-1}) was calculated according to the following equation (Craig, 1957):

$$CHO_{ing} = VCO_2 \left(\frac{\delta \mathrm{Exp} - Exp_{bkg}}{\delta \mathrm{Ing} - Exp_{bkg}} \right) \cdot \left(\frac{1}{0.07467} \right)$$

Where δ Exp represents ^{13}C enrichment of expired gas sample, δ Ing represents ^{13}C represents enrichment of ingested carbohydrate, Exp_{bkg} represents enrichment of expired gas sampled during the familiarisation session at the corresponding timepoint, and 0.7467 VCO_2 of 1 g glucose oxidation.

Carbohydrates provided during the recovery period in Chapters 5 and 6 were either naturally low (familiarisation session) or naturally high (experimental session) in ^{13}C atoms. This allowed for a determination ^{13}C enrichment in the body pool (familiarisation session) and thus correction of final carbohydrate oxidation rates for the enrichment already present in the body.

3.4. Glycogen reducing (depleting) exercise protocol

All three experimental chapters of this thesis employed a glycogen reducing exercise protocol with an aim of depleting muscle glycogen stores. However, due to lack of measurement of muscle glycogen concentrations in Chapters 4 and 5, it was deemed

that a protocol should be called glycogen reducing rather than glycogen depleting exercise bout.

A variation of a commonly used high-intensity-interval exercise protocol was utilised (Kuipers *et al.*, 1989). This protocol, as described below, was previously shown to successfully and reliably reduce muscle glycogen concentrations to ≤ 150 mmol · kg^{-1} DM (Wallis *et al.*, 2008). After a 5-min warm-up at 50% Wmax participants cycled at alternating workloads of 90% and 50% Wmax, respectively, each lasting 2 minutes. Once 90 % workload was deemed too demanding for participants to be able to cycle at a cadence of more than 60 revolutions per minute (RPM) despite strong verbal encouragement, 90 % intensity was first reduced to 80 % and then to 70 %. When blocks at 70 % Wmax could not be completed at the cadence > 60 RPM, the exercise session was terminated.

3.5. Blood sampling and Plasma analysis

Venous blood samples (~6 mL) were collected into tubes coated with ethylenediaminetetraacetic acid (also known as EDTA tubes). EDTA present in the tube binds with calcium in the blood and prevents the blood from clotting. Upon collection, tubes were stored on ice and then centrifuged at 4°C and 1006 × g for 15-min. Aliquots of plasma were then stored at −70°C and later analysed.

Plasma samples were analysed using automated clinical analysers (iLAB 650, Clinical Chemical Analyser, Instrumentation Laboratory, Warrington, UK (Chapter 4) and RX Daytona+ Randox, London, UK (Chapters 5 and 6) as well as immunoenzymatic assays (Chapters 5 and 6).

3.5.1. Analysis of plasma using automated clinical analysers

Prior to each analysis, a calibration (when necessary) and quality checks were conducted for each metabolite as per manufacturer's instructions. Both automated clinical analysers operate on the principles of measuring the absorbance of light.

For a better understanding of how an automated clinical analyser operates, analysis of glucose concentration using Glucose Kit (Randox, London, UK) is provided as an example. The plasma sample was initially incubated with hexokinase that breaks down glucose to glucose-6-phosphate. Subsequently glucose-6-phosphate is oxidized by glucose-6-phosphate dehydrogenase to form NADH. NADH in turn shows a strong absorbance at 450 nm. The automated analyser measures absorbance at this wavelength and based on the calibration curve provides the concentration of the metabolite in the solution (i.e., plasma).

Using the same principles, samples in experimental chapters (described in each chapter) were analysed for glucose (Glucose kit; Randox, London, UK), lactate (Lactate kit; Randox, London, UK), glycerol (Glycerol kit; Randox, UK) and non-esterified fatty acids (NEFA) (NEFA kit; Randox, UK). Intra-assay coefficients of variation of each of the machines based on 20 duplicate samples can be seen in the Table 3.1.

Table 3.1. Intra-assay coefficient of variation

Analyte	(iLAB 650)	(RX Daytona)
Glucose	0.87%	0.58%
NEFA	0.53%	1.26%
Lactate	1.13%	0.59%
Glycerol	/*	1.85%

**Glycerol analysis was not performed on the iLab 650 analyser*

3.5.2. Analysis of insulin and galactose

ELISA kits (ultrasensitive Elisa kit; Mercodia AB, Uppsala, Sweden and Human Insulin Elisa Kit; Invitrogen, Life Technologies, California, USA; in Chapters 4 and 6, respectively) were used for analysis of insulin in studies 4 and 6 of this thesis. The

protocol for Human Elisa Kit (Invitrogen, Life Technologies, California, USA) is described. ELISA plates provided by the manufacturer are coated with antibodies directed towards antigens of insulin. Standards, controls and samples were added in duplicates to the wells so that insulin is captured by the antibody. Then, a detecting antibody labelled with horseradish peroxidase was added and incubated for 30 minutes. After incubation, the plate was washed to remove unbound enzyme-labelled antibody. This was followed by incubation of a substrate solution tetramethylbenzidine. The reaction was stopped by HCl and the microtiter plate was read spectrophometrically by a Biotek 800 Absorbance Reader (Biotek Instruments, USA) with absorbance read at a wavelength of 450 nm. Standard curves were fitted, and this was then used to determine insulin concentrations in the samples. The intra-assay coefficient of variation based on 20 duplicate samples was 3.5% in Chapter 4 and 5.2% in Chapter 6.

In a similar manner as above, galactose (Sigma-Aldrich, St. Louis, USA) was analysed in the samples of the study described in Chapter 6. Namely, standards, controls and samples were added in duplicates to the wells. After, a master reaction mix (i.e., galactose assay buffer, galactose probe, galactose enzyme mix and horseradish peroxidase) was added to each plate. After incubation at 37ºC the absorbance was measured at 570 nm colorimetrically using Biotek 800 (Biotek Instruments, USA). The intra-assay coefficient of variation based on 20 duplicate samples was 5.0 %.

3.6. Time Trial

In Chapters 4 and 5, a performance test in the form of a simulated time trial (TT) on a cycle ergometer was employed. The cycle ergometer (Lode, Groningen, Netherlands) was connected to a personal computer running a custom-made software that is able to read and store the information from the cycle ergometer (i.e., power output).

Participants had to perform a certain amount of work (equal to ~40 min of cycling at 65% W_{max}) as quickly as possible.

The amount of work for each participant was calculated according to the following equation:

$$\text{Total amount of work} = 0.65\ W_{max} \times 2{,}400\ \text{J}$$

The ergometer was set in the linear mode and the linear factor calculated according to the formula:

$$L = W / (RPM)^2$$

Where L is a linear factor, W is predicted power and RPM is the cycling cadence. RPM was set to 80, whereas W represented 65% Wmax.

On the computer monitor visual information were provided to participants, namely the amount of work performed, target work and the percentage of work completed relative to the target work. As per recommendations (Currell and Jeukendrup, 2008b) there was no verbal encouragement provided and the TT took place in silence (i.e., no music) due to potentially influencing the outcomes.

This type of a TT was previously used by Currell et al. (2006) who reported the test to have a coefficient of variability of 4.5 %.

4. High rates of fat oxidation are maintained after the sleep low approach despite delayed carbohydrate feeding during exercise

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Abstract

While it is well documented that a high carbohydrate (CHO) availability is important for optimal performance, evidence suggests that performing certain training sessions with limited carbohydrate availability might positively influence training adaptations. However, due to low carbohydrate availability such an approach could compromise training volume.

The purpose of this study was to investigate whether delayed carbohydrate feeding during exercise undertaken the morning after a glycogen reducing exercise session would alter whole body metabolism (i.e., fat oxidation rates, NEFA availability) and recover some of the performance lost as a result of low carbohydrate availability as compared to a placebo and a high carbohydrate availability conditions.

Nine cyclists took part in this double-blinded randomised study (9 men; 1 woman; VO_2peak: 58.8 ± 5.5 mL $kg^{-1} \cdot min^{-1}$). Each trial started in the afternoon with a glycogen reducing exercise bout. The following morning 1-h of steady-state cycling (SS) was followed by a time trial (TT). Carbohydrates were not ingested during recovery of a glycogen reducing protocol or during next day exercise in the Placebo trial (PLA); CHO were not ingested during recovery but were fed (15 g every ~15-min) from 30-min into SS and continued during the TT in the delayed feeding trial (DELAY); CHO were provided during recovery (1.2 g $\cdot$ $kg^{-1} \cdot h^{-1}$ for 7 hrs) and next day exercise (as in DELAY) in a third condition (CHO). Exercise metabolism was assessed using indirect calorimetry and blood sampling.

Fat oxidation rates during SS were similar in PLA (0.83 ± 0.17 g $\cdot$ min^{-1}) and DELAY (0.78 ± 0.14 g $\cdot$ min^{-1}) ($p = 0.805$) and were both higher than CHO (0.57 ± 0.27 g $\cdot$ min^{-1}) ($p < 0.05$). However, plasma NEFA concentrations were supressed at 60-min time point in DELAY (0.8 ± 0.5 mmol $\cdot$ L^{-1}) as compared to PLA (1.4 ± 0.8 mmol $\cdot$ L^{-1}; p = 0.001) and comparable to CHO (0.6 ± 0.4 mmol $\cdot$ L^{-1}; p = 0.994). There were no significant differences in TT performance (49.1 ± 10.7, 43.4 ± 7.6, 41.0 ± 7.9 min in PLA, DELAY and CHO, respectively; $p > 0.05$).

Delayed carbohydrate feeding could be a strategy to maintain high-fat oxidation rates typically associated with exercise undertaken after the sleep-low approach to training, but the acute performance effects remain inconclusive.

4.1. Introduction

Recently, the concept of carbohydrate periodization for endurance athletes has emerged whereby dietary carbohydrate intake is tailored to support the varying carbohydrate demands and goals of different types of training and competition (Burke *et al.*, 2011; Impey *et al.*, 2018). This stems in part from research showing that strategically restricting carbohydrate availability around certain training sessions can enhance metabolic (e.g., increase fat oxidation rates) and/or functional adaptations (e.g., improved performance) (Yeo *et al.*, 2008; Hulston *et al.*, 2010; Marquet, Brisswalter, *et al.*, 2016) by augmenting the acute and cumulative adaptive (i.e., molecular) response to exercise (Impey *et al.*, 2018). However, exercising with low carbohydrate availability can decrease exercise capacity (Bergström *et al.*, 1967; Impey *et al.*, 2016b) and intensity (Yeo *et al.*, 2008; Hulston *et al.*, 2010). Despite suggestions of increased training efficiency (i.e., comparable molecular signalling response with a lower training volume) (Impey *et al.*, 2016b), this strategy might not achieve optimal adaptations due to reduced overall training volume. Maintaining capacity to undertake intense and long duration training sessions whilst in a state of reduced endogenous carbohydrate availability would most likely lead to the most favourable adaptations.

Carbohydrate feeding during exercise can improve exercise performance and capacity (Coyle *et al.*, 1983; Coggan and Coyle, 1989; Stellingwerff and Cox, 2014).

However, such a practice is not consistent with the principles of exercising with low carbohydrate availability. Carbohydrate ingestion before and/or during exercise can supress signalling of key molecular pathways thought to be responsible for skeletal muscle oxidative adaptation (Civitarese *et al.*, 2005; Akerstrom *et al.*, 2006) and in the long term reduce the training response when training under conditions of low carbohydrate availability (Morton *et al.*, 2009). For example, Morton et al. observed a blunted increase in succinate dehydrogenase activity after 6 weeks of training that included high intensity interval exercise sessions commenced with reduced muscle glycogen stores but with carbohydrate intake just before and during the training sessions as compared to when no carbohydrates were ingested before and during the training sessions. Collectively, it appears that some of the proposed metabolic (adaptive) signals associated with exercise with low muscle glycogen (e.g., elevated lipid metabolism, increased catecholamines) (Philp, Hargreaves and Baar, 2012) are suppressed with carbohydrate provision, further underpinning why carbohydrate feeding during exercise might impede training adaptation.

Prior studies concerned with optimizing training adaptation have not considered the potential for delaying the feeding of carbohydrates during exercise commenced with a low carbohydrate availability (i.e., low muscle glycogen concentrations). Delaying feeding of carbohydrate until 135-min into a strenuous exercise bout, a time when liver and muscle glycogen content is likely reduced, enhanced exercise capacity but did not alter respiratory exchange ratio (RER) or non-esterified fatty acids (NEFA)

concentrations, indicating unaltered metabolic environment (Coggan and Coyle, 1989). Further, carbohydrate feeding immediately after the onset of exercise commenced with reduced muscle glycogen enhances exercise performance (Widrick *et al.*, 1993; Ali *et al.*, 2016). Thus, delaying carbohydrate feeding during exercise commenced under conditions of low glycogen availability has the potential to maintain the metabolic (adaptive signals) but not compromise performance typically associated with exercise with low glycogen.

It is an established practice for world class athletes to start training sessions after an overnight fast when liver but not muscle glycogen stores are reduced and start ingesting carbohydrates later in the exercise bout (Froome and Walsh, 2015; Morton and Fell, 2016; Levitch, 2018). Furthermore, a recommended approach for training with low carbohydrate availability is to perform a glycogen depleting session in the afternoon and avoid carbohydrate intake before completing the next training session in the morning without carbohydrate provision (i.e., the 'sleep-low' strategy, low liver and muscle glycogen) (Marquet, Brisswalter, *et al.*, 2016). We hypothesised that carbohydrate feeding commenced 30 minutes after the start exercise would not alter the metabolic environment (e.g., substrate utilisation, plasma NEFA) thought to be critical for training adaptations during exercise performed after the sleep low strategy. We chose to delay carbohydrate feeding by 30 minutes because this strategy has previously been shown to maintain fat oxidation at similar rates to those observed during overnight fasted state moderate intensity exercise (Horowitz *et al.*, 1999). Further we

hypothesized that delayed feeding would enhance performance, that is typically compromised under conditions of low carbohydrate availably when exercise is commenced following the sleep-low strategy.

4.2. Materials and methods

4.2.1. Participants

Ten healthy, endurance-trained participants (9 men, 1 woman) accustomed to cycling exercise, provided written informed consent and completed the study that was approved by a Local Ethics Committee. The participants' characteristics were as follows: mean age: 27 ± 5 years, body mass: 67.7 ± 5 kg, height: 176 ± 7 cm, maximal oxygen uptake (VO_2peak): 4.0 ± 0.4 L· min^{-1} (58.8 ± 4.9 mL · kg^{-1} · min^{-1}), and maximal cycle ergometer power output (Wmax): 351 ± 46 W (5.2 ± 0.8 W · kg^{-1}).

4.2.2. Experimental design

After preliminary testing, each participant completed a familiarisation trial and three experimental trials each consisting of two exercise sessions; a glycogen-reducing exercise bout in the afternoon of Day 1 and a 1-h steady state exercise bout (SS) immediately followed by a time trial (TT) with a predicted duration of 40-min on the morning of Day 2. The exercise protocol was adapted from a previous study (Currell, Jentjens and Jeukendrup, 2006). The experimental trials differed in the diet provided

for the remainder of the Day 1 (7 hrs refeeding period after the glycogen reducing exercise session) and during the SS and the TT on Day 2. On one occasion participants received carbohydrates at a rate of 1.2 g · kg^{-1} · h^{-1} during a 7 hrs re-feeding period and carbohydrates at a rate of 15 g every 15 minutes during the SS of the second exercise bout commencing 30-min after exercise onset (i.e., at 30, 45 and 60-min time points) and $^{1}/_{3}$ and $^{2}/_{3}$ into the TT (CHO). On the other two occasions they received a noncaloric placebo food in the 7 hrs re-feeding period and carbohydrates during exercise on Day 2 as described above (DELAY) or they were given noncaloric placebo both during re-feeding (Day 1) and during exercise on Day 2 (PLA). The study adopted a double-blinded crossover design in which the order of the trials was randomized using an online research tool (www.randomizer.com). Experimental trials were separated by 6-14 days.

4.2.3. Preliminary testing and familiarization trial

Participants performed an incremental test to exhaustion to determine VO_2peak and Wmax on a cycle ergometer as described in the General methods chapter. Participants meeting the study's minimal VO_2peak criteria (≥ 50 mL · min^{-1} · kg^{-1}) were then scheduled for the familiarisation trial that, with the exception of blood sampling, followed the same protocol as the PLA trial (see below).

4.2.4. Experimental trials

A schematic overview of the study is depicted in Figure 4.1.

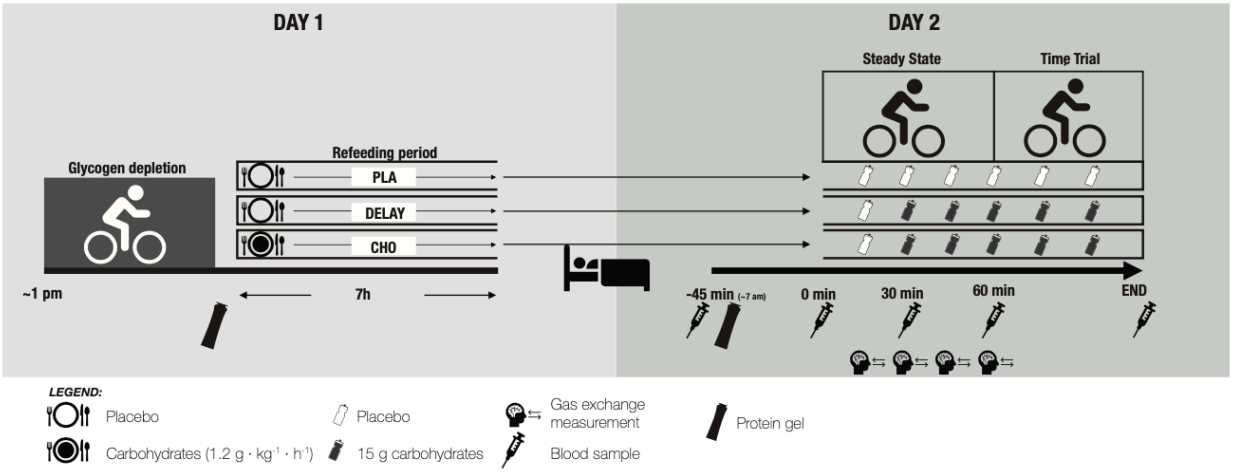

Figure 4.1 A schematic representation of the experimental design. In the afternoon of day 1, participants completed a muscle glycogen reducing protocol followed by ingestion of 20 g of whey protein in the form of a gel and a blinded diet either consisting of carbohydrates (1.2 g · kg^{-1} · h^{-1}) or a placebo for the 7 hrs period. The next morning participants entered the laboratory at ~7 am and first blood sample was obtained. After, a protein gel was ingested which was followed by 45-min of rest after which an exercise session commenced. During the steady state part, they received a drink every 15 minutes that either contained carbohydrates (15 g) or was a placebo. Blood sample was obtained every 30-min, while breath was analysed every 15-min. During the time trial participants received a drink with or without carbohydrates 1/3 and 2/3 into the time trial. At the end, a final blood sample was obtained.

Day 1. Prior to entering the laboratory at ~1 pm participants were asked to replicate the diet and activity patterns on the day of the visit and on the day preceding each experimental trial. A glycogen reducing exercise protocol was run as described in the General methods Chapter was run. Immediately post-exercise participants were

given a protein gel which contained 20 g protein (WHEY 20, Science in Sport, Nelson, UK) and the 7 hrs feeding period (explained below) was initiated. The protein gel was provided to minimize hunger in order to further support the blinding of the study.

Day 2. The next morning (i.e., ~7 am) participants reported to the laboratory after an overnight fast. Upon arrival an indwelling cannula was placed in an antecubital arm vein and a baseline blood sample taken. Immediately after participants received a further identical protein gel and rested for 45 minutes, after which a second blood sample was obtained and the SS part of exercise at 50% Wmax commenced. Ingestion of 20 g of protein 45-min before exercise has previously been shown not to influence NEFA availability and fat oxidation rates as compared to a fasted condition (Impey *et al.*, 2015). During the SS, VO_2 and VCO_2 were quantified every 15 minutes (i.e., 15, 30, 45 and 60 min) by participants breathing into the mouthpiece for 3 minutes, while blood samples were collected at 30 and 60-min time points. Immediately on completion of the SS the TT started as described in the General methods chapter. Following the TT, a final blood sample was collected.

