UNIVERSITY OF BIRMINGHAM

EXPLORING NOVEL APPLICATIONS FOR LACTOSE IN HUMAN NUTRITION

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Abstract:

Lactose is a disaccharide of glucose and galactose, found exclusively in dairy. Despite its common presence in the human diet, little is known about its possible roles in sports nutrition, and whether there are any potential applications for this carbohydrate. The aim of the thesis was to identify possible applications for lactose in a sports nutrition context. Therefore, Chapter 3 was conducted to determine whether ingested lactose can be readily oxidised during moderate intensity exercise compared to sucrose, which is a commonly ingested carbohydrate in sports nutrition. Both carbohydrates exhibited similar exogenous carbohydrate oxidation rates, demonstrating that lactose can act as a viable source of substrate for exercise metabolism. Interestingly, lactose resulted in less