4.2.5. Nutritional manipulation

The nutritional manipulation on Day 1 after the glycogen reducing exercise involved receiving 1.2 $g \cdot kg^{-1} \cdot h^{-1}$ of a 2:1 maltodextrin and fructose (MyProtein, Cheshire, UK) mixture (CHO) or the same volume of placebo (PLA and DELAY) for 7 hours. The

intervention was delivered every 30-min (0.6 g · kg^{-1} · h^{-1} at each time point) by incorporation of both sugars in the above ratio into a noncaloric beverage (Robinsons, Herts, UK) and a sugar free jelly (Hartley's, Leeds, UK). Apart from the food provided, participants were not allowed to consume any other food. The second part of the nutritional manipulation involved intake during the SS and the TT exercise on Day 2. During the SS, participants received 200 ml of a non-caloric beverage at 15-min time point in all 3 conditions. In PLA participants kept receiving the same volume at 30, 45 and 60-min time points and at $^{1}/_{3}$ and $^{2}/_{3}$ of the completed amount of work during the TT, whereas in DELAY and CHO, 15g of maltodextrin (MyProtein, Cheshire, UK) was added to the beverage at these time points (7% concentration). After each trial, a questionnaire was given to participants asking them to state the condition they believed they had undertaken. Less than 50% of participants correctly guessed the condition, showing that blinding was successful.

4.2.6. Blood analyses

Blood samples were collected, treated and analysed as described in the General methods chapter for glucose, NEFA, lactate and Insulin. Area under the curve (AUC) was calculated between time points 'baseline' and 60-min of SS.

4.2.7. Gas exchange measurements

Fat and carbohydrate oxidation rates were calculated using stoichiometric equations of Jeukendrup and Wallis (2005) as described in the General methods chapter.

4.2.8. Heart rate and ratings of perceived exertion

Heart rate (HR) values were obtained at 15-min intervals during the SS. Simultaneously every 15-min participants were asked to report the rate of perceived exertion (RPE) using 6-20 scale (Borg, 1982).

4.2.9. Statistics

The sample size was selected to be comparable with previous research that has investigated metabolic and performance responses to acute train low interventions (Impey *et al.*, 2015, 2016b; Hearris *et al.*, 2019). Data were initially tested for sphericity using Mauchly's test. Then, a two-way ANOVA for repeated measures was used to compare differences in substrate utilization and blood metabolites. When necessary, analyses were adjusted using the Greenhouse–Geisser correction. A one-way ANOVA was used to compare AUC and time for the TT completions. Where significant effects were observed by ANOVA, post-hoc pair-wise comparisons were

made with paired t-tests with the Tukey test applied to account for multiple comparisons. Effect sizes (ES) for TT performance were calculated using Hedge's g, where 0.2-0.5 represented a small, 0.5-0.8 moderate and >0.8 a large effect. All values are presented as mean ± SD. Statistical significance was set at $p < 0.05$. Statistics were performed using SPSS (Version 21; SPSS Inc., Chicago, IL, US) and Prism (Version 8; GraphPad Software, San Diego, CA, US).

4.3. Results

4.3.1. Glycogen-reducing session (Day 1)

Time to complete the glycogen reducing sessions in in CHO, PLA and DELAY, respectively, were 124 ± 31; 126 ± 35 and 123 ± 42 minutes, without any statistically significant differences between the trials ($p = 0.920$). Participants completed 1701 ± 429, 1750 ± 512 and 1693 ± 595 kJ of mechanical work during the glycogen reducing sessions in CHO, PLA and DELAY, respectively, without any statistically significant differences between the trials ($p = 0.966$). Neither were there any differences in the number of completed stages at 90, 80 and 70 % W_{max} between all three conditions ($p = 0.920$).

4.3.2. Fat and carbohydrate oxidation rates, VO_2, RER, RPE and HR during steady state exercise (Day 2)

Fat and carbohydrate oxidation rates are presented in Figure 4.2 whereas % VO_2peak, HR and RPE during the SS exercise bout are presented in Table 4.1 and grouped into time frames before (0-30 min) or after (30-60 min) a time point at which in DELAY and CHO carbohydrates started to be ingested.

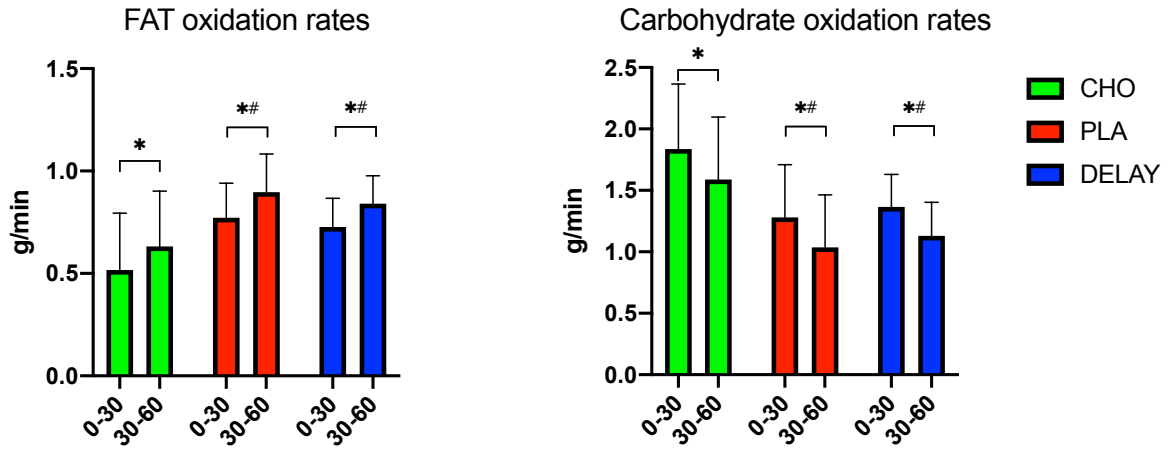

Figure 4.2 Fat a) and carbohydrate b) oxidation rates during the steady state part of exercise bout on Day 2.

**Significantly different between first- and second-time frame ($p < 0.05$)*
#Significantly different from CHO ($p < 0.05$)

As shown in Figure 4.2, carbohydrate oxidation was lower and fat oxidation higher throughout the SS in PLA ($p = 0.014$ and $p = 0.012$; for carbohydrate and fat oxidation, respectively) and DELAY ($p = 0.041$ and $p = 0.045$; for carbohydrate and fat oxidation,

respectively) as compared with CHO, while there was no difference between PLA and DELAY ($p = 0.870$ and $p = 0.805$; for carbohydrate and fat oxidation, respectively). In all conditions, carbohydrate oxidation decreased, while fat oxidation increased over time ($p < 0.001$). Furthermore, there was no significant difference in RER values between DELAY (0.82 ± 0.03) and PLA (0.81 ± 0.04) ($p = 0.915$), while both differed as compared to CHO (0.87 ± 0.06) ($p = 0.039$ and $p = 0.016$ for DELAY and PLA, respectively).

Table 4.1 Exercise intensity, rate of perceived exertion and heart rate data during the steady state part of exercise bout on Day 2.

Trial	Time (min)	% VO_2peak	HR (bpm)	RPE (6-20)
CHO	0-30	63.8 ± 5.4	134 ± 8	12 ± 2
	30-60	64.5 ± 6.2*	136 ± 7	13 ± 2*
PLA	0-30	65.4 ± 4.2	137 ± 9	13 ± 2
	30-60	66.4 ± 4.5*	137 ± 8	15 ± 3*
DELAY	0-30	64.9 ± 4.2	135 ± 8	14 ± 2
	30-60	65.9 ± 4.5*	138 ± 9	15 ± 3*

Data are presented as mean ± SD; N=10, CHO carbohydrate in recovery and in 30-60 min time frame; PLA no carbohydrate in recovery and during; DELAY no

carbohydrate in recovery and carbohydrate intake in 30-60 min time frame. *Significantly different from previous time point ($p < 0.05$)

As shown in Table 4.1, there were no differences in %VO_2peak between conditions ($p = 0.220$), but it increased to a similar extent in all conditions over time ($p = 0.025$). Also, there was no effect of time ($p = 0.552$) or condition ($p = 0.338$) for HR. RPE increased over time in all 3 conditions ($p = 0.006$). It was significantly higher in DELAY (14±3) when compared to CHO (13 ± 2; $p = 0.036$) and tended to be higher in PLA (14 ± 3) than CHO ($p = 0.055$), whilst being similar between and PLA and DELAY (0.975).

4.3.3. Plasma, NEFA, insulin, glucose and lactate during exercise (Day 2)

Results for NEFA, Insulin, glucose and lactate are presented in Figure 4.3.

NEFA concentrations (Figure 4.3a) were lower at the baseline in CHO (0.9 ± 0.5 mmol · L^{-1}) as compared to PLA (1.5 ± 0.4 mmol · L^{-1}; $p < 0.001$) and DELAY (1.6 ± 0.8 mmol · L^{-1}; $p < 0.001$). NEFA concentrations dropped from the baseline to 0-min time point in all conditions ($p < 0.05$) and there were no differences between conditions in absolute concentrations ($p > 0.05$). After 30-min of SS, NEFA concentrations increased in all conditions ($p < 0.05$). However, the increase was less pronounced in CHO in comparison to PLA and DELAY, where values were significantly higher at this

time point at 0.7 ± 0.5, 1.2 ± 0.6 and 1.1 ± 0.7 mmol · L^{-1} in CHO, PLA and DELAY, respectively ($p < 0.05$). Concentrations did not further change neither in PLA and CHO ($p > 0.05$), whereas insignificantly dropped to 0.8 ± 0.5 mmol · L^{-1} in DELAY from 30 to 60-min time point ($p = 0.165$) so that at 60-min time point DELAY and CHO values were not statistically significantly different ($p = 0.994$). AUC for NEFA was significantly lower in CHO as compared with PLA ($p = 0.007$) and DELAY ($p = 0.042$), without being different between DELAY and PLA (0.678).

Insulin concentrations (Figure 4.3b) did not differ at the baseline ($p > 0.05$) and were only marginally increased just before the SS ($p > 0.05$). At the 30-min time point insulin concentrations dropped similarly in all conditions as compared to 0-min, although the decrease was only significant in PLA (-4.1 ± 2.3 mU · L^{-1}; $p < 0.001$) and CHO (-3.5 ± 3 mU · L^{-1}; $p = 0.003$) and not in DELAY (-2.5 ± 2 mU · L^{-1}; $p = 0.178$) condition. Insulin concentrations did not change significantly between 30-min and 60-min in any condition ($p > 0.05$). Nonetheless, they were significantly higher in DELAY (+ 3.6 ± 3.5 mU · L^{-1}; $p = 0.003$) and CHO (+ 4.7 ± 3.0; mU · L^{-1} $p < 0.001$) as compared with PLA, whereas there was no difference between DELAY and CHO ($p > 0.999$) at 60-min. AUC for Insulin was significantly higher in CHO as compared with PLA ($p = 0.034$), whereas there was no difference between CHO and DELAY ($p = 0.194$) or PLA and DELAY ($p = 0.619$).

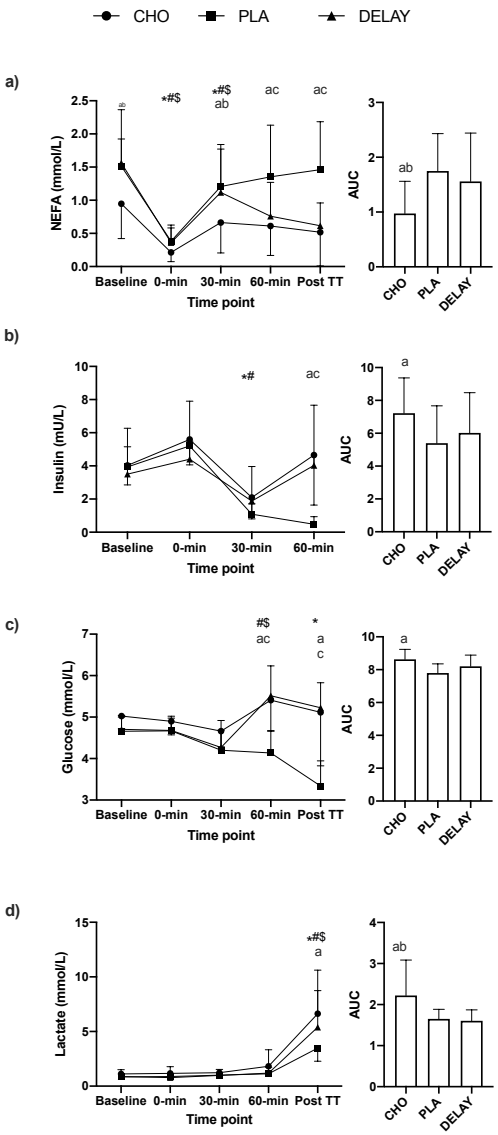

Figure 4.3 Plasma a) NEFA, b) insulin, c) glucose and d) lactate before, during and just after exercise bout on Day 2. Area under the curve (AUC) was calculated for period between baseline and 60-min time point.

** Significantly different from previous time point in PLA ($p < 0.05$);*
 # Significantly different from previous time point in CHO ($p < 0.05$);
$ Significantly different from previous time point in DELAY ($p < 0.05$);
a Significant difference between CHO and PLA ($p < 0.05$);
b Significant difference between CHO and DELAY ($p < 0.05$);
c Significant difference between PLA and DELAY ($p < 0.05$).

At baseline, before the SS (0-min) and at mid-point of the SS (30-min) concentrations of glucose (Figure 4.3c) were not different between conditions ($p > 0.05$). Concentrations remained stable for the rest of the SS in PLA ($p > 0.05$), whereas glucose concentration increased by 1.3 ± 0.6 mmol · L^{-1} in DELAY from 30-min to 60-min time point ($p < 0.001$) and by 0.7 ± 0.6 mmol · L^{-1} in CHO ($p = 0.009$). Concentrations did not change at the end of the TT in CHO and DELAY, whereas concentrations significantly decreased (-0.8 ± 0.3 mmol · L^{-1}) at the end of the TT in PLA ($p < 0.001$). Glucose concentrations were higher in CHO (5.4 ± 0.8 and 5.1 ± 1.3 mmol · L^{-1}) and DELAY (5.6 ± 0.7 and 5.2 ± 0.6 mmol · L^{-1}) as compared to PLA (4.2 ± 0.6 and 3.3 ± 0.6 mmol · L^{-1}) at 60-min and post TT time points ($p < 0.05$) with no difference between CHO and DELAY ($p > 0.999$) conditions. AUC for glucose was significantly higher in CHO as compared with PLA ($p = 0.006$), whereas there was no difference between CHO and DELAY ($p = 0.189$) or PLA and DELAY ($p = 0.228$).

Lactate concentrations (Figure 4.3d) remained constant during the SS and only significantly increased post TT in all three conditions ($p < 0.05$) with only significant difference between CHO and PLA ($p < 0.001$), without differences between PLA and DELAY ($p = 0.127$) or DELAY and CHO ($p = 0.774$). AUC for lactate was significantly higher in CHO as compared with PLA ($p = 0.029$) and DELAY ($p = 0.019$), whereas there was no difference between PLA and DELAY ($p = 0.974$).

4.3.4. TT performance

Participants completed the TT in 41.0 ± 7.9, 49.1 ± 10.7 and 43.4 ± 7.6 (minutes in CHO, PLA and DELAY conditions, respectively, with no statistically significant differences between the trials ($p = 0.094$). ES comparisons for DELAY vs. PLA, CHO vs. PLA and CHO vs. DELAY were 0.57 (moderate), 0.8 (large) and 0.3 (small). TT results with mean values and individual data points are presented in Figure 4.4. Only 9 participants successfully finished all TTs, while one participant could not finish the TT in PLA condition reporting blurred vision and light-headedness. Later analysis showed that his plasma glucose concentrations had dropped to 2.7 mmol · L^{-1} at the point of fatigue. This participant's data was not included in the analysis of performance responses.

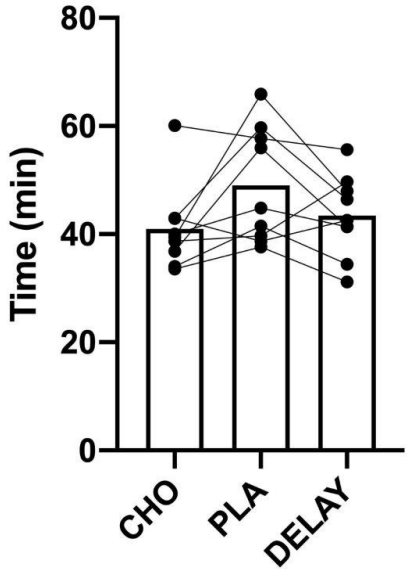

Figure 4.4 Time to complete the TT. Bars represent the mean, while dots and connecting lines represent individual participants.

4.4. Discussion

The main aim of this study was to explore how delayed carbohydrate feeding during subsequent exercise, when following the sleep-low approach to training with low carbohydrate availability, affected whole-body metabolism. A primary finding was that delayed carbohydrate feeding did not compromise the high fat oxidation rates typically observed during exercise commenced with low carbohydrate availability. Secondly, we explored how exercise performance was affected by delayed carbohydrate feeding during exercise following an acute sleep-low intervention. The present study did not show any significant differences in TT performance when exercise was undertaken with high carbohydrate availability or commenced with low carbohydrate availability combined with or without delayed carbohydrate feeding.

Overall fat oxidation rates during exercise on Day 2 were higher in both sleep-low conditions (PLA and DELAY) as compared to when carbohydrates were provided in recovery (CHO). Furthermore, during exercise on Day 2 delayed carbohydrate feeding in DELAY did not prevent an increase in rates of fat oxidation so that in PLA and DELAY they remained comparable. This provides further support for a concept that low carbohydrate availability and particularly low muscle glycogen determine fat oxidation rates during exercise (Arkinstall *et al.*, 2004). The elevated fat oxidation in DELAY occurred despite NEFA concentrations being reduced to concentrations similar to those observed in CHO and thus below those seen in PLA. The reduction

in NEFA concentrations most likely occurred as a result of the reduction of adipose tissue lipolysis because of insulin (Campbell *et al.*, 1992). Our results therefore show that delayed feeding in DELAY caused a divergence between fat oxidation rates and NEFA availability. This contrasts some previous work showing that a suppression of NEFA availability is associated with reduced fat oxidation rates (Horowitz *et al.*, 1997). While speculative, intramuscular triacylglycerol (IMTG) utilization could have been increased (Watt, Holmes, *et al.*, 2004; van Loon, Manders, *et al.*, 2005) and become an important source of fatty acids in DELAY partially replacing plasma borne NEFA.

From the perspective of training adaptations, the significance of the divergence in fat oxidation and NEFA availability during DELAY is unclear. Delayed carbohydrate feeding in DELAY increased fat oxidation rates as compared to CHO, but supressed plasma NEFA availability as compared with PLA. As implied in the present study fat oxidation during exercise not only relies on plasma NEFA, but also on IMTG. Thus a high flux through lipid metabolism pathways could be sustained by IMTG utilization which could also act as a signal for molecular adaptations (Philp, Hargreaves and Baar, 2012; Meex *et al.*, 2015). There are multiple proposed mechanisms on why training with reduced muscle glycogen content might promote desirable molecular signalling (e.g., AMPK), which include elevated plasma NEFA concentrations and glycogen depletion (Philp, Hargreaves and Baar, 2012). It has been implied that increased NEFA could directly cause augmentation of molecular signalling (e.g., PPAR and p38MAPK) that would in the long term lead to favourable adaptations (Philp *et al.*, 2013; Zbinden-Foncea *et al.*, 2013). Even though NEFA concentrations

declined with delayed carbohydrate feeding overall exposure as assessed by NEFA AUC was similar between PLA and DELAY, thus it could be speculated that the overall NEFA stimulus is maintained with DELAY. Further research is required to better understand whether the crucial signal is NEFA availability and/or high muscle fat utilisation per se (i.e., high fat oxidation rates) that are most important for promoting training adaptations when exercising under conditions of low muscle glycogen. It has to be acknowledged that apart from different carbohydrate availability, overall energy availability was different as well.

Undertaking exercise sessions in a muscle glycogen depleted state compromises ability to exercise at high intensities (Yeo *et al.*, 2008; Hulston *et al.*, 2010). We delayed the feeding in conditions of low carbohydrate availability in an attempt to maximise the lipid metabolic response to exercise, however in this context we were unable to discriminate performance differences between any of the study conditions. We based our protocol on a previous study showing a good reliability of the TT performance in the state of low carbohydrate availability (Currell, Jentjens and Jeukendrup, 2006). Unfortunately, the sensitivity of the test was not assessed in that study and could thus be a reason for lack of differences in performance observed in the present study. Despite familiarization, a large variability in TT performance was observed in response to the experimental conditions between participants which undoubtedly also contributed to the failure to reveal clear performance differences. Another explanation for lack of significant findings might be a small sample size. Indeed, a post-hoc power calculation showed that there was a 60 %, 35 % and 13

% chance of detecting a significant difference between CHO vs. PLA, DELAY vs. PLA and CHO vs DELAY, respectively. Nonetheless, the direction of the change in exercise performance and the effect sizes observed were in line with what might have been predicted thus indicating a potential for rescuing of performance in DELAY. This would be in line with a recent study demonstrating a better capacity to sustain high intensity efforts with higher muscle glycogen content at the start of the exercise (Hearris *et al.*, 2019).

Although there were no clear performance differences, the plasma glucose concentration data is potentially revealing. Maintenance of circulating glucose concentrations during exercise is often considered one of the key mechanisms underpinning the ergogenic effect of carbohydrate feeding, particularly in studies of exercise capacity (Christensen and Hansen, 1939b; Coyle *et al.*, 1986; Coggan and Coyle, 1989). Our results showed diminishing plasma glucose concentrations in the PLA condition, and indeed one participant failed to complete the TT in PLA which could be attributed to hypoglycaemia (plasma glucose 2.7 mmol · L^{-1}). In contrast, plasma glucose concentrations were maintained in DELAY at comparable levels to those seen in CHO. This raises the possibility that had exercise capacity been assessed, and not TT performance, endurance could have been increased more consistently with delayed feeding. This notion is a speculation, but it is noteworthy that the participant unable to finish the trial in PLA was able to complete the other trials without difficulty. While further research is required, delayed feeding could potentially enable athletes to increase the duration of the training sessions

undertaken in glycogen depleted state. This could be beneficial for athletes seeking to increase total duration of training at lower intensities, or for those wishing to maximise the metabolic benefits of training under conditions with elevated fat oxidation rates. It has to be acknowledged that elite athletes train in excess of 20 hours a week with training sessions lasting up-to 6 hours (Jeukendrup, Craig and Hawley, 2000) and thus limited duration of training with the conventional sleep low approach without delayed carbohydrate intake might not be desirable.

In summary, the present study demonstrates that delayed feeding with a moderate dose of carbohydrates did not prevent an increase in fat oxidation rates during exercise typically observed with training under conditions of low carbohydrate availability. Delayed carbohydrate feeding during exercise could therefore be an effective way of undertaking endurance training in a state of muscle glycogen depletion with an aim to achieve high fat oxidation rates and to prevent hypoglycaemia response with avoidance of carbohydrates in recovery and training bouts. Nonetheless, further research is required to understand muscle metabolic and molecular responses to such an intervention, its potential to impact exercise capacity or performance and ultimately the impact on long-term training adaptations.

As stated in the introduction of this chapter, a high carbohydrate availability is important for optimal performance. While this chapter has focused on the training aspect of the recovery period (i.e., manipulation of carbohydrate availability during recovery and subsequent exercise and its impact on metabolism), in scenarios when

there is limited time available between two consecutive high intensity training sessions planned or races, restoration of carbohydrate stores within the body are important for recovery of performance. Therefore, the next two experimental chapters address some of the pertinent research questions in this area.

5. Impact of post-exercise fructose-maltodextrin ingestion on subsequent endurance performance

This chapter was published in the **Frontiers in nutrition**; doi: 10.3389/fnut.2020.00082

Abstract

Current sports nutrition guidelines recommend athletes to ingest carbohydrates at a rate 1.0-1.2 g · kg^{-1} · h^{-1} to optimise repletion of muscle glycogen during short-term recovery from endurance exercise. However, they do not provide specific advice on monosaccharides (e.g. fructose or glucose) other than to ingest carbohydrates of moderate to high glycaemic index. Recent evidence suggests that combined ingestion of fructose and glucose in recovery leads to enhanced liver glycogen synthesis and that this translates into improvement of subsequent endurance capacity.

The purpose of the present study was to investigate whether consuming a combination of fructose and glucose as opposed to glucose alone during short-term recovery (i.e., 4 hrs) from exhaustive exercise would also improve subsequent pre-loaded cycle time trial (TT) performance.

Eight participants (7 men, 1 woman; VO_2peak: 56.8 ± 5.0 mL $O_2 \cdot min^{-1} \cdot kg^{-1}$) participated in this randomised double-blind study. Each experimental session involved a glycogen reducing exercise bout in the morning, a 4 hrs recovery period and 1-h of steady state (SS) exercise at 50% Wmax followed by a ~40-min simulated TT. During recovery carbohydrates were ingested at a rate of 1.2 $g^{-1} \cdot h^{-1}$ in the form of fructose and maltodextrin (FRU+MD) or dextrose and maltodextrin (GLU+MD) (both in 1:1.5 ratio). Substrate oxidation rates, including ingested carbohydrate oxidation, were determined during the SS. Blood samples were collected during recovery, during the SS exercise and at the end of the TT for determination of glucose and lactate concentrations.

There were no differences in TT performance (37.41 ± 3.45 (GLU+MD); 37.96 ± 5.20 minutes (FRU+MD), $p = 0.547$). During the first 45-min of SS oxidation of ingested carbohydrates was greater in FRU+MD ($p < 0.05$) and there was a trend towards higher overall carbohydrate oxidation rates in FRU+MD ($p = 0.08$). However, at 60-min of SS, differences in substrate oxidation disappeared.

Ingestion of combined fructose and glucose compared to glucose only during recovery from an exhaustive exercise bout increased the ingested carbohydrate oxidation rate during subsequent exercise. Under the conditions studied, subsequent TT performance was not improved with fructose-glucose.

5.1. Introduction

Numerous top level athletes undertake multiple training sessions daily (Solli, Tønnessen and Sandbakk, 2017; Etxebarria, Mujika and Pyne, 2019) and it is common in certain sports that competitive elements follow each other with limited (i.e. a few hours) time for recovery. During prolonged strenuous exercise carbohydrate oxidation accounts for a large proportion of energy expenditure (van Loon *et al.*, 2001) and muscle and liver glycogen content can be substantially reduced (Stepto *et al.*, 2001; Fuchs *et al.*, 2016). From a metabolic perspective, if recovery of the ability to perform strenuous exercise is a priority, it is important to replenish both muscle and liver glycogen stores. Guidelines suggest that in order to maximise short term post-exercise muscle glycogen synthesis moderate to high glycaemic carbohydrates should be ingested as soon as possible at a rate 1.0-1.2 $g \cdot kg^{-1} \cdot h^{-1}$ (Burke *et al.*, 2011). However, evidence suggests that carbohydrate type can also influence liver glycogen synthesis and thereby impact post-exercise recovery (Wallis and Wittekind, 2013; Fuchs, Gonzalez and van Loon, 2019).

The most recent evidence indicates that combining fructose- and glucose- based carbohydrate sources (e.g. a combination of fructose and maltodextrin, or sucrose) leads to higher total glycogen storage as compared to when only glucose-based carbohydrates are ingested. This is thought to be due to the preferential storage of fructose and glucose as liver and muscle glycogen, respectively (Nilsoon and

Hultman, 1974; Casey *et al.*, 2000; Wallis *et al.*, 2008; Décombaz *et al.*, 2011; Fuchs *et al.*, 2016; Trommelen *et al.*, 2016). Recently, co-ingestion of fructose and maltodextrin during a 4 hrs recovery period enhanced recovery of subsequent endurance running capacity as compared to glucose and maltodextrin (Maunder, Podlogar and Wallis, 2018). This has now been verified in a cycling model (Gray *et al.*, 2020). A metabolic mechanism was inferred in the study by Maunder and colleagues through observations of increased exogenous and better maintained total carbohydrate oxidation during the second exercise bout. However, the relatively short duration second exercise bout (i.e., exogenous oxidation could be compared between trials for just 30 min of exercise) challenged assumptions related to retention of the ^{13}C label in the body CO_2 pools when using ^{13}C labelled carbohydrates for determination of exogenous carbohydrate oxidation (Jeukendrup and Jentjens, 2000). In addition, it was not possible to completely discount a role for gastrointestinal (GI) distress in explaining the differences in subsequent exercise capacity when fructose-glucose or glucose only sources were provided.

The purpose of the present study was to test the hypothesis that combined ingestion of fructose and glucose as opposed to glucose alone during short-term recovery from exhaustive exercise improves subsequent pre-loaded cycle time trial performance. Exercise performance was selected as the primary outcome as some have questioned the external validity of measuring exercise capacity that has been assessed in previous studies by Maunder and colleagues and Gray and colleagues (Currell and Jeukendrup, 2008b). Cycling was used as the exercise mode given that

GI distress is less prevalent in cycling than in running (Peters *et al.*, 2000) thus improving the potential to isolate metabolic effects from GI influences. In a parallel trial, breath $^{13}CO_2$ appearance from ingested ^{13}C labelled bicarbonate was determined as a marker of label retention within the bicarbonate pool during exercise, in order to attribute the metabolic effects of post-exercise carbohydrate feeding with more certainty.

5.2. Materials and Methods

5.2.1. Participants

Eleven healthy, endurance-trained participants (8 men, 3 women) accustomed to cycling exercise, provided written informed consent and met the eligibility criteria to undertake the study that was approved by the Science, Technology, Engineering and Mathematics Ethics Committee, University of Birmingham, UK (Ethics code: ERN_17-1236). Their mean age, body mass, height, maximal oxygen uptake (VO_2peak), and maximal cycle ergometer power output (Wmax) were 31 ± 5 years, 68 ± 9 kg, 173 ± 7 cm, 3.86 ± 0.52 L $O_2 \cdot min^{-1}$ (56.9 ± 4.8 mL $O_2 \cdot min^{-1} \cdot kg^{-1}$), and 338 ± 47 W (5.0 ± 0.5 W $\cdot$ kg^{-1}), respectively. These participants all completed preliminary testing and their data are included for estimation of ^{13}C bicarbonate elimination (further details below). Three participants dropped out after the preliminary or familiarisation visits: 2 due to time commitments; 1 due to GI discomfort during the familiarisation trial. Thus 8 participants successfully finished all trials. Their mean age, body mass, height,

maximal oxygen uptake (VO_2peak), and maximal cycle ergometer power output (Wmax) were 31 ± 5 years, 71 ± 7 kg, 175 ± 8 cm, 4.04 ± 0.44 L $O_2 \cdot min^{-1}$ (56.8 ± 5.0 mL $O_2 \cdot min^{-1} \cdot kg^{-1}$), and 352 ± 41 W (5.0 ± 0.5 W $\cdot kg^{-1}$), respectively.

5.2.2. Experimental design

Participants underwent preliminary testing that consisted of a VO_2peak test and a steady state exercise bout. The latter was used to estimate elimination rate of ingested ^{13}C bicarbonate. Participants then completed a familiarisation trial and two experimental trials consisting of a glycogen-reducing exercise bout in the morning followed by a 4 hrs recovery period and a 1-h steady state exercise bout (SS) immediately followed by a time trial (TT) with a predicted duration of 40-min. The experimental trials differed in the diet provided during the 4 hrs recovery window. On both occasions participants received carbohydrates at a rate of 1.2 g $\cdot kg^{-1} \cdot h^{-1}$ during a 4 hrs re-feeding period with a difference in the composition of carbohydrates. On one occasion they received a mixture of glucose-based carbohydrates, namely dextrose and maltodextrin (GLU+MD) and on another fructose and maltodextrin (FRU+MD), both in a 1:1.5 ratio. The order of the trials was randomized, the study was double-blinded, and the trials were separated by at least 6 days.

5.2.3. Preliminary testing and determination of bicarbonate elimination

Participants came to the laboratory at ~07:00 hrs after an overnight fast. They performed an incremental test to exhaustion to determine VO_2peak and Wmax on a cycle ergometer (Excalibur Sport; Lode, Groningen, Netherlands) as described in the General methods chapter.

Participants then rested for 30-min before undertaking an exercise session assessing kinetics of ^{13}C labelled bicarbonate elimination as previously described (Leese *et al.*, 1994). Stable isotope methodology is commonly used to assess oxidation rates of ingested carbohydrates. However, after their oxidation, labelled carbon atoms previously constituting carbohydrate molecules can be for some time held within the body pool of bicarbonate and thus oxidation rates of ingested carbohydrates underestimated. Thus, it is common to calculate the time required for the bicarbonate pool to be turned over. Briefly, immediately after ingestion of 0.23 ± 0.08 mg · kg body weight of ^{13}C labelled bicarbonate (99% purity, Cambridge Isotope Laboratories, Inc.; Andover, USA) participants exercised at 50% Wmax for 60 minutes. $^{13}CO_2$ production, reflecting ^{13}C labelled bicarbonate excretion was determined throughout the exercise by measurement of CO_2 production (as described above using indirect calorimetry) and by quantification of $^{13}CO_2/^{12}CO_2$ ratio in expired breath using isotope ratio mass spectrometry (IsoAnalytical Ltd., Crewe, UK). Breath was collected into 10-mL evacuated tubes (Exetainer Breath Vial, Labco

Ltd.; Buckinghamshire, UK). Cumulative excretion of labelled bicarbonate was quantified at 2, 5, 10, 20, 30, 45 and 60 minutes.

5.2.4. Familiarisation and experimental trials

A familiarisation trial was performed ~7 days after the preliminary testing. It followed the same protocol as the experimental trials in GLU+MD condition without blood sampling. As well as familiarisation, this trial was used to quantify any background shift in $^{13}CO_2$ production during the SS exercise period to enable a more accurate determination of ingested carbohydrate oxidation rates (further described below).

Participants entered the laboratory at ~7:00 after not eating from 22:00 the day before. They were asked to replicate the diet and activity patterns of the day preceding each experimental trial. On entering the laboratory, a blood sample was taken using venepuncture from an antecubital vein. Then they performed a glycogen reducing exercise protocol as described in the General methods section. Immediately post-exercise an indwelling cannula was placed in an antecubital arm vein and a blood sample taken. Participants then started the 4 hrs recovery period.

During recovery, participants passively rested for 4 h, during which time sedentary activities such as reading and use of laptops were permitted. Immediately upon obtainment of the blood sample post exercise, participants commenced ingesting a

15% carbohydrate containing drink every 30 minutes with a carbohydrate ingestion rate of 1.2 $g \cdot kg^{-1} \cdot h^{-1}$. Composition of the drink was either glucose (Roquette, Lestrem, France or Myprotein, The Hut Group, Cheshire, UK for familiarisation and experimental trials, respectively) and maltodextrin (Avebe, Veendam, The Netherlands or Myprotein, The Hut Group, Cheshire, UK for familiarisation and experimental trials, respectively) (GLU+MD) or fructose (Peak Supps, Bridgend, UK) and maltodextrin (FRU+MD) in a 1:1.5 ratio. Carbohydrates ingested during recovery of the familiarization trial were of naturally low ^{13}C abundance (–26.17δ $^{13}C_{V\text{-}PDB}$ (‰)) whereas during the experimental trials they were naturally high ^{13}C abundance (–11.18 δ $^{13}C_{V\text{-}PDB}$ (‰) for FRU+MD and –11.14 δ $^{13}C_{V\text{-}PDB}$ (‰) for GLU+MD). A venous blood sample was obtained every 1 hour during the recovery with the cannula flushed with saline every 30 minutes to maintain patency.

During the 1-hr long SS at 50% Wmax, participants breathed for 3 minutes every 15 minutes into a mouthpiece connected to the metabolic cart (as described above) for the determination of VO_2 and VCO_2 and breath samples were collected into Exetainers as described above for subsequent mass spectroscopy analysis of ^{13}C enrichment in the expired breath. In addition to that, blood samples were collected every 15 minutes during the SS and at the end of the TT. TT was run as described in the General methods section. Following the TT, a final blood sample was collected.

5.2.5. Blood analyses

Venous blood samples (~6 mL) were collected into EDTA tubes, stored and analysed as described in the General methods chapter for glucose and lactate.

5.2.6. Gas exchange measurements

Fat and carbohydrate oxidation rates were calculated using stoichiometric equations as described in the General methods chapter.

The isotopic enrichment was expressed as δ per millilitre difference between the $^{13}C/^{12}C$ ratio of the sample and a known laboratory reference standard.

The percentage of elimination of ingested ^{13}C bicarbonate was calculated according to the following equation (Leese *et al.*, 1994):

$$\%^{13}C_{elimination} = \frac{85 \cdot V_{^{13}CO_2}}{0.0224 \cdot m_{bicarb}} \cdot 100$$

Where 85 represents molecular weight of ingested sodium bicarbonate, $V_{^{13}CO_2}$ volume of expired $^{13}CO_2$ (L), 22.4 L is volume of air occupied by 1 mol of CO_2 and m_{bicarb} mass (mg) of ingested bicarbonate. $V_{^{13}CO_2}$ was calculated by multiplying the volume of expired gas and the atom per cent excess of $^{13}CO_2$.

The ingested carbohydrate oxidation rate during SS, representing oxidation of the carbohydrates ingested during recovery, were calculated as described in the General methods chapter.

Heart rate, rate of perceived exertion and gastrointestinal comfort

During recovery, gastrointestinal comfort (GC) was assessed every hour using a 10-point Likert scale (Thorburn *et al.*, 2006) that included assessment of experience of nausea, stomach fullness and abdominal cramping. Heart rate (HR) values were obtained at 15-min intervals during the SS via a heart rate strap (H7, Polar, Kempele, Finland) which was connected via Bluetooth® to a watch (Ambit 3 Sport, Suunto, Vantaa, Finland). Simultaneously, every 15-min participants were asked to report the rate of perceived exertion (RPE) using 6-20 scale (Borg, 1982) and GC using the same questionnaire as during the recovery.

5.2.7. Statistics

Sample size was determined using g*power software (Faul *et al.*, 2007) assuming an effect size of 1.84 as observed previously for differences in subsequent exercise capacity between the two carbohydrate conditions (Maunder, Podlogar and Wallis, 2018). It was calculated that to achieve statistical power of 80% to detect differences between the two conditions, a minimum of 7 participants should complete both experimental trials. A two-way ANOVA for repeated measures was used to compare

differences in substrate utilization and blood metabolites at different time points. Where significant effects were observed by ANOVA for time x condition interaction, post-hoc comparisons were made with paired t-tests with the Tukey test applied to account for multiple comparisons. TT performance and amount of work completed during glycogen reducing exercise sessions were initially tested for normality using Shapiro-Wilk test. If normality was met, a t-test was used to analyse the data, otherwise the nonparametric Wilcoxon signed-rank test was used. All values are presented as mean ± SD. Statistical significance was set at $p < 0.05$. Data analysis was performed using SPSS (Version 24; SPSS Inc., Chicago, IL, US), Prism (Version 8; GraphPad Software, San Diego, CA, US) and Microsoft Excel (Microsoft, Redmond, Washington, USA).

5.3. Results

5.3.1. Bicarbonate elimination

The D-max method (Cheng *et al.*, 1992) was used to determine the point when a plateau in excretion of ^{13}C atoms had been reached. This point occurred at 21.04 ± 0.87 minutes. Example of the calculation for one participant can be seen in Figure 5.1.

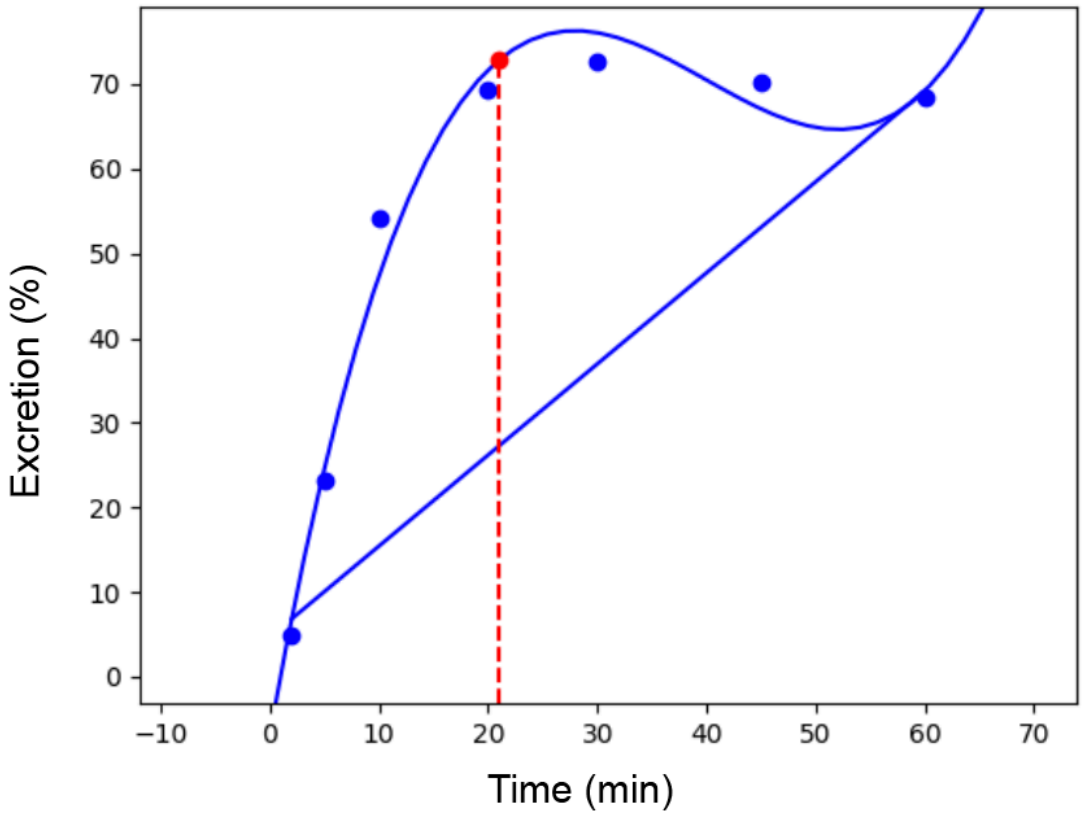

Figure 5.1. Elimination of ingested ^{13}C labelled bicarbonate.

5.3.2. Glycogen-reducing session

Participants completed 1367 ± 421 and 1298 ± 571 kJ of work during the glycogen reducing sessions in GLU+MD and FRU+MD, respectively, without any statistically significant differences between the trials ($p = 0.662$). Neither were there any differences in the number of completed stages at 90, 80 and 70 % Wmax between all three conditions ($p = 0.458$).

5.3.3. TT Performance

Results for TT can be seen in Figure 5.2, Participants finished the TT in 37.41 ± 3.45 (GLU+MD) and 37.96 ± 5.20 minutes (FRU+MD) without any significant difference between them ($p = 0.547$).

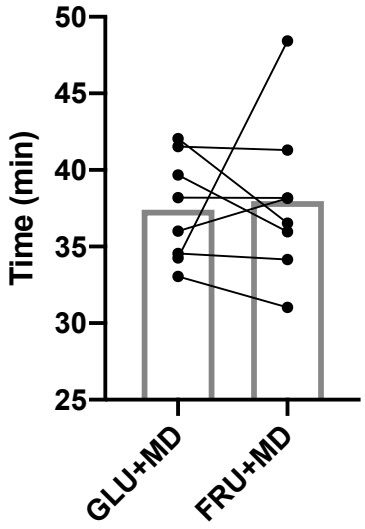

Figure 5.2. Time required to complete the TT. Bars represent mean, while dots and connecting lines represent individual participants.

Carbohydrate and fat oxidation rates, VO_2, RPE and HR during steady state exercise

Ingested and endogenous (i.e. carbohydrates stored in the body before the onset of carbohydrate ingestion during recovery) carbohydrate oxidation rates are presented in Figure 5.3. There was no difference in total carbohydrate oxidation rates between GLU+MD and FRU+MD ($p = 0.080$). There was only a main effect of time for overall carbohydrate oxidation ($p < 0.001$). Based on the findings that oxidation of ingested

carbohydrates could only be accurately determined after ~21 minutes of exercise, statistical analysis for oxidation of ingested and endogenous carbohydrates has only been calculated from 30-min time point onwards. There was a time x condition interaction in ingested carbohydrate oxidation rates ($p = 0.003$) with higher oxidation rates of ingested carbohydrates at 30 and 45-min time points in FRU+MD as compared to GLU+MD ($p < 0.001$). Inversely, there was a trend towards higher endogenous carbohydrate oxidation rates in GLU+MD at 30-min time point during SS (time x condition interaction; $p = 0.061$) Furthermore, there was a tendency for higher fat oxidation rates in GLU+MD versus FRU+MD ($p = 0.054$) and an overall main effect of time for fat oxidation ($p < 0.001$).

There was no difference in oxygen uptake (VO_2) ($p = 0.157$), RPE ($p = 0.366$) or HR ($p = 0.570$) between both conditions (Table 5.1). However, there was a main effect of time for RPE ($p < 0.001$).

Table 5.1. Exercise intensity, fat oxidation rates, rate of perceived exertion and heart rate during the steady state part of exercise bout.

Time (min)	Trial	VO_2 ($L \cdot min^{-1}$)	RPE (6-20)	HR (bpm)
15	GLU+MD	2.53 ± 0.26	12 ± 1	137 ± 12
	FRU+MD	2.51 ± 0.27	12 ± 1	137 ± 10
30	GLU+MD	2.56 ± 0.31	13 ± 2	138 ± 11
	FRU+MD	2.52 ± 0.27	13 ± 1	138 ± 11
45	GLU+MD	2.53 ± 0.26	13 ± 1*	139 ± 11
	FRU+MD	2.50 ± 0.30	13 ± 2*	135 ± 11
60	GLU+MD	2.63 ± 0.29	14 ± 2	138 ± 10
	FRU+MD	2.51 ± 0.27	14 ± 1	135 ± 12

* Significantly different to a previous time point

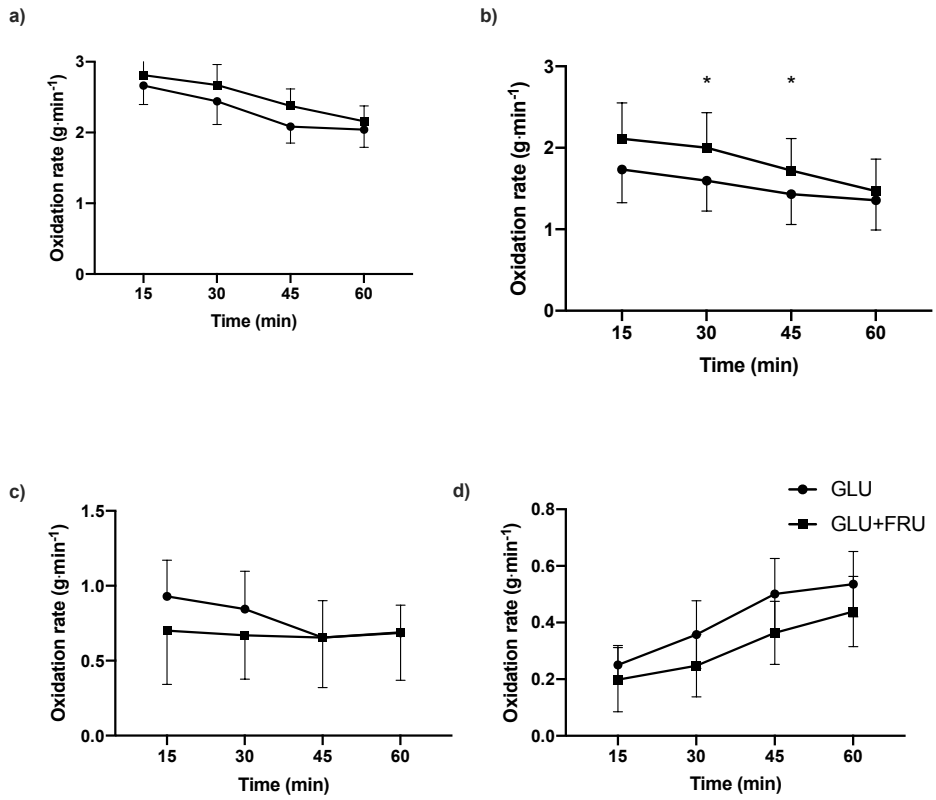

Figure 5.3. a) Total carbohydrate oxidation rates, b) ingested carbohydrate oxidation rates and c) endogenous carbohydrate oxidation rates d) fat oxidation rates during SS.

* Significantly different between GLU and GLU+FRU

5.3.4. Blood metabolites

Plasma metabolites (Figure 5.4) were analysed separately for the recovery and subsequent exercise bout. Although care was taken to maintain patency of inserted cannulas, it was sometimes not possible to obtain blood samples from all

participants. Thus, we were only able to analyse samples for 5 and 6 participants' during recovery and the subsequent exercise bout, respectively.

There were no differences in glucose concentrations between trials during recovery ($p = 0.163$), however there was a main effect of time ($p < 0.001$). For lactate concentrations there was a significant interaction between condition and time ($p = 0.006$) and post-hoc analysis showed significantly higher concentrations 1-h ($p = 0.03$), 2 hrs ($p < 0.001$), and 3 hrs ($p = 0.01$) into recovery in FRU+MD.

During the SS, there was a condition x time interaction for glucose ($p < 0.001$). Concentrations of plasma glucose were higher in GLU+MD at 45-min ($p < 0.001$), 60-min ($p < 0.001$) and at the end of the TT ($p < 0.001$), whereas there was only a significant effect of time for lactate ($p < 0.001$).

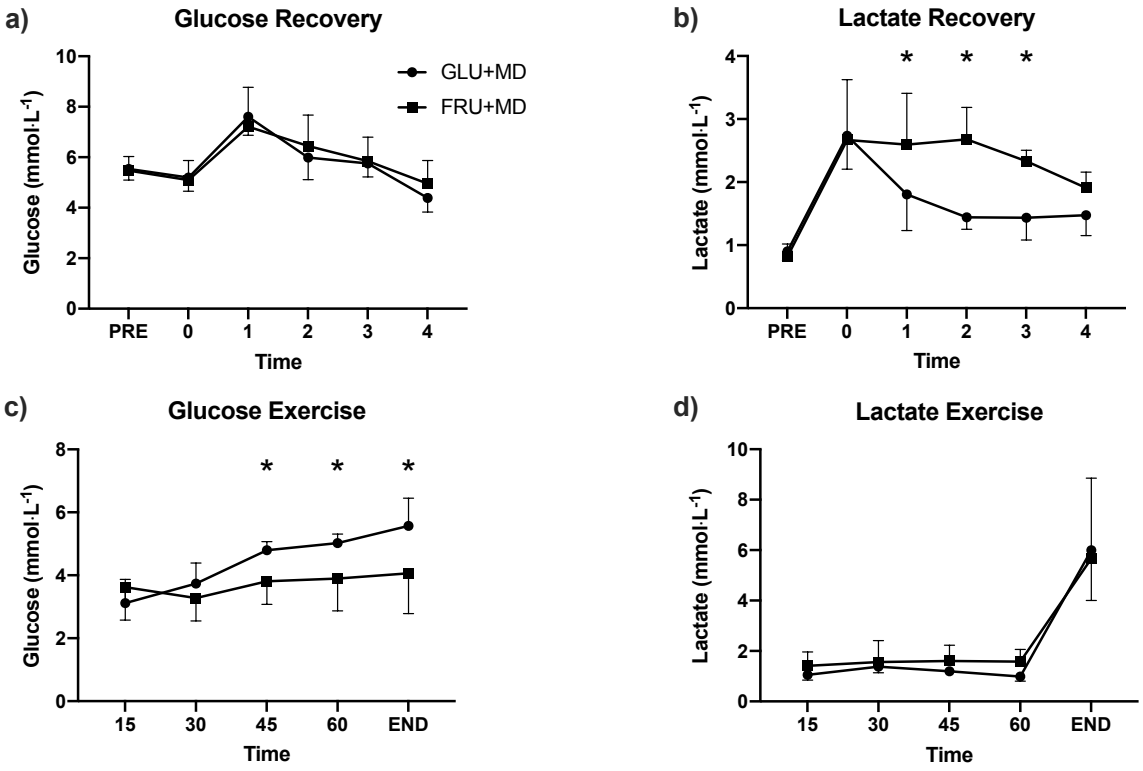

Figure 5.4. Plasma a) glucose during the 4 hrs recovery period, b) lactate during the 4 hrs recovery period, c) glucose during the steady state exercise and d) lactate during steady state exercise.

* Significantly different between GLU and GLU+FRU

5.3.5. GI comfort

There was no difference in GI comfort between trials in any of the measures ($p > 0.05$). During recovery average values for nausea, stomach fullness and abdominal cramping were 1.0 ± 0.0 and 1.0 ± 0.0; 1.2 ± 0.4 and 1.0 ± 0.1; and 1.6 ± 1.6 and 1.9 ± 1.7 for GLU+MD and FRU+MD, respectively. Average values during exercise for

nausea, stomach fullness and abdominal cramping being 1.3 ± 0.4 and 1.2 ± 0.4; 1.0 ± 0.1 and 1.1 ± 0.2; and 1.8 ±1.3 and 1.6 ± 1.0 for GLU+MD and FRU+MD, respectively.

5.4. Discussion

The main aim of the present study was to assess whether ingestion of a combination of fructose and glucose as compared to glucose only during a 4 hrs recovery period after a glycogen reducing exercise protocol would improve subsequent pre-loaded cycle TT performance. Contrary to expectations, which were based on a previous observation of improved subsequent exercise capacity with post-exercise fructose-glucose versus glucose only provision (Maunder, Podlogar and Wallis, 2018), there were no differences in performance outcomes between the two conditions. Accordingly, these data are discussed in the context of previous work and the metabolic and perceptual observations also made in the present study.

Maunder et al. (2018) investigated effects of fructose and glucose-based carbohydrates on exercise recovery estimated oxidation rates of carbohydrates ingested in recovery during subsequent exercise. However, due to the duration of the subsequent exercise bout, comparisons between conditions were limited to the first 30 minutes of exercise. Most researchers report exogenous carbohydrate oxidations rates from 60 minutes of exercise onwards citing the potential to underestimate

oxidation rates due to retention of ^{13}C atoms within the body's bicarbonate pool (Jeukendrup and Jentjens, 2000). In the present study, breath $^{13}CO_2$ recovery from ingested ^{13}C labelled bicarbonate was determined as a proxy for the turnover of the bicarbonate pool and it was found that on average ~21 minutes were needed for the ingested ^{13}C label to be eliminated. This observation is interpreted to mean that during moderate intensity exercise the aforementioned caution in reporting exogenous carbohydrate oxidation rates during the first 60 minutes of exercise is likely unnecessary. At least from a perspective of ^{13}C retention within the body's bicarbonate pool, exogenous carbohydrate oxidation rates can be accurately determined from as early as 30 min after ^{13}C labelled carbohydrate ingestion during exercise. This approach has been adopted in the present study and the finding will be useful for other researchers wishing to assess exogenous carbohydrate oxidation whilst employing protocols with a similar exercise intensity.

During SS higher oxidation rates of ingested carbohydrates in FRU+MD as compared to GLU+MD were observed from 30 to 45 min). Previous research would suggest the higher ingested oxidation rates were subsequent to higher carbohydrate availability in FRU+MD due to enhanced replenishment of liver (Décombaz *et al.*, 2011; Fuchs *et al.*, 2016) and similar replenishment of muscle glycogen stores (Wallis *et al.*, 2008; Trommelen *et al.*, 2016). There was a trend towards higher endogenous carbohydrate oxidation rates in the first half of the SS in GLU+MD. The differential pattern in exogenous and endogenous carbohydrate oxidation in FRU-MD versus GLU-MD converged to be similar by the end of the SS exercise bout, as was total CHO

oxidation at that point. Furthermore, there was a clear trend for higher carbohydrate oxidation rates in FRU+MD and that might have offset the potential benefits of higher carbohydrate storage. In contrast, in the studies by Maunder et al. (2018) and by Gray et al. (2020), which used a slightly higher exercise intensity (70% VO_2max) than the present study, carbohydrate oxidation rates were comparable between both treatments. The extension in endurance running capacity with FRU+MD in the study by Maunder et al. was temporally associated with higher total and ingested CHO oxidation at the point of fatigue in the GLU+MD condition, while this has not been observed by Gray et al. Whilst it is not possible to say with certainty, it could be that at higher exercise intensities used by Maunder and colleagues and Gray and colleagues the larger storage of carbohydrates in FRU+MD enabled participants to exercise for longer.

As expected, during the recovery period plasma lactate concentrations were elevated in FRU+MD which is consistent with previous studies (Wallis *et al.*, 2008; Fuchs *et al.*, 2016; Maunder, Podlogar and Wallis, 2018; Gray *et al.*, 2020), likely a result of fructose conversion into lactate by the liver (Tappy and Le, 2010; Sun and Empie, 2012). Higher plasma lactate concentrations likely led to higher total carbohydrate oxidation rates during the recovery (not measured in present study) as observed previously (Rosset *et al.*, 2017; Gray *et al.*, 2020), which could contribute to lower net carbohydrate availability during the subsequent exercise bout in FRU+MD even if overall carbohydrate storage was higher. This difference in plasma lactate levels subsided by the end of recovery and there was no difference in lactate concentrations

during the SS exercise. However, this does not necessarily mean that lactate flux did not continue to be elevated in FRU+MD, as increased flux could potentially explain higher oxidation rates of ingested carbohydrates during the SS exercise (Lecoultre *et al.*, 2010) especially considering the splanchnic-to-muscle lactate shuttle and given that lactate's preferential fate is oxidation (Brooks, 1986). On the other hand, glucose concentrations did not differ during the recovery, remained stable on average during the SS in FRU+MD but were higher in the second part of the SS and at the end of TT in GLU+MD. A similar finding was observed previously (Maunder, Podlogar and Wallis, 2018) where glucose concentrations did not differ during the first 30-min of the exercise, but were higher in GLU+MD at the point of fatigue. That glucose concentrations in GLU+MD diverged from FRU+MD could have been a result of an ongoing absorption that is a consequence of a limited transport of glucose across the intestinal membrane (Jeukendrup and Jentjens, 2000) and whereas in FRU+MD absorption was complete during the recovery period, whilst it might not have been in GLU+MD. In the absence of performance differences between conditions, it thus seems unlikely that different plasma glucose concentrations influenced the performance outcomes. However, looking at the TT performance results, there appears to be an outlier whose performance was drastically worse in FRU+GLU as compared to GLU. This participant's plasma glucose concentrations reached a nadir of 2.1 mmol $\cdot$ L^{-1} at 30-min time point and stayed below 3 mmol $\cdot$ L^{-1} for the whole SS part of exercise. At the end of TT, concentration increased to 3.11 mmol $\cdot$ L^{-1}. Low plasma glucose concentrations could have played a role in the performance

outcomes of this participant, however, removing him from the results does not change the lack of significant differences in performance outcomes of the study ($p = 0.156$).

Previous studies of carbohydrate feeding during exercise (Jentjens, Venables and Jeukendrup, 2004; Currell and Jeukendrup, 2008a; Triplett *et al.*, 2010) have typically observed improved gut comfort with combined fructose and glucose feeding, possibly due to greater absorption than with glucose only feeding. Some observed performance benefits of fructose-glucose feeding during exercise have thus been attributed to improved gut comfort (Rowlands *et al.*, 2015). Although suggested as a potential contributor towards improvements in exercise running capacity in FRU+MD in previous work (Maunder, Podlogar and Wallis, 2018), it has to be noted that the study did not find any statistically significant differences in GI comfort. The authors only speculated that this might contribute to overall findings due to an observed trend towards higher reported GI distress in GLU+MD condition. Reduced gut comfort would likely be more prevalent among runners than cyclists likely due to a higher mechanical stress as a result of horizontal movement of the body during running (De Oliveira, Burini and Jeukendrup, 2014), and this has been documented (Peters *et al.*, 2000). In the present study cycling was used as an exercise modality to try to minimise the effects that GI comfort might have on the study outcomes. In fact, the incidence of GI discomfort was practically non-existent in both conditions. This is in line with a recent work (Gray *et al.*, 2020) that observed no differences in gastrointestinal discomfort. Collectively it seems that current evidence does not

support the argument that short-term recovery of exercise performance/capacity is affected by GI comfort as a result of ingestion of different types of sugars.

This study is not without limitations. The menstrual cycle of the single woman participant was not controlled and given well documented effects menstrual cycle on metabolism, this could have influenced the results (Oosthuyse and Bosch, 2010). However, it is important to note that neither her performance, substrate oxidation nor plasma metabolite responses were deviant from the rest of the sample. Nonetheless, caution is needed before generalising the results of the present study to women athletes. Furthermore, it has to be acknowledged that plasma metabolite responses are not representative of the whole sample due to difficulties obtaining blood samples in 3 participants. However, close inspection of these participants' TT and substrate oxidation data reveals trends similar to those observed in other participants, which likely means that plasma metabolite responses would have been similar. In addition to that, lack of muscle and liver glycogen measurements present a limitation as well as assuming that protein oxidation during exercise was negligible.

In summary, the present study demonstrates that ingestion of a combination of fructose and glucose as opposed to glucose only in recovery after an exhaustive exercise bout increases oxidation rates of ingested carbohydrates during subsequent exercise. However, in the conditions of the present study subsequent pre-loaded cycle time trial performance was not improved when fructose-glucose was provided during exercise recovery. Further research is required to better understand if and

when combined ingestion of fructose and glucose during short term recovery from exhaustive exercise can improve subsequent exercise performance.

This study shed more data on metabolism and performance effects of fructose-glucose ingestion during a recovery period after an exhaustive exercise bout. As already discussed, the research area of fructose-glucose co-ingestion has been relatively well researched. On the other hand, very little is known about the effects that in recovery ingestion of another monosaccharide, namely galactose. The next experimental chapter explored the effects of galactose ingestion alone or in combination with glucose on restoration of muscle glycogen content as well as effects on metabolism during the subsequent exercise bout.

6. Effectiveness of combined galactose-glucose ingestion as compared to galactose or glucose only on post-exercise muscle glycogen repletion and metabolism during the subsequent exercise bout

This study was funded by a grant awarded to Dr Gareth Wallis by the USA National Dairy Council. As part of the grant, bio-statistical support was provided by Prof. David Rowlands (Massey University, New Zealand. Accordingly, the statistical analysis of the data presented herein was undertaken by Prof. Rowlands. Tim Podlogar carried out the experiments, analysed the samples and interpreted the data.

Abstract

It is important to replenish glycogen stores as efficiently as possible in the post-exercise period. Research shows that combining different sugar types (i.e., fructose and glucose) results in optimal replenishment of total body glycogen stores. While galactose has been shown to expedite repletion of liver glycogen when combined with glucose, there is no data available on muscle glycogen.

The aim of this study was to assess the effectiveness of galactose intake alone, in combination with glucose and glucose only on post-exercise muscle glycogen

repletion and explore the whole-body metabolism during the subsequent exercise bout.

Nine participants took part in this double-blinded randomised study (7 men, 2 women; VO_2peak: 51.1 ± 8.7 ml · kg^{-1} · min). Each of the three experimental trials consisted of a glycogen reducing exercise bout followed by 4 hrs of recovery and a 90-min steady state cycling exercise bout (SS) at 50% Wmax. During recovery, carbohydrates were provided at a rate of 1.2 g · kg^{-1} · h^{-1} in different compositions: glucose only (GLU), galactose-glucose (GAL+GLU; 1:2 ratio) and galactose only (GAL). Muscle glycogen concentration was determined from skeletal muscle biopsies of the vastus lateralis obtained at the start and at the end of recovery period. Substrate oxidation rates were determined during the SS. Venous blood samples were analysed for blood metabolites.

Replenishment of muscle glycogen was superior with GLU (+155 ± 22 mmol · kg dry mass $(DM)^{-1}$) relative to GAL+GLU (+97 ± 82 mmol · kg DM^{-1}; p = 0.010) and GAL (+118 ± 66 mmol · kg DM^{-1}; p = 0.024), but was not different between GAL and GAL+GLU (p = 0.426). During recovery, plasma glucose and insulin concentrations were significantly higher in GLU and GAL+GLU as compared to GAL. Plasma galactose concentration in recovery was higher in GAL as compared to GLU and GAL+GLU with no significant difference between GAL+GLU and GLU. During SS, oxidation of during recovery ingested carbohydrates was highest in GLU, followed by

GAL+GLU and was lowest in GLU. Total carbohydrate oxidation was lowest in GAL, followed by GAL+GLU and GLU.

Superior muscle glycogen synthesis was observed with GLU, suggesting GAL+GLU may not be optimal for the short-term post-exercise replenishment of total body glycogen stores. Interestingly, GAL was equally effective as GAL+GLU despite absence of increased plasma glucose or insulin concentrations.

6.1. Introduction

Carbohydrates stored within the body (primarily located in the liver and muscle as glycogen) are a predominant fuel for prolonged strenuous exercise (van Loon *et al.*, 2001) and depletion of those stores has been shown to coincide with fatigue (Bergström *et al.*, 1967; Casey *et al.*, 2000). Because glycogen stores are finite, sports nutrition research has focused on approaches on how to reduce reliance on glycogen during exercise and how to promote a quick recovery of the stores in the post-exercise period (Burke *et al.*, 2011). Current sports nutrition guidelines advise athletes to consume moderate to high glycaemic index carbohydrates as soon as practical in the post-exercise period at a rate of 1.0-1.2 $g \cdot kg^{-1} \cdot h^{-1}$. These guidelines are based on studies assessing replenishment of muscle glycogen content and do not consider different rates of liver glycogen restoration as a consequence of ingestion of different types of sugars (i.e., fructose and galactose). This, as discussed in Chapter 1, plays

an important role in the post-exercise recovery period. Before being transported from the intestine to the bloodstream, all carbohydrates are broken down to monosaccharides which differ in subsequent metabolism and differentially impact different glycogen storage sites (i.e., fructose and galactose preferentially affect liver glycogen synthesis).

Recent research indicates that the most effective post-exercise nutrition strategy would be the one that optimises both liver and muscle glycogen synthesis (Wallis and Wittekind, 2013; Fuchs, Gonzalez and van Loon, 2019). Combining different sugar types (i.e., fructose and glucose) has been shown to result in a similar muscle glycogen, but higher liver glycogen repletion in the post-exercise recovery period (Nilsoon and Hultman, 1974; Casey *et al.*, 2000; Wallis *et al.*, 2008; Décombaz *et al.*, 2011; Fuchs *et al.*, 2016; Trommelen *et al.*, 2016). However, when fructose is ingested or infused alone, it leads to inferior replenishment rates of muscle glycogen in the post exercise period as compared to isoenergetic provision of glucose or fructose-glucose combinations (or sucrose) (Bergström and Hultman, 1967; Blom *et al.*, 1987). More recently, combining fructose and glucose has also been found to positively affect subsequent exercise capacity (Maunder, Podlogar and Wallis, 2018; Gray *et al.*, 2020) as compared to glucose, but such benefits have so far not been shown for subsequent exercise performance (Chapter 3).

In contrast, little is known about the monosaccharide galactose. Galactose is most commonly consumed as part of lactose that is present in milk and consists of glucose

and galactose bound together with a glycosidic bond. In the intestine, galactose is absorbed using the same transporters as glucose (i.e., sodium glucose-linked transporter 1 - SLGT1 and glucose transporter 2 – GLUT2 (Daniel and Zietek, 2015)), however subsequent metabolism diverges. It is currently believed that galactose first needs to be taken up by the liver and be metabolised primarily via the Leloir pathway to glucose-6-phosphate (Hansen and Gitzelmann, 1975) and thus in this respect has similar characteristics to fructose, which also needs to be metabolised by the liver. Interestingly, galactose-glucose ingestion also results in enhanced repletion of liver glycogen stores in a similar way as fructose-glucose co-ingestion (Décombaz *et al.*, 2011). This implies that combining galactose and glucose could affect muscle glycogen synthesis in a similar way as a fructose-glucose combination (i.e., similar repletion rates as glucose) (Wallis *et al.*, 2008). In support of this hypothesis is the evidence that chocolate milk consumption in the post-exercise recovery period can lead to comparable replenishment of muscle glycogen content as an isocaloric maltodextrin containing beverage (Ferguson-Stegall *et al.*, 2011).

The purpose of the present study was to test the hypothesis that galactose and glucose co-ingestion would be as effective in replenishment of muscle glycogen stores as glucose, while ingestion of galactose only would result in suboptimal replenishment, so being similar in this respect to when fructose is consumed in isolation. A second aim of the study was to explore how the ingestion of those carbohydrate sources affect metabolism (i.e., substrate utilisation) during subsequent moderate intensity steady state exercise.

6.2. Methods

6.2.1. Participants

Nine healthy, endurance-trained participants (7 men, 2 women) accustomed to cycling exercise, provided written informed consent and completed the study that was approved by West Midlands – Black Country Research Ethics Committee (REC/NRES Study Number: 19/WM/0150). The participant's mean age, body mass, height, maximal oxygen uptake (VO_2peak), and maximal cycle ergometer power output (Wmax) were 26 ± 8 years, 70.6 ± 8.6 kg, 175 ± 10 cm, 3.6 ± 0.8 L · min^{-1} (51.1 ± 8.7 mL · kg^{-1} · min^{-1}) and 330 ± 80 W (4.7 ± 1.0 W · kg^{-1}), respectively.

6.2.2. Experimental design

After preliminary testing, each participant completed a familiarisation and three experimental trials. Each experimental trial commenced with an exhaustive, glycogen reducing exercise bout, followed by a 4 hrs of recovery period and then a (up to) 90 min steady state (SS) exercise bout. Carbohydrate drinks were provided during the recovery period in each trial at a rate of 1.2 g · kg^{-1} · h^{-1}. Trials differed in the type of carbohydrates provided to participants. They either received galactose (GAL), glucose (GLU) or galactose and glucose (GAL+GLU) in a 1:2 ratio. The study was double-blinded, and the order of the trials was randomized. Muscle glycogen

concentrations were determined from skeletal muscle biopsies obtained from the vastus lateralis muscle before a glycogen reducing exercise bout during the familiarisation session (i.e., estimate of typical resting glycogen content) and then in each experimental trial immediately after the exhaustive exercise bout and following the 4 hrs recovery period. Venous blood samples were collected during the recovery period and during the SS and subsequently analysed for blood metabolites. During SS the oxidation rate of the carbohydrates that were ingested during recovery period was determined via the use of ^{13}C label isotope methodology.

6.2.3. Preliminary testing and familiarization trial

Participants performed an incremental test to exhaustion to determine VO_2peak and Wmax on a cycle ergometer using the protocol described in the General methods chapter.

Participants meeting the study's minimal VO_2peak criteria (≥ 45 $mL \cdot kg^{-1} \cdot min^{-1}$ for men and ≥40 $mL \cdot kg^{-1} \cdot min^{-1}$ for women) were then scheduled for the familiarisation trial. This trial differed from the experimental trials in that only one muscle biopsy taken, and it was obtained before the glycogen reducing exercise session to obtain baseline glycogen concentration. Further, there was no blood sampling involved. The trial was also used to quantify any background shift in $^{13}CO_2$ production during the SS exercise

to enable a more accurate determination of ingested carbohydrate oxidation rates (in detail described in the general methods chapter).

6.2.4. Experimental trials

Participants entered the laboratory at ~7:00 after not eating from 22:00 the day before. They were asked to replicate the diet and activity patterns of the day preceding each experimental trial. The trial started with a glycogen reducing exercise as described in detail in General methods chapter.

Participants then dismounted the ergometer and lay supine on a bed and the first muscle biopsy was obtained using the suction-modified Bergström needle biopsy technique (Evans, Phinney and Young, 1982) from the vastus lateralis muscle through a skin incision under a local aesthesia (1% lidocaine). Following the procedure, an indwelling catheter was placed in an antecubital arm vein and the first venous blood sample was obtained.

Immediately upon obtainment of the blood sample, participants ingested an initial bolus (460 mL) of one of the three carbohydrate drinks. During recovery, participants passively rested for 4 hrs, during which sedentary activities such as reading and use of laptops were permitted. 220 mL doses of the test beverage were provided every 30-min during exercise such that the total fluid intake during the recovery period was

2.0 L. Composition of the drink was either glucose (Roquette, Lestrem, France or BulkPowders, Essex, UK for familiarisation and experimental trials, respectively) (GLU), galactose (Galaxtra, Solace Nutrition, Connecticut, USA) (GAL) or galactose (Galaxtra, Solace Nutrition, Connecticut, USA) and glucose (BulkPowders, Essex, UK) mixture in a 1:2 ratio. Carbohydrates ingested during the recovery of the familiarization trial were of naturally low ^{13}C abundance (–26.42 δ $^{13}C_{V\text{-}PDB}$ (‰)) whereas during the experimental trials they were either naturally high in ^{13}C abundance (–11.65 δ $^{13}C_{V\text{-}PDB}$ (‰)) for GLU or a small amount of galactose tracer (D-Galactose, Cambridge Isotope Laboratories, Inc; Massachusetts, USA) was added to achieve a comparable ^{13}C abundance to GLU (–11.98 ± 0.19 δ $^{13}C_{V\text{-}PDB}$ (‰) for GAL and (–11.76 ± 0.09 δ $^{13}C_{V\text{-}PDB}$ (‰) for GAL+GLU).

A venous blood sample was obtained every hour during the recovery with the cannula flushed with saline every 30-min to maintain patency. Further, a gastrointestinal comfort (GC) was assessed every hour using a 10-point Likert scale (Thorburn *et al.*, 2006) that included assessment of experience of nausea, stomach fullness and abdominal cramping. 4 hrs after the ingestion of the first carbohydrate containing drink a second muscle biopsy was obtained from the contralateral leg.

Immediately after the obtainment of the second muscle biopsy, participants mounted the stationary bicycle and started the second exercise session that consisted of 90-min of riding at 50% Wmax. Every 30-min participants breathed for 3-min into a mouthpiece connected to a metabolic cart. Concomitantly a blood sample was

obtained. Every 15-min heart rate (HR) values were obtained via a heart rate strap (H7, Polar, Kempele, Finland) which was connected via Bluetooth® to a watch (A300, Polar, Kempele, Finland) and participants were asked to report the rate of perceived exertion (RPE) (Borg, 1982) and GC.

6.2.5. Muscle glycogen analysis

On collection, muscle biopsy samples were dissected free of visible fat and connective tissue, immediately frozen in liquid nitrogen, and stored at −70°C until further analysis for muscle glycogen concentration. For this muscle tissue was powdered and freeze-dried. 2-5 mg of dry muscle (DM) tissue was then hydrolysed by adding 500 µL of 1 mmol · L^{-1} HCl and subsequently incubated for 2 hrs in an oven at 95°C. After cooling to room temperature, samples were neutralized with 2M NaOH. This was followed by centrifugation and the supernatant was analysed in duplicate for glucose using an automated photometric based clinical chemistry analyser (described below). Where tissue size permitted (~50 %), muscle glycogen concentration was determined in duplicate. The intraassay coefficient of variation for muscle glycogen determination was <10%.

6.2.6. Blood analyses

Venous blood samples (~6 mL) were collected into EDTA tubes, stored and analysed for glucose, lactate, glycerol, NEFA, galactose and insulin as described in the General methods section.

6.2.7. Gas exchange measurements

Fat and carbohydrate oxidation rates during SS were calculated using the approach described in the General methods chapter. Alike, oxidation of the ingested carbohydrate during SS wad determined using stable isotope mass spectroscopy as used in Chapter 5 and described in detail in the General methods chapter.

6.2.8. Statistics

The sample size was estimated from the values taken from a previous study observing muscle glycogen repletion rates of ~40 ± 20 mmol · kg^{-1} · DM^{-1}· h^{-1} and where the test-retest within-participant error was 9 mmol · kg^{-1} · DM^{-1}· h^{-1} (Wallis *et al.*, 2008). Using equations for crossover trials, and controlling for type I error (α = 0.05) at 5%, and type II error of 20% (β = 0.8), a sample size of 12 yielded the power to detect the smallest critical value of change in muscle glycogen concentration of 8.25 mmol · kg^{-1} · DM^{-1}· h^{-1}. At n=9, the critical value was a 25% increase in glycogen

resynthesis rate or 0.52xSD. Assuming galactose alone would exhibit a similar effect on muscle glycogen as fructose (i.e., muscle glycogen synthesis is ~45% lower with fructose vs. glucose), a sample size of minimum of 9 participants was considered sufficient to show different muscle glycogen synthesis rates in combined galactose-glucose or glucose alone as compared to galactose alone.

Results are presented for the Recovery period and the Subsequent Exercise bout separately. For the Recovery period muscle glycogen and blood metabolite data are presented in graphical form as raw mean ± SD. The effects of treatment on outcomes were estimated from linear mixed models (Proc Mixed, SAS 9.4, Cary, NC) with adjustment for baseline covariates for the recovery time period datasets. Fixed effects were treatment, period, and sex, with treatment adjusted for baseline concentration where necessary (i.e., immediate post-exercise sample) and for the recovery data, treatment*time; participant was a random effect, with additional variation modelled for period = 1 and sex = male. Glycogen and recovery data are analysed as difference scores with the baseline covariate. Statistical summaries are presented in tabular form expressed as baseline adjusted least squares means estimates ± 95% confidence interval (CI) and the treatment effect estimate as the least square means difference (± 95% CI), effect size and corresponding P value. Effect size was calculated as the standardised difference i.e., changes score/SD for immediate post-exercise concentrations.

For the SS substrate utilisation and blood metabolite data are presented in graphical form as raw means ± SD. The effects of treatment on outcomes were estimated from repeated-measures linear mixed model analysis of variance (Proc Mixed, SAS 9.4, Cary, NC). Fixed effects were treatment, treatment*time, period, sex; participant was a random effect, with additional variation modelled for period=1 and sex=male. Statistical summaries are presented in tabular form expressed as least squares means estimates ± 95% confidence intervals (CI), treatment effects as the least square means estimate (± 95% CI), effect size and corresponding P value. Effect size was calculated as the standardised difference, i.e., change score/pooled SD for glucose (control condition).

6.3. Results

6.3.1. Glycogen-reducing session

Participants completed 1226 ± 716, 1134 ± 458 and 1148 ± 616 kJ of mechanical work during the glycogen reducing sessions in GLU, GAL and GAL+GLU, respectively, without any statistically significant differences between the trials ($p = 0.941$). Neither were there any differences in the number of completed stages at 90, 80 and 70 % W_{max} between all three conditions ($p = 0.769$).

6.3.2. Muscle glycogen

Muscle glycogen concentration measured in rested participants during the familiarisation session was 472 ± 123 mmol · kg dry muscle mass (DM) $^{-1}$, which is in consistent with data from a recent meta-analysis for this population (Areta and Hopkins, 2018).

Figure 6.1 and Table 6.1 display the absolute and net changes in muscle glycogen content during recovery and statistical summaries, respectively. All data are n=9 with the exception of the 4 hrs post-exercise sample in GAL+GLU where, due to a participant's withdrawal part way through the recovery related to gastrointestinal discomfort, sample size is n=8.

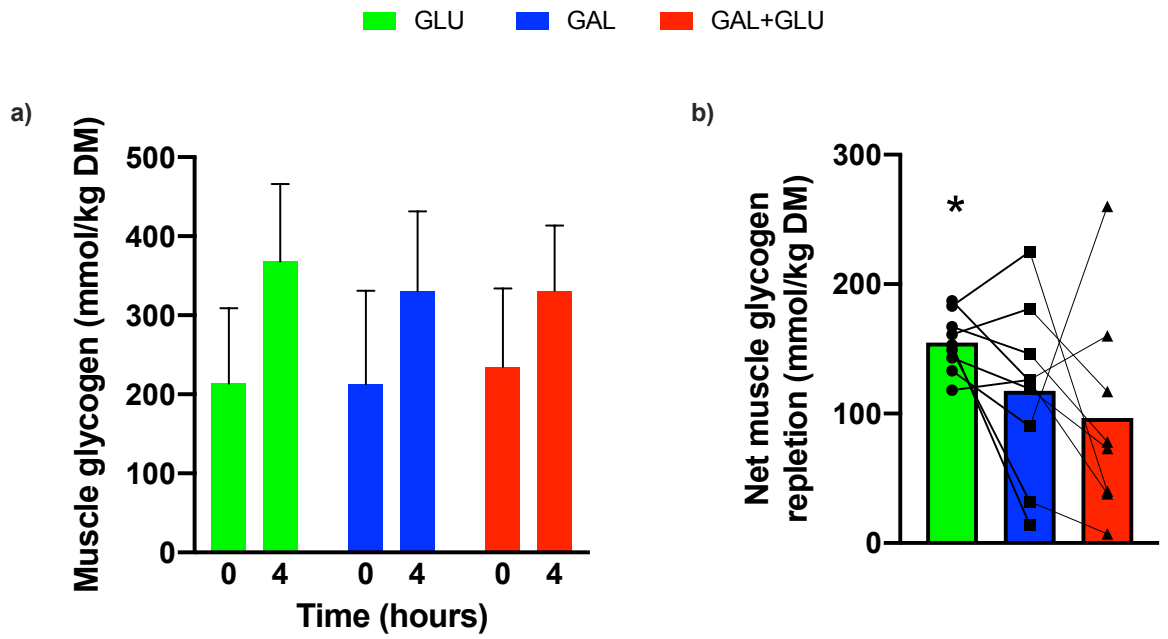

Figure 6.1. Absolute a) muscle glycogen concentrations immediately and 4 hrs post-exercise and net change b) in muscle glycogen concentration during the 4 hrs recovery period. Data are Raw ± SD, n=8-9.

* Significantly different between GLU and the other two conditions (GAL and GAL+GLU)

The increased skeletal muscle glycogen concentration in response to carbohydrate feeding during recovery from exercise was 1.3 to 1.6-fold higher with glucose feeding (155 ± 22 mmol · kg DM^{-1}) relative to combined galactose-glucose (97 ± 82 mmol · kg DM^{-1}) and galactose only (118 ± 66 mmol · kg DM^{-1}) (Figure 6.1b). There was no significant difference in net increase in muscle glycogen between GAL+GLU and GAL.

Table 6.1. Statistical summary of net change in muscle glycogen concentration during the 4 hrs post-exercise recovery period.

	Baseline adjusted LSM (95% CI)			Treatment effect as LSM difference (95% CI)		
Trial, contrast	GLU	GLU+GAL	GAL	GAL+GLU – GLU	GAL – GLU	GAL+GLU – GAL –
Estimate	165 (128, 202)	94 (57, 132)	111 (75, 148)	-72 (-121, -23)	-55 (-101, -9)	-17 (-66, 31)
Effect size	-	-	-	-0.64 (-1.46, -0.26)	-0.49 (-1.14, -0.14)	-0.15 (-0.65, 0.23)
P value	-	-	-	0.010	0.024	0.426

Units are mmol · kg DM^{-1}. LSM – Least squares means. N=8-9. Effect size is the standardised difference, i.e., change score/SD for baseline concentrations.

6.3.3. Plasma metabolites during recovery

Plasma metabolites during the recovery period are presented in Figure 6.2, whereas statistical summary is presented in Table 6.2. Blood was obtained from 8 participants due to issues with obtaining blood samples from one participant.

The plasma glucose response was similar for GLU and GAL+GLU, but 0.8 to 1.6 mmol · L^{-1} higher as compared to GAL (Figure 6.2a). Plasma insulin concentrations stayed remarkably unchanged in GAL, but rose in GLU and GAL+GLU, without being significantly different between each other in GLU and GAL+GLU (Figure 6.2b). Plasma galactose concentrations increased only in GAL and peaked at 5.6 ± 1.3 mmol · L^{-1} at 1-h time point but stayed below 0.5 mmol · L^{-1} throughout the recovery in GAL+GLU and GLU (Figure 6.2c). Plasma NEFA and glycerol concentrations were suppressed after the carbohydrate feeding was initiated but were least suppressed in GAL feeding as compared to GLU or GAL+GLU (Figures 6.2e and 6.2d). Further, concentrations were slightly, but significantly higher in GAL+GLU as compared to GLU. Plasma lactate concentrations were slightly higher in GAL and GAL+GLU feeding as compared to GLU, but not different between GAL and GAL+GLU (Figure 6.2f).

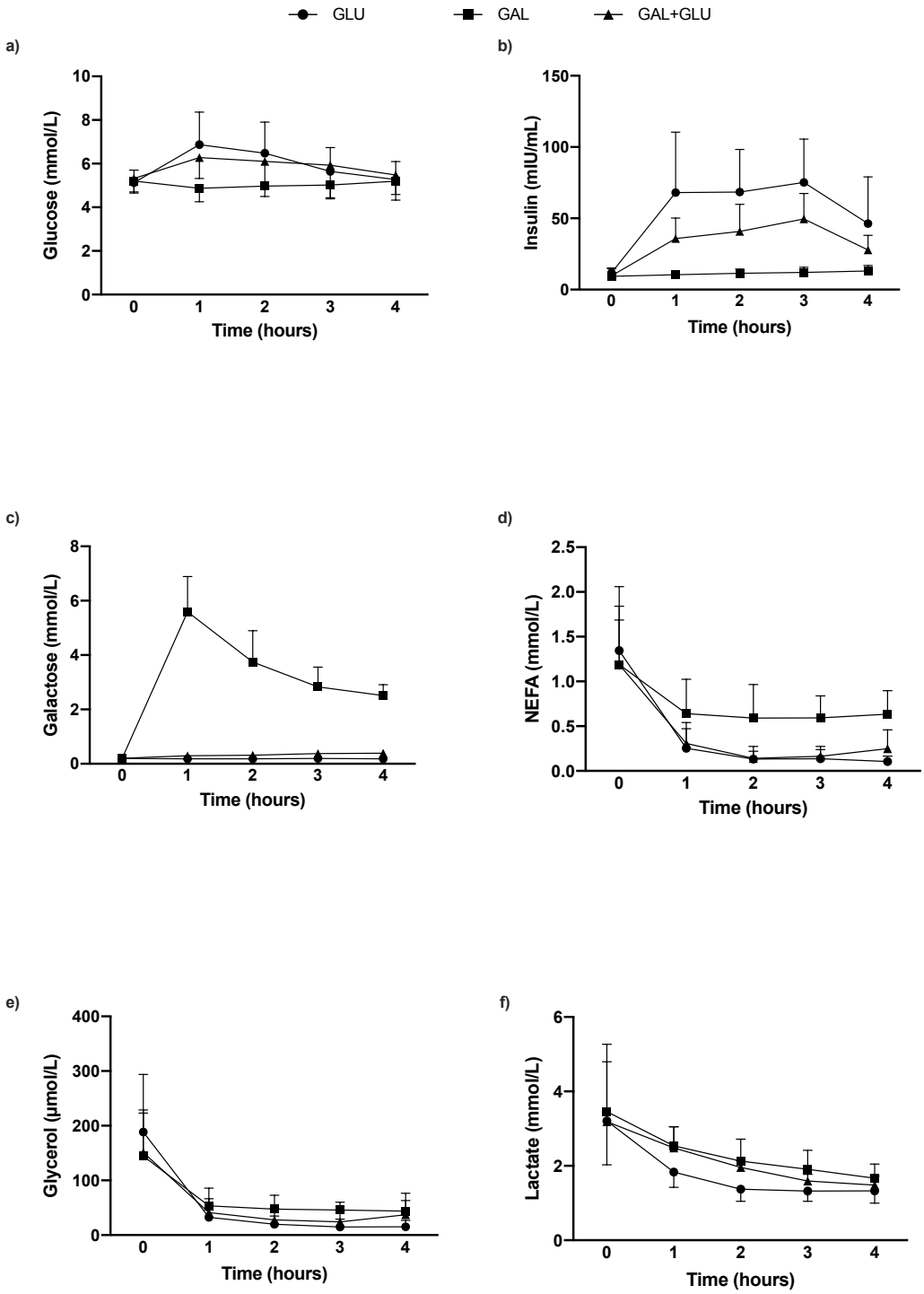

Figure 6.2. Plasma glucose a), insulin b), galactose c), NEFA d), glycerol e) and lactate f) concentration responses during the 4 hrs post-exercise recovery period. Data are raw mean ± SD. n=8.

Table 6.2. Statistical summary of plasma concentrations during the 4 hrs post-exercise recovery period.

	Baseline adjusted LSM (95% CI)			Treatment effect as LSM difference (95% CI)		
Treatment, contrast	GLU	GAL+GLU	GAL	GAL+GLU – GLU	GAL – GLU	GAL+GLU – GAL –
Glucose ($mmol \cdot L^{-1}$)						
Estimate	0.99 (0.26, 1.7)	1.01 (0.29, 1.72)	-0.61 (-1.40, 0.19)	-0.01 (-0.49, 0.52)	-1.59 (-2.17, -1.0)	-1.60 (0.96, 2.23)
Effect size	-	-	-	0.02 (-0.88, 0.92)	-2.82 (-4.06, -1.14)	2.84 (1.70, 4.18)
P value	-	-	-	0.959	<0.001	<0.001

Insulin ($\mu IU \cdot mL^{-1}$)						
Estimate	41	33	8	-8	-33	26
	(25, 57)	(22, 44)	(-9, 24)	(-23, 8)	(-47, -19)	(10, 41)
Effect size	-	-	-	-1.95	-8.53	6.56
				(-5.92, 1.89)	(-12.6, -4.95)	(2.61, 10.99)
P value	-	-	-	0.320	<0.001	0.002
Galactose ($mmol \cdot L^{-1}$)						
Estimate	0.07	0.20	3.46	0.13	3.38	-3.25
	(-0.27, 0.40)	(0.14, 0.54)	(3.10, 3.83)	(-0.11, 0.37)	(3.11, -3.66)	(-3.56, -2.94)
Effect size	-	-	-	1.7	43.5	-41.8
				(-1.3, 4.9)	(36.9, -52.5)	(-50.7, 35.1)

P value	-	-	-	0.272	<0.001	<0.001
Lactate (mmol · L^{-1})						
Estimate	-1.69 (-1.86, -1.52)	-1.23 (-1.43, -1.06)	-1.39 (-1.53, -1.24)	0.30 (0.11, 0.49)	0.44 (0.24, 0.65)	-0.14 (-0.35, 0.06)
Effect size	-	-	-	0.2 (0.07, 0.35)	0.3 (0.16, 0.46)	-0.1 (-0.24, 0.04)
P value	-	-	-	0.003	<0.001	0.168
NEFA (mmol · L^{-1})						
Estimate	-1.11 (-1.18, -1.03)	-1.00 (-1.08, -0.92)	-0.61 (-0.70, -0.52)	0.11 (0.01, 0.21)	0.50 (0.40, 0.60)	-0.39 (-0.50, -0.28)

Effect size	-	-	-	0.18 (0.01, 0.35)	0.83 (0.62, 1.10)	-0.65 (-0.90, -0.46)
P value	-	-	-	0.037	<0.001	<0.001
Glycerol ($\mu mol \cdot L^{-1}$)						
Estimate	-142 (-153, -132)	-120 (-130, -110)	-106 (-118, -93)	22 (12, 32)	37 (26, 48)	-14 (-26, -3)
Effect size	-	-	-	0.26 (0.14, 0.39)	0.42 (0.29, 0.59)	-0.17 (-0.31, -0.04)
P value	-	-	-	0.037	<0.001	0.016

LSM – Least squares means. N=8. Effect size is the standardised difference, i.e., change score/SD for baseline concentrations.

6.3.4. Substrate utilisation during subsequent exercise

Figure 6.3 and Table 6.3 display ingested, total and endogenous carbohydrate oxidation and total fat oxidation rates during the (up to) 90-min SS and statistical summaries, respectively. Datasets include 6-9 participants, as some participants could not complete the whole 90-min SS in all conditions. In none of the conditions was the occurrence of premature fatigue more frequent than in others. Total carbohydrate oxidation rates were higher in GLU, followed by GAL+GLU and were lowest in GAL (Figure 6.3a). Inversely, fat oxidation rates (Figure 6.3b) were highest in GAL, but markedly lower in GLU and GAL+GLU, where GLU resulted in slightly lower fat oxidation rates as compared with GAL+GLU (Figure 6.3b). Oxidation of the carbohydrates ingested during the recovery period during the subsequent exercise bout (Figure 6.3c) was highest in GLU, closely followed by GAL+GLU and was markedly lower in GAL. There was a reverse relationship for oxidation of endogenous carbohydrates (Figure 6.3d), as they were highest in GAL and much lower in GAL+GLU and GLU, without being different to each other.

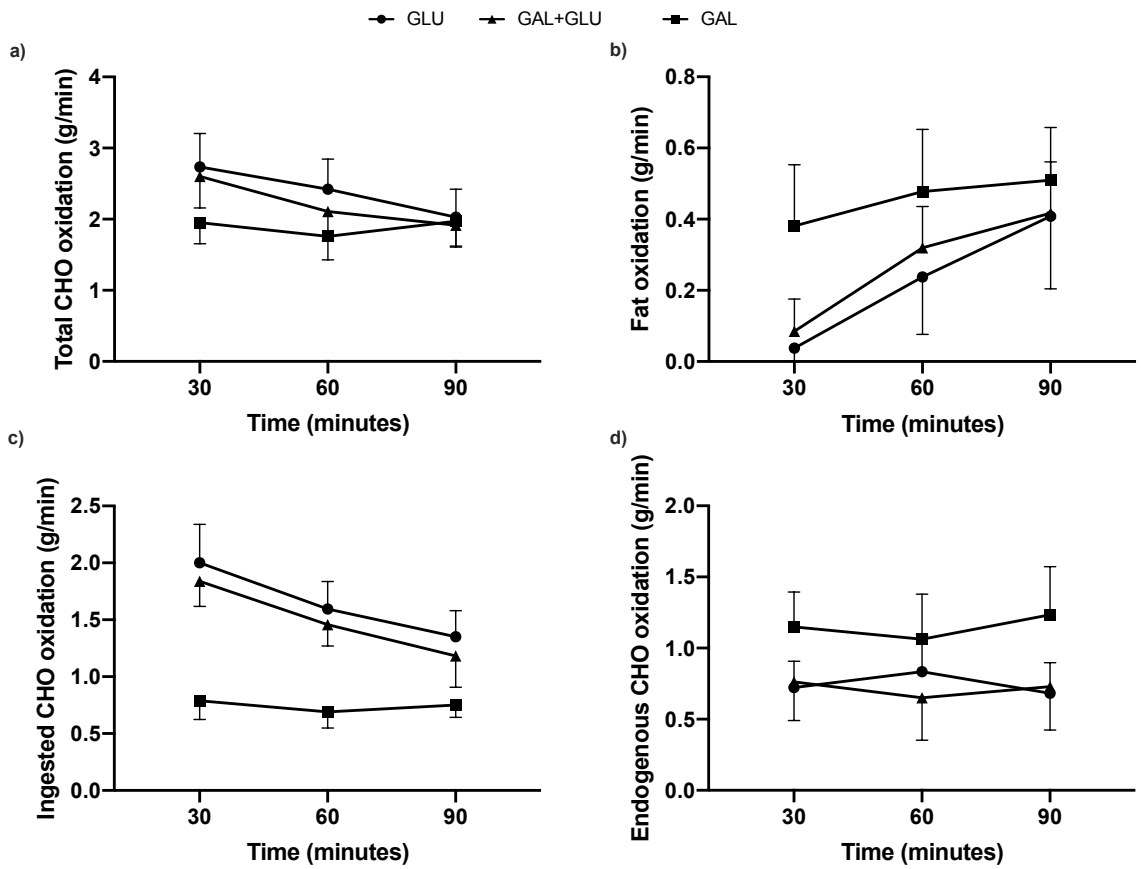

Figure 6.3. Substrate utilisation during the SS. Total carbohydrate (CHO) oxidation a); fat oxidation b); during recovery ingested CHO oxidation c); and endogenously stored CHO oxidation d) rates. Data are raw mean ± SD. n=6-9.

Table 6.3. Statistical summary of ingested and endogenous substrate oxidation during the SS.

	LSM (95% CI)			Treatment effect as LSM difference (95% CI)		
Treatment, contrast	GLU	GAL+GLU	GAL	GAL+GLU – GLU	GAL – GLU	GAL+GLU – GAL –
Total CHO ($g \cdot min^{-1}$)						
Estimate	2.27 (2.14, 2.41)	2.10 (1.96, 2.23)	1.70 (1.55, 1.84)	-0.18 (-0.33, -0.03)	-0.56 (-0.71, -0.40)	0.38 (0.23, 0.52)
Effect size	-	-	-	-0.34 (-0.68, -0.06)	-1.08 (-1.56, -0.75)	--0.74 (0.45, 1.11)
P value	-	-	-	0.024	<0.001	<0.001

Ingested CHO ($g \cdot min^{-1}$)						
Estimate	1.58 (1.50, 1.66)	1.45 (1.37, 1.53)	0.64 (0.56, 0.73)	-0.13 (-0.22, -0.03)	-0.92 (-1.01, -0.82)	0.79 (0.70, 0.88)
Effect size	-	-	-	-0.36 (-0.61, 0.10)	-2.36 (-3.24, -1.83)	2.04 (1.60, 2.75)
P value	-	-	-	0.009	<0.001	<0.001
Endogenous CHO ($g \cdot min^{-1}$)						
Estimate	0.69 (0.57, 0.80)	0.63 (0.52, 0.75)	1.04 (0.92, 1.16)	-0.05 (-0.17, 0.06)	0.35 (0.23, 0.47)	-0.41 (-0.52, -0.29)
Effect size	-	-	-	-0.25 (-0.80, 0.28)	1.59 (1.03, 2.29)	-1.83 (-2.55, -1.28)

P value	-	-	-	0.358	<0.001	<0.001
Fat ($g \cdot min^{-1}$)						
Estimate	0.17 (0.11, 0.24)	0.25 (0.18, 0.31)	0.42 (0.36, 0.49)	0.07 (0.01, 0.14)	0.24 (0.17, 0.31)	-0.17 (-0.23, -0.10)
Effect size	-	-	-	0.38 (0.04, 0.76)	1.25 (0.88, 1.73)	-0.87 (-1.27, -0.54)
P value	-	-	-	0.034	<0.001	<0.001

LSM – Least squares means. N=6-9

6.3.5. Plasma metabolites during subsequent exercise

Plasma metabolite responses during the SS are displayed in Figure 6.4, whereas statistical summaries are reported in Table 6.4. Displayed data are from n=5-8 as some participants could not complete the entire 90 min exercise in all conditions and/or difficulties were experienced obtaining blood samples during the trial.

Plasma glucose concentrations (Figure 6.4a) were significantly higher in GAL as compared to GAL+GLU and trended to be higher as compared to GLU as well. While insulin concentrations (Figure 6.4b) were lowest in GAL+GLU as compared to GLU and GAL, they were nonetheless relatively similar. Galactose concentrations (Figure 6.4c) on the other hand differed markedly between GAL and GLU/GAL+GLU. They were significantly higher in GAL+GLU as compared to GLU as well. NEFA and glycerol concentrations (Figures 6.4d and 6.4e, respectively) increased over time in all three conditions but were highest in GAL from the start.

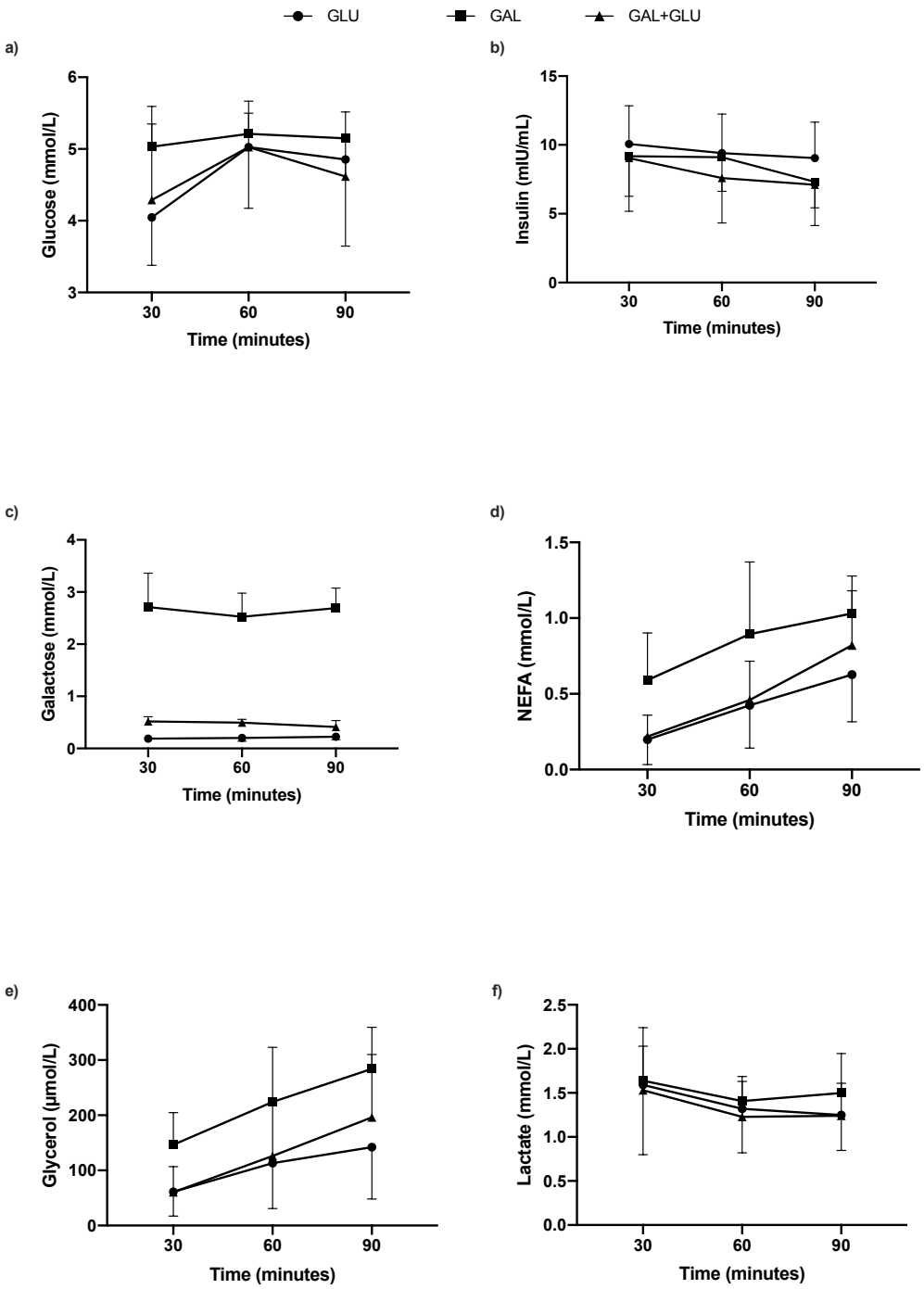

Figure 6.4. Plasma glucose a), insulin b), galactose c), NEFA d), glycerol e) and lactate f) concentration responses during SS. Data are raw mean ± SD. n=5-8.

6.3.6. Gastrointestinal comfort, rates of perceived exertion and heart rate

Gastrointestinal discomfort was virtually non-existent in all three trials given as shown by mean GC ratings on 1-10 scales (nausea, stomach fullness and abdominal cramping). Both during the recovery and during the SS mean ratings never exceeded 2. This indicates that such a high intake of carbohydrates and different sugar combinations did not negatively and differently affect gut comfort.

RPE values were very similar across GLU, GAL and GAL+GLU with mean values 13 ± 1, 14 ± 2 and 13 ± 2, respectively.

Mean HR during the SS were very similar across all three conditions with values (beats · min^{-1}) 144 ± 13 in GLU, 145 ± 12 in GAL and 146 ± 13 in GAL+GLU.

Table 6.4. Statistical summary of plasma metabolite responses during the SS.

	Least squares mean (95% CI)			Treatment effect as LSM difference (95% CI)		
Treatment, contrast	GLU	GAL+GLU	GAL	GAL+GLU – GLU	GAL – GLU	GAL+GLU – GAL –
Glucose ($mmol \cdot L^{1}$)						
Estimate	4.98 (4.64, 5.31)	4.85 (4.52, 5.18)	-5.41 (5.01, 5.81)	-0.17 (-0.56, 0.21)	0.39 (0.02, 0.79)	-0.56 (-0.97, -0.14)
Effect size	-	-	-	-0.19 (-0.61, 0.22)	0.42 (-0.01, 0.89)	-0.60 (-1.11, -0.17)
P value	-	-	-	0.374	0.064	0.01

Insulin ($\mu IU \cdot mL^{1}$)						
Estimate	11.3 (10.1, 12.5)	8.7 (7.6, 9.9)	10.3 (8.9, 11.8)	-2.6 (-4.1, -1.2)	-1.0 (-2.5, 0.6)	-1.7 (-3.2, -0.1)
Effect size	-	-	-	-0.93 (-1.53, -0.42)	0.34 (-0.91, 0.20)	-0.59 (-1.21, -0.04)
P value	-	-	-	<0.001	0.216	0.041
Galactose ($mmol \cdot L^{1}$)						
Estimate	0.21 (0.06, 0.37)	0.45 (0.29, 0.60)	2.73 (2.55, 2.92)	0.23 (0.06, 0.39)	2.52 (2.34, 2.69)	-32.29 (-2.46, -2.29)
Effect size	-	-	-	3.42 (1.02, 6.15)	37.54 (30.82, 47.54)	-34.10 (-43.70, -27.70)

P value	-	-	-	0.007	<0.001	<0.001
Lactate (mmol · L^{1})						
Estimate	1.29 (1.02, 1.56)	1.49 (1.23, 1.75)	1.68 (1.36, 2.00)	0.20 (-0.11, 0.52)	0.40 (0.06, 0.73)	-0.19 (-0.54, 0.15)
Effect size	-	-	-	0.33 (-0.17, 0.87)	0.65 (0.12, 1.24)	-0.32 (-0.91, 0.24)
P value	-	-	-	0.201	0.020	0.269
NEFA (mmol · L^{1})						
Estimate	0.19 (0.03, 0.36)	0.53 (0.36, 0.69)	0.75 (0.56, 0.94)	0.34 (0.18, 0.50)	0.55 (0.39, 0.72)	0.21 (-0.38, -0.05)

Effect size	-	-	-	1.07 (0.58, 1.66)	1.74 (1.19, 2.45)	-0.67 (-0.16, -1.25)
P value	-	-	-	<0.001	<0.001	0.014
Glycerol ($\mu mol \cdot L^{1}$)						
Estimate	82 (41, 123)	135 (95, 175)	212 (165, 259)	55 (13, 96)	131 (87, 176)	77 (-121, -32)
Effect size	-	-	-	0.64 (0.17, 1.19)	1.54 (1.00, 2.27)	-0.90 (-1.53, 0.39)
P value	-	-	-	0.012	<0.001	<0.001

6.4. Discussion

The primary aim of the present study was to investigate the effects of ingesting different carbohydrate types, namely glucose only, a galactose-glucose combination and galactose only, on muscle glycogen repletion during a 4 hrs recovery period after glycogen reducing exercise. In contrast with the initial hypothesis, combined ingestion of galactose and glucose in a 1:2 ratio at the rate of 1.2 $g \cdot kg^{-1} \cdot h^{-1}$ resulted in lower repletion rates of muscle glycogen as compared to ingesting an isocaloric amount of glucose only. As hypothesised, ingestion of galactose only was shown to be a suboptimal source for muscle glycogen repletion as compared to glucose only, but unexpectedly resulted in comparable repletion rates to combined galactose-glucose ingestion.

Due to similarities between fructose and galactose metabolism (i.e., it is typically considered that both first need to be metabolised by the liver) it was hypothesised that muscle glycogen repletion rate would have been similar in GLU and GAL+GLU, while both being superior to GAL. This hypothesis was based on previous observations that combined ingestion of fructose and glucose results in similar, while ingestion of fructose only in inferior repletion rates of muscle glycogen content in the post-exercise period as compared to glucose. However, the results showed enhanced muscle glycogen repletion in GLU as compared to GAL+GLU and GAL and no difference between GAL and GAL+GLU. While there was no significant difference

in any of the blood metabolites during the recovery between GAL+GLU and GLU, there was a trend for plasma insulin concentrations to be higher in GLU as compared to GAL+GLU which could contribute to differences in muscle glycogen repletion rates as observed previously (Young *et al.*, 1988; van Loon *et al.*, 2000). A lower insulin response would be in line with results of a previous study investigating liver glycogen synthesis when ingesting glucose only, galactose and glucose or fructose and glucose, that showed a trend for lower insulin with galactose-glucose co-ingestion as compared to the other two conditions (Décombaz *et al.*, 2011). Some studies show a similar pattern for plasma insulin concentrations when ingesting a combination of fructose and glucose or glucose only after exhaustive exercise (Wallis *et al.*, 2008; Trommelen *et al.*, 2016), while others do not (Fuchs *et al.*, 2016). Thus, it could be that a combination of galactose and glucose as compared to glucose only is slightly less insulinogenic and consequently this might have caused a reduction in muscle glycogen repletion rate.

In contrast to the initial hypothesis, GAL resulted in muscle glycogen repletion to a magnitude similar to that observed when 0.8 $g \cdot kg^{-1} \cdot h^{-1}$ of glucose was provided in GAL+GLU condition. This occurred in an absence of any discernible change of either plasma insulin or glucose concentrations, which have previously been shown to explain 94% of variance in muscle glycogen replenishment (Doyle, Sherman and Strauss, 1993). At this point, it remains unclear as to how this was achieved, and this requires further research. Given that plasma glucose and insulin concentrations remained stable throughout the recovery in GAL, while it cannot be discounted on

the basis of the present data, it seems unlikely that galactose was extracted by the splanchnic tissue, converted to glucose and released into the blood circulation from where it entered the muscle tissue and was converted to glycogen as would have been assumed based on the current understanding of galactose metabolism.

It is plausible that galactose actually entered the muscle cells and was directly converted to glycogen. Galactose could be transported via GLUT 4 (Kase *et al.*, 2013), the presence of which is increased at the muscle membrane in the post-exercise period (Richter and Hargreaves, 2013). Given that plasma galactose concentrations were highly elevated (on average to ~3.5 mmol $\cdot$ L^{-1}), a high concentration gradient was created which is important as GLUT4 operates under principles of diffusion gradient (Wasserman, 2009). After the uptake, galactose would need to be converted to glucose-6-phosphate to be able to be converted to glycogen. Evidence from 7-28 week old human foetuses indicates that two of the important enzymes of the Leloir pathway (galactokinase and galactose 1-phosphate uridylyltransferase) are present at least in the early stages of human development in muscle tissue (Shin-Buehring *et al.*, 1977) and could thus be still present in adulthood as well. In addition to that, evidence that human muscle primary cells can be differentiated in galactose medium indicates that galactose can be utilised as an energy source within the muscle cells (Aguer *et al.*, 2011).

In this respect it may be that galactose is similar to fructose, for which it has been previously been shown that it can be taken up and oxidised or stored as glycogen in

human muscle tissue provided that concentrations of fructose in the blood were elevated (Ahlborg and Björkman, 1990; Zierath *et al.*, 1995). Ahlborg and Björkman infused fructose and its concentrations in serum reached ~4 mmol · L^{-1} and it is presently unknown whether such high concentrations could be achieved by fructose ingestion. Current evidence shows that when fructose is ingested in combination with glucose or fat and protein plasma concentrations of fructose do not exceed 0.4 mmol · L^{-1} (Theytaz *et al.*, 2014; Francey *et al.*, 2018). Nonetheless, given that galactose concentrations are highly elevated with galactose only ingestion, it is plausible to think that galactose could directly contribute to muscle glycogen resynthesis, however further research is required to establish this.

While it is logical that galactose concentrations did not rise in GLU, lack of an increase of galactose concentrations in GAL+GLU can be explained by enhanced splanchnic galactose uptake when galactose is co-ingested with glucose (Sunehag and Haymond, 2002). In addition to that, lactate concentrations were higher in GAL+GLU and GAL as compared to GLU, indicating that some of the galactose might have been converted to glucose, however it seems unlikely that this might have had a substantial influence on muscle glycogen repletion (Bangsbo *et al.*, 1997).

A secondary purpose of the study was to explore whole-body metabolism during the steady state exercise bout that followed a 4 hrs refeeding period. The highest rates of carbohydrate oxidation and lowest fat oxidation rates were observed in GLU and

were accompanied with the highest plasma insulin and lowest NEFA and glycerol. On the other hand, the lowest carbohydrate and highest fat oxidation rates were observed in GAL, while plasma NEFA and glycerol concentrations were the highest. Differences in substrate oxidation rates can be in part explained by different muscle glycogen concentrations at the start of exercise (Arkinstall *et al.*, 2004) and in part different metabolic environment (i.e., plasma NEFA, glycerol and insulin concentrations) (Horowitz *et al.*, 1997). Collectively, the metabolic environment in GLU was most facilitative of all for high rates of carbohydrate utilisation.

Using stable isotope mass spectrometry methodology, we were also able to quantify oxidation rates of the carbohydrate that was ingested in the recovery period and that which remained after the glycogen reducing exercise bout (endogenous stores). Oxidation of carbohydrates ingested during the recovery period was highest in GLU and lowest in GAL, while the reverse was true for endogenously stored carbohydrates.

Only two galactose studies to-date measured oxidation rates of ingested and endogenously stored carbohydrates. Leijssen and colleagues had participants perform two moderate intensity cycling exercise bouts (Leijssen *et al.*, 1995). Galactose or glucose were ingested just before (~50 g) and during (~12.5 g every 15-min) the first 120-min exercise bout. This was followed by 60-min of rest and the second 30-min long exercise bout during which no carbohydrates were ingested. During the first exercise bout, total carbohydrate oxidation rates were similar,

however, oxidation of ingested galactose was significantly lower than oxidation of ingested glucose. Galactose concentrations in plasma peaked at 12 mmol · L^{-1} at the end of the first bout. During the second exercise bout, which would be most comparable to the present study, they observed only slightly higher carbohydrate oxidation rates with glucose, but comparable oxidation rates of ingested carbohydrates. This appears to contrast the result of the present study that observed significantly lower both total and in recovery ingested carbohydrate oxidation rates in GAL as compared to GLU. However, the second exercise bout started in notably different metabolic environments. Namely, because no carbohydrates were ingested during the recovery, plasma insulin concentrations were low in both conditions. This likely explains similar total carbohydrate oxidation rates for both conditions in the study by Leijssen and colleagues. Secondly, oxidation rates of carbohydrates ingested in the form of galactose during the second exercise bout were twice as high as during the first exercise bout and are actually comparable to those observed in the present study.

In the second study O'Hara and colleagues investigated the effects of different pre-exercise carbohydrate feedings (75 g of glucose or galactose 30-min before the onset of exercise) on metabolism during the 120-min long moderate intensity cycling exercise (O'Hara *et al.*, 2012). They observed similar total carbohydrate oxidation rates. However, in the first 60-min of exercise oxidation of ingested carbohydrates was higher in glucose condition, so similar as in the present study. In the second 60-

min, oxidation of ingested carbohydrates noticeably declined in glucose condition, while oxidation in the galactose condition remained stable so that throughout the whole 120 minutes there was no difference in oxidation rates of ingested carbohydrates. The reduction in oxidation of ingested glucose is likely a result of near complete oxidation of carbohydrates ingested before exercise.

In conclusion, this study demonstrated that galactose-glucose co-ingestion results in suboptimal replenishment of muscle glycogen stores and thus from a performance standpoint likely could not be recommended for post-exercise recovery period. Intake of galactose on the other hand was shown to result in substantial, though suboptimal, rates of muscle glycogen replenishment without any discernible elevations in glucose and insulin concentrations. Further research is required to better understand the mechanisms by which muscle glycogen synthesis occurs when galactose is ingested alone.

7. General Discussion

The main goal of this thesis was to investigate the effects of (or absence of) carbohydrate feeding during the recovery period following a glycogen reducing exercise bout on aspects of recovery metabolism and of metabolism and performance during a subsequent exercise bout, undertaken with or without carbohydrate feeding.

More specifically, the aims of the thesis were:

1. To explore a novel approach to sleeping and training with reduced carbohydrate availability whereby carbohydrates are ingested with a delay during a subsequent morning exercise bout.
2. To compare glucose only or fructose-glucose co-ingestion during the post-exercise recovery period on metabolism and performance during a subsequent exercise bout.
3. To investigate the efficacy of galactose ingestion alone or in combination with glucose during recovery after glycogen reducing exercise on muscle glycogen synthesis, and metabolism during a subsequent exercise bout.

Accordingly, the major new findings generated by this thesis, in light of the aforementioned aims are:

Chapter 2:

- Delayed carbohydrate feeding during exercise following a sleep low approach to training did not supress high fat oxidation rates typically observed under conditions of low carbohydrate availability.
- Delayed carbohydrate feeding led to a suppression of NEFA and elevation of insulin concentrations while as compared to no carbohydrate intake, glucose concentrations were maintained.
- Performance outcomes of the study were unclear, however, there was a trend towards improved performance with delayed carbohydrate feeding.

Chapter 3:

- Exogenous carbohydrate oxidation rates can be determined after 30-min of moderate intensity exercise.
- Fructose-glucose co-ingestion as compared with glucose only led to higher oxidation rates of during recovery ingested carbohydrates during subsequent exercise, hinting at increased whole-body post-exercise glycogen storage.
- There was no benefit of fructose-glucose consumed during exercise recovery over glucose only on subsequent exercise performance.

Chapter 4:

- Glucose was a superior source for post-exercise muscle glycogen repletion over galactose or a combination of galactose and glucose.
- Galactose ingestion resulted in substantial replenishment of muscle glycogen stores similar to those observed with a moderate glucose intake (galactose-glucose combination) without a rise in glucose and insulin levels.

The results of the experimental work described in this thesis have so far been discussed in the context of each study separately. However, there are some common themes that could benefit from a joint discussion and this will follow in the next sections. Namely, “carbohydrate availability and training adaptations” as well as the “role of monosaccharide blends in recovery” will be discussed. This will be followed by a section describing the implications of the present work for practitioners, a section describing major improvements that would benefit the present work and finally, conclusions and future research directions.

7.1. Carbohydrate availability and training adaptation

In the past decade evidence has accumulated showing that high carbohydrate availability is important for optimal performance as well as post exercise recovery

especially in endurance sports (Burke *et al.*, 2011) but that high carbohydrate availability might not always be desirable due to potentially negatively affecting training adaptations (Bartlett, Hawley and Morton, 2015). Thus, an approach termed as carbohydrate periodisation has emerged that recommends fuelling training sessions according to their energy demands and performing certain training sessions, mostly those of lower exercise intensity with reduced carbohydrate availability (Impey *et al.*, 2018).

The most simplistic way of periodizing carbohydrate intake is to perform certain training sessions after an overnight fast. However, there is limited evidence available supporting efficacy of such an approach. For example, Van Proeyen and colleagues investigated moderately trained individuals performing 6 weeks of endurance training in the fasted as compared to fed (carbohydrate intake before and during exercise) state and observed that fasted training resulted in an upregulation of some of the important enzymes (e.g., CS) (Van Proeyen *et al.*, 2011). However, the study did not observe any statistically significant performance benefits of fasted training. The lack of substantial positive differences when comparing fed and fasted training could be a consequence of similar muscle glycogen content at the onset of exercise given that AMPK activity appears to be regulated by glycogen content which can in turn upregulate downstream molecular pathways responsible for mitochondrial biogenesis (Philp, Hargreaves and Baar, 2012). Another contributing factor could be relatively low-fat oxidation rates as compared to training with reduced muscle glycogen content as described in the following paragraph.

It is well documented that fat oxidation is elevated when training commences after an overnight fast (Vieira *et al.*, 2016), but only a few studies to-date compared fat oxidation rates when an exercise session is commenced with normal versus reduced muscle glycogen content (Arkinstall *et al.*, 2004; Bartlett *et al.*, 2013; Hearris *et al.*, 2019). In the study by Arkinstall and colleagues investigators initially reduced participants' muscle glycogen stores with a high intensity interval training after which a low carbohydrate and high fat or high carbohydrate and low fat diets were consumed in the subsequent 48 hrs. After that, steady state exercise was performed. They found that when muscle glycogen stores were reduced, fat oxidation rates were higher and proposed that muscle glycogen stores are an important determinant of fuel utilisation during exercise. Similar observations came from Bartlett and colleagues and Hearris and colleagues employing a high intensity interval training protocol for the subsequent exercise bout. Although none of the experimental conditions in Chapter 2 could be described as training in the fasted state due to the intake of a protein gel, the metabolism (i.e., fat oxidation rates) during the exercise bout should not be affected by its ingestion and should be comparable to training after an overnight fast (Impey *et al.*, 2015). The study described in Chapter 2 (Podlogar, Free and Wallis, 2020) thus supports findings by Arkinstall and colleagues and expands them by providing evidence that delayed carbohydrate feeding does not supress high fat oxidation rates. Thus, it appears that in this regard delayed carbohydrate feeding could be a feasible approach to training with low carbohydrate availability between two training sessions.

Accordingly, the metabolic environment created with an overnight fast (i.e., normal muscle glycogen stores coupled with modestly elevated fat oxidation rates) as compared to training after a carbohydrate rich breakfast does not necessarily create an environment required for enhanced molecular signalling. Thus, from this standpoint training commenced with reduced carbohydrate availability (i.e., low muscle and liver glycogen content) likely offers bigger advantages as recently observed in a literature review (Impey *et al.*, 2018). Impey and colleagues analysed studies investigating training with reduced muscle glycogen content and found that such approach to training positively affects activation of acute cell signalling pathways and in the long term this could result in favourable metabolic (e.g., increased fat oxidation) and performance outcomes as previously discussed in the Literature Review chapter. However, training with reduced muscle glycogen content has to be understood as a "work-efficient approach to training" as has been phrased previously by Impey and colleagues (Impey *et al.*, 2016a, 2018) given that low muscle glycogen levels and likely highly elevated fat oxidation rates could be achieved by prolonging duration of a training session as well (Areta and Hopkins, 2018). It is indeed well documented that overall training volume is associated with improved exercise performance and it is commonly observed that athletes are striving to achieve as high training volumes as possible (Seiler, 2010; Bishop, Botella and Granata, 2019). Thus, it could be that the approach that would benefit athletes would be the one that enables them to accumulate maximal training volumes and that

metabolic environment in which these sessions are undertaken would be most supportive.

While to-date most of the research interest has been directed towards manipulation of glycogen stores, recent evidence suggests that at least some of the benefits attributed to training with reduced muscle glycogen stores could be down to the timing between two exercise training sessions (Diaz-Lara, Botella and Reisman, 2020). Some studies, including the seminal work by Hansen and colleagues were designed so that in the condition in which second exercise session was commenced with reduced muscle glycogen content two exercise sessions were performed in close proximity to each other, whereas in the other condition, ~24 hours were in between (Hansen *et al.*, 2005). Recently, two studies were published comparing the sleep-low approach to training with undertaking two training sessions in close proximity with one another one looking at long-term adaptations and the other at acute responses (Ghiarone *et al.*, 2019; Andrade-Souza *et al.*, 2020). Andrade-Souza and colleagues investigated performing a high intensity interval exercise on its own (control condition), 2 (same day) or 15 (next day) hrs after glycogen reducing exercise bout. They observed enhanced molecular signalling (e.g., PGC-1α) with twice daily approach as compared to once-daily exercise session even though carbohydrate intake was limited between both sessions, so in both scenarios the subsequent training session was undertaken with low carbohydrate availability. Ghiarone and colleagues on the other hand used a similar model of exercise but investigated long-term effects. After 3-weeks of training intervention, mitochondrial efficiency was

improved to a higher degree in the group that trained twice daily as compared to the group that trained once but with reduced muscle glycogen content in the second session. However, these changes did not translate to an improvement in aerobic fitness or higher fat oxidation rates during 100-min steady state exercise.

The emerging question from this debate is whether carbohydrate availability during the recovery period affects responses to performing two training sessions in close proximity with one another. To-date two studies investigated this (Cochran *et al.*, 2010; Hammond *et al.*, 2019). Both studies employed high intensity interval training sessions as an exercise modality for both daily training sessions. While Cochran and colleagues found some beneficial changes on the molecular level (i.e., higher activation of p38MAPK) for performing second exercise session with low carbohydrate availability, Hammond and colleagues did not observe enhanced molecular signalling associated with regulation of mitochondrial biogenesis or lipid metabolism. However, different biopsy timings, different exercise intensities and durations and nutrition protocols could have caused apparently contradicting findings.

Collectively, it is likely that both timing and carbohydrate availability have an influence on the molecular responses to training and consequently training effects as well. However, It has to be noted that these studies were designed so that total training duration and usually volume as well were matched, meaning that in the best case scenario positive adaptations associated with given training approach have to be

understood as a more time efficient training rather than more optimal training. The reason is that a training approach with more time for recovery and/or higher carbohydrate availability would very likely enable athletes to achieve higher training volumes. Indeed, the study from Chapter 2 (Podlogar, Free and Wallis, 2020) provides a support that delayed carbohydrate feeding after limited carbohydrate availability during recovery from previous exercise bout might, at least for some athletes, enable them to train at higher intensities and, based on blood glucose levels, perhaps also for longer if that had been measured. This would be in line with previous findings of the effects of carbohydrate feeding during exercise as discussed in Chapter 1.

Although the evidence is not strong, it was suggested that elevated NEFA concentrations in the plasma play an important role in mediating positive molecular signalling associated with training under low carbohydrate availability (Philp, Hargreaves and Baar, 2012). Based on this, it could be speculated that galactose ingestion in between two training sessions could be recommended when exercise is of lower intensity given that galactose, as found in Chapter 4, does not suppress fat oxidation and NEFA concentrations in the same way as does ingestion of glucose.

7.2. Role of monosaccharides blends and recovery

The aims of chapters 3 and 4 were to uncover some of the outstanding questions regarding the role of different monosaccharides in the short term (i.e., ≤4 hrs) recovery

after an intense glycogen reducing exercise bout. As discussed in Chapter 1, the role of glucose ingestion in the recovery period is well established and is part of current recommendations (Burke *et al.*, 2011). However, evidence has accumulated showing benefits of complementing glucose with fructose and even galactose on repletion of especially liver glycogen stores in the post-exercise recovery period (Wallis and Wittekind, 2013; Gonzalez and Betts, 2019). Given the relationship between muscle glycogen storage and exercise capacity (Bergström *et al.*, 1967), it was hypothesised that enhanced glycogen storage as a result of adding fructose/galactose to glucose (albeit mostly in the liver) would subsequently lead into enhanced exercise capacity and/or performance.

As discussed in the introduction, in the postabsorptive phase, liver glycogen is broken down to provide glucose especially to organs without glycogen stores (e.g., brain) and maintain stable blood glucose levels (Young, 1957). Coupling this with a well-known relationship that low blood sugar levels coincide with fatigue (Christensen and Hansen, 1939b; Coyle *et al.*, 1983), this provides a clear rationale why optimising both liver and muscle glycogen storage in the post-exercise recovery period could positively affect exercise capacity and performance.

Previous evidence has demonstrated that combining glucose with fructose or galactose leads to an enhanced liver glycogen storage as compared to glucose only (Décombaz *et al.*, 2011). In addition to that, research also shows that fructose combined with glucose is as effective in replenishment of muscle glycogen stores as

compared to glucose only (Wallis *et al.*, 2008; Fuchs, Gonzalez and van Loon, 2019). In contrast, data on galactose and glucose combinations and their effects on muscle glycogen have been to-date non-existent.

While positive effects of fructose-glucose combinations on total glycogen storage are well demonstrated, the outstanding question is whether this translates into improved exercise performance/function. To-date two studies compared ingestion of glucose only with a combination of fructose-glucose during short term post-exercise recovery period and found positive effects on recovery of exercise capacity when both monosaccharides were ingested simultaneously both in running and cycling exercise (Maunder, Podlogar and Wallis, 2018; Gray *et al.*, 2020). Both studies saw participants exercise until exhaustion at ~70% VO_2peak. Although the relative intensity in relation to VO_2peak was similar, plasma lactate concentrations indicate that in the study by Maunder and colleagues participants exercised below the lactate threshold, whereas in the study by Gray and colleagues they exercised at the intensity above it. Furthermore, in neither of the studies was fatigue preceded by a decrease in blood glucose levels as would be hypothesised based on the theoretical background on how the liver would limit exercise capacity.

In contrast with these positive findings, the study described in Chapter 3 did not observe any performance benefits of a monosaccharide mix. The study design was different in that a ~40-min long time trial was preceded by 1 hour long moderate steady state exercise (~63% VO_2peak), which could have influenced the outcomes.

Mechanisms on why exercise capacity was improved, and performance is not clear. For instance, Maunder and colleagues observed that fatigue was preceded by a suppression in carbohydrate oxidation rates, whereas this was not observed in the study by Gray and colleagues. Maunder and colleagues also observed higher oxidation rates of ingested carbohydrate and the study in Chapter 3 confirms these findings. In addition to that, carbohydrate oxidation rates in the study described in Chapter 3 found a trend towards higher carbohydrate oxidation rates during the steady state exercise period. While none of the studies found any negative effects on performance on fructose-glucose combination, it looks as fructose-glucose is at least as effective as glucose only in the post-exercise recovery period. It is also clear that in certain situations it can be beneficial, however both the mechanisms and the exact situations where the mix is beneficial requires further investigation.

There are a few substantial differences between the three studies which could have impacted the results and require further investigation. Firstly, a time trial with variable power output versus a fixed intensity time to exhaustion. Secondly, it could be that the duration of the steady state in the study described in Chapter 3 was too long so that by the time trial was initiated, participants started with similar glycogen availability. Thirdly, a difference in intensities in the steady state part of exercise could have affected the outcomes. For example, it could be that the lower energy demands in the study described in Chapter 3 created an environment where different substrate oxidation rates were possible, and that lack of significant differences in substrate utilisation was actually a statistical type II error.

On the other hand, the work in the present thesis demonstrates that ingestion of galactose only or in combination with glucose during the post-exercise recovery period results in lower muscle glycogen repletion rates as compared to glucose only. This was surprising given the apparent positive effects of milk on post-exercise recovery (James *et al.*, 2019) as well as the hypothesis that galactose behaves in a similar way as fructose as has been described in more detail in chapters 2 and 6. Current knowledge of the effectiveness of repletion of muscle/liver glycogen stores is depicted in Figure 7.1.

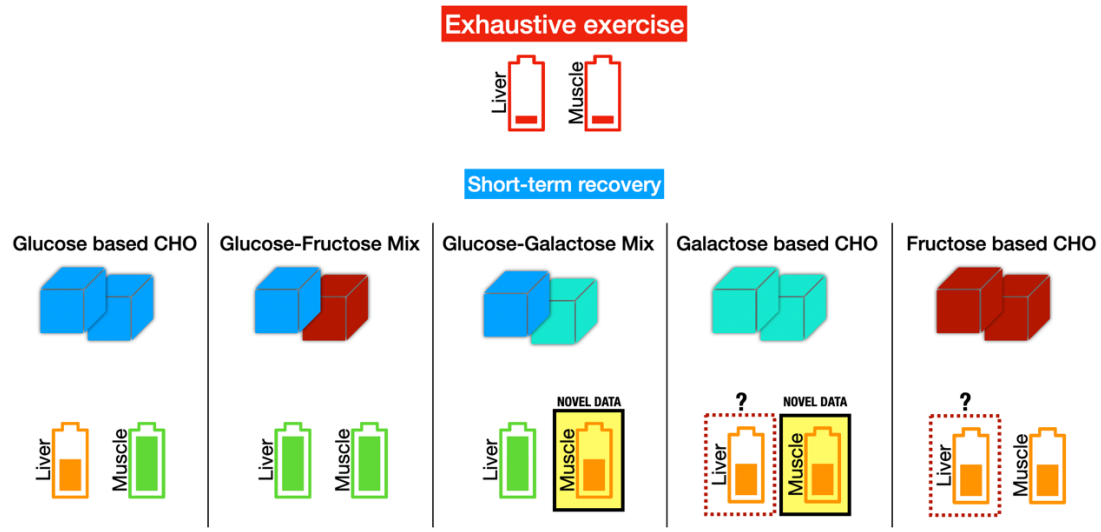

Figure 7.1. Current understanding on how different monosaccharides and their combinations affect post-exercise synthesis of liver and muscle glycogen. Glucose based carbohydrates appear to result in optimal rates of muscle but not liver glycogen synthesis. Glucose-fructose mixes have been shown to optimally stimulate liver and muscle glycogen replenishment. Data from this thesis demonstrates that glucose-galactose mixtures are not optimal for muscle glycogen synthesis, whereas previous research demonstrated that liver repletion rates are similar to glucose-fructose mixes. In addition to that, this thesis showed that galactose results in suboptimal replenishment of muscle glycogen, whereas direct data is lacking for liver glycogen assessment. Fructose appears to be suboptimal for replenishment of muscle glycogen, while data for how effective it is in replenishment in liver glycogen is unknown.

Presently, it is difficult to give a comprehensive explanation of the findings described in the Chapter 6. The most plausible reason for suboptimal replenishment of muscle glycogen with galactose-glucose combination as discussed in Chapter 4 could be that an insufficient insulin response caused a suboptimal replenishment of muscle glycogen stores. One of the previous studies, although without glycogen reducing exercise before feeding, postulated an existence of an insulin threshold, whereby a certain level of insulin is required for muscle glycogen synthesis (Young *et al.*, 1988). It has to be noted that this model likely is not completely appropriate given that exercise per-se causes an upregulation of GLUT4 transporters on the cell membrane and that insulin likely is not crucial (Richter and Hargreaves, 2013). However, it is known from a protein-carbohydrate co-ingestion studies that additional insulin stimulation could enhance muscle glycogen replenishment in the presence of a lower carbohydrate intake (van Loon *et al.*, 2000; Alghannam, Gonzalez and Betts, 2018). A close look at the variability of the glycogen repletion data reveals that there was much higher variability in both galactose conditions as compared to glucose only. In the case of combined galactose-glucose, insulin response was highly variable as well and this could have had an impact on the repletion rates. However, further research is required to investigate if this is the case. In addition to that, galactose-glucose combination is rarely ingested in isolation, but is usually present in milk products, thus with some fat and most importantly protein (Pritchett and Pritchett, 2013; James *et al.*, 2019), which could potentially differently affect muscle glycogen replenishment rates.

A surprising observation was that galactose was as effective in replenishment of muscle glycogen stores as galactose-glucose combination in an absence of any discernible rise in insulin or glucose concentrations. This rejected the initial hypothesis that galactose alone would be a very poor source for replenishment of muscle glycogen. Presently, it is difficult to explain why galactose ingestion resulted in substantial muscle glycogen replenishment rates, however, as discussed in the Chapter 4, there is actually some evidence that conversion of galactose into the glycogen in the skeletal muscle could take place, provided that concentration gradient between plasma and muscle cells would be sufficient in order the galactose to be transported across the muscle membrane. Further research is needed to explore if this is the case.

Drawing comparisons with fructose is difficult as research on fructose ingestion only is scarce, likely as a result of a high prevalence of gastrointestinal discomfort occurring when only fructose is ingested (Jones, Butler and Brooks, 2011). Although it was found that fructose ingestion in the post-exercise recovery period results in markedly lower rates of muscle glycogen repletion (Blom *et al.*, 1987), a more recent study in which fructose was combined with fat and protein found similar muscle glycogen repletion rates (Rosset *et al.*, 2017). Whether the reason for a discrepancy in the results is addition of other nutrients that could potentially reduce fructose malabsorption requires further investigation. Inversely, galactose malabsorption is likely to be much less prevalent given that it uses the same transporters as glucose.

Further, direct evidence that when fructose is ingested in isolation it preferentially replenishes liver glycogen stores is limited as it comes only from an infusion study (Nilsoon and Hultman, 1974). In contrast with this finding is a recent study hinting at a potential for a lower liver glycogen storage when fructose only is ingested in the post-exercise recovery period (Rosset *et al.*, 2017). This evidence comes from estimating total glycogen storage and deducing storage of measured muscle glycogen. Similarly, there is no evidence that galactose only results in enhanced rates of liver glycogen synthesis as compared to glucose. Inversely, splanchnic galactose extraction is lower when galactose is ingested alone as compared to when it is combined with glucose hinting at a possibility that galactose only ingestion is inferior from a liver glycogen synthesis as well this time as compared to galactose-glucose combination (Sunehag and Haymond, 2002).

Although difficult to ascertain, it could be speculated that combined liver and muscle glycogen storage influence substrate utilisation rates during subsequent exercise and that the role of muscle storage is more important than the liver. This speculation stems from observations from studies described in chapter 3 and 4 and previous studies in this area described earlier. Firstly, when fructose-glucose (optimal source for repletion of both glycogen storage sites) is ingested total carbohydrate oxidation rates trended to be the highest. Secondly, galactose-glucose ingestion (Chapter 4) resulted in lower carbohydrate oxidation rates as compared with glucose only. In this case, muscle glycogen stores were replenished to a lower degree with galactose-glucose as compared to glucose only, while the reverse was likely true for liver

glycogen. Thirdly, galactose only ingestion resulted in lowest carbohydrate oxidation rates coupled with lower muscle glycogen storage as compared to glucose and likely a lower liver glycogen storage as compared to galactose-glucose. Whether this is really the case and whether this is only a consequence of different pre-exercise insulin concentrations, requires further investigation.

7.3. Implications for practice

The work in the present PhD thesis provided answers to some of the questions pertaining to the field of sports nutrition which can be used when working with athletes.

- Delayed carbohydrate feeding after a sleep low approach could be incorporated into athletes' training plan when increasing fat oxidation is a priority and likely when trying to keep the training volume (exercise and/or intensity) relatively high.
- Combining fructose and glucose in the post-exercise period could potentially lead to favourable effects on the post-exercise recovery. However, in certain cases co-ingestion could lead to low blood sugar levels and a decreased performance. Thus, it could be advised that such strategy is tested before being used during competitions.

- Galactose-glucose blends are not optimal source of carbohydrates in the post-exercise recovery period.

An overview of how carbohydrate availability and different types of carbohydrates can affect performance and recovery with different goals in mind (i.e., training adaptations or performance) can be seen in Figure 7.2.

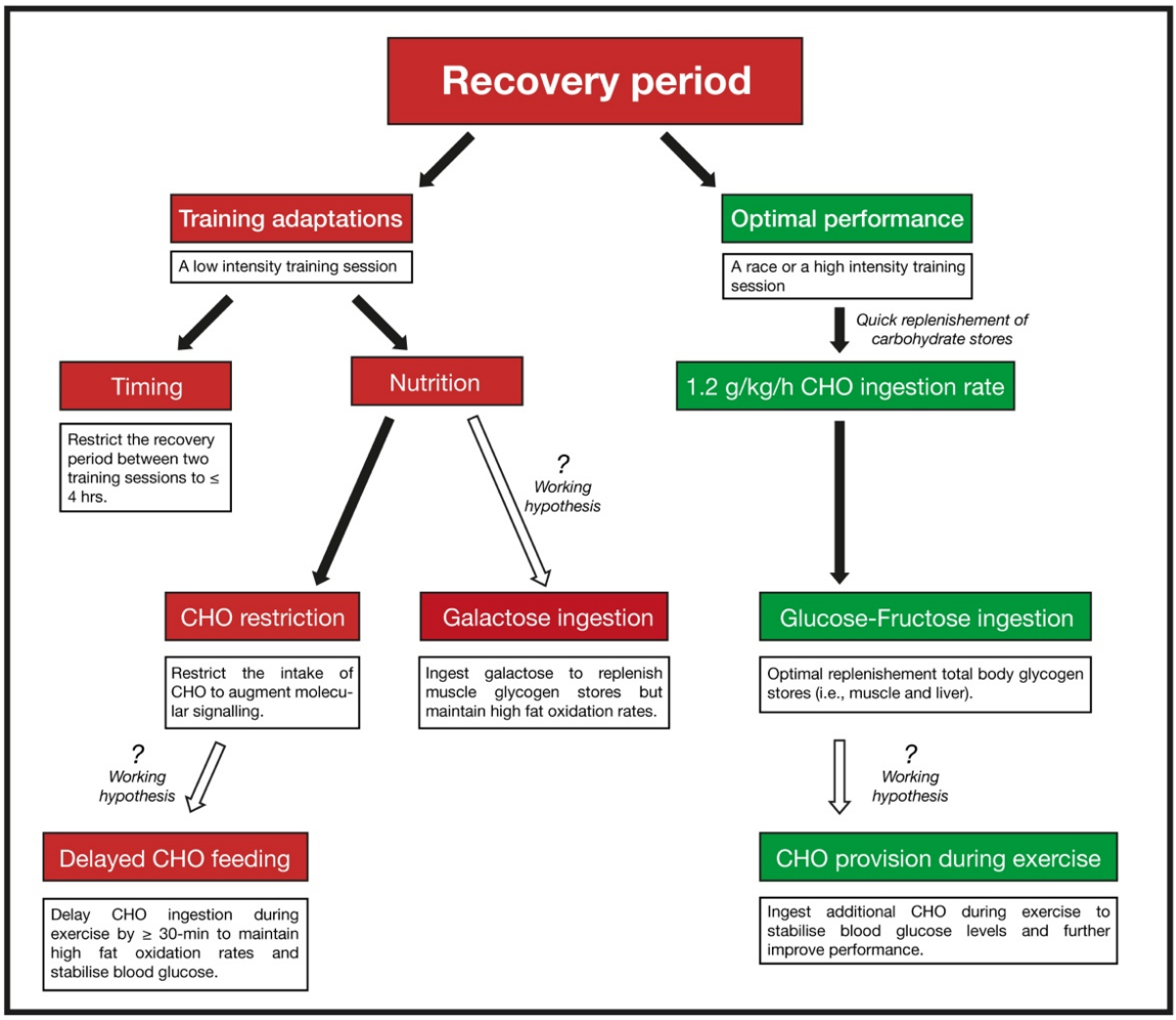

Figure 7.2. Recovery period decision diagram. Recovery period in endurance athletes has one of the two goals in mind – promotion of training adaptations or recovering for optimal performance during subsequent intense efforts. Different strategies have been shown to be effective in promoting training responses and this thesis provides two more potential ways on how to achieve that – delayed carbohydrate feeding and galactose ingestion. However further work is required to see if this is the case. On the other hand, when optimal performance is desired it has clearly been demonstrated that a high carbohydrate diet is preferential, and that fructose-glucose co-ingestion leads to optimal replenishment rates of whole-body carbohydrate stores. However, due to potential of hypoglycemia with fructose ingestion, provision of carbohydrates during the exercise bout could be advisable as well.

7.4. Major improvements

Most of the limitations of the studies conducted have already been mentioned in the corresponding chapters, so this section will propose on how the studies conducted as part of this thesis could be improved.

1. Addition of muscle biopsies in the first experimental chapter coupled with an array of specific analyses, such as measurement of muscle glycogen concentration and cell signalling would provide a more comprehensive overview of the effects of delayed carbohydrate feeding after a sleep low approach to training.
2. Measurement of muscle and liver glycogen in the second experimental chapter would allow to directly compare effectiveness of replenishment of glycogen stores with either of the two experimental conditions and speculations based on the previous studies would have been unnecessary.
3. Assessment of sensitivity and reliability of the performance tests in the first two experimental chapters would aid better sample size calculations and prevent speculations about the test not being reliable or sensitive enough. This could have been done as a separate study or the familiarisation session would have been repeated to ascertain an estimate of reproducibility.

4. Analysis of biopsy samples for activities of certain enzymes responsible for galactose metabolism as well as appearance of GLUT4 transporters on muscle membranes would aid with the understanding on how substantial replenishment of muscle glycogen stores were achieved with ingestion of galactose even in the absence of rise in glucose and insulin concentrations. In addition to that, inclusion of assessment of recovery (i.e., exercise performance or capacity) would allow giving a more complete overview on what do different galactose mixtures mean in terms of muscle function.
5. All three studies would benefit from larger sample sizes so that the possibility of type II statistical error in certain measures would be ruled out.
6. A rigorous a priori sample size calculation using the same principles and the same statistical analysis method in all three experimental chapters would a) aid to the quality of the present work and b) prevent doubts regarding the possibility of Type 2 statistical error.

7.5. Conclusions and future directions

This thesis, consisting of three experimental chapters investigated the effects of carbohydrate feeding of different combinations in recovery of glycogen reducing exercise on subsequent metabolism during exercise as well as performance. The first experimental chapter provides evidence that delayed carbohydrate feeding after a sleep low approach to training does not supress fat oxidation rates. The second

experimental chapter observed higher oxidation rates of fructose-glucose ingestion during the post-exercise recovery period as compared to glucose only, but this did not translate in improved performance. The third experimental chapter found galactose-glucose and galactose only ingestion to result in similar rates of muscle glycogen synthesis but be inferior to the repletion rates observed when the same amount of glucose was ingested.

Most of the pertinent research questions that have opened up as a result of the present work have already been discussed in the corresponding chapters. Stemming from the discussion in this chapter are some of the most important research questions answering which would provide important insights into the field of sports nutrition.

1. How would molecular signalling thought to be responsible for mitochondrial biogenesis and long-term adaptations be affected by delayed carbohydrate feeding in a sleep low approach to training in both a work matched endurance training as well as work-unmatched training (training until exhaustion or at the highest possible exercise intensity). This would advance our understanding of "time efficiency" of training as well as provide answers on how delayed carbohydrate feeding actually affects training responses.
2. Further investigate the scenarios when fructose-glucose co-ingestion during the post-exercise recovery period would positively affect exercise performance. Namely, manipulate the duration of exercise and its intensity.

3. Investigate whether continuous ingestion of carbohydrates during subsequent exercise would differently affect performance/capacity after fructose-glucose or glucose only are ingested during the post-exercise recovery period.
4. Investigate how galactose ingestion in the post-exercise recovery period affects liver glycogen stores and explore the mechanisms for replenishment of muscle glycogen stores.
5. Finally, it would be interesting to compare the molecular signalling associated with improved oxidative metabolism with training preceded by galactose ingestion given that it results in replenishment of muscle glycogen but does not cause an elevation of insulin and glucose concentrations and thus does not substantially supress NEFA availability.

8. References

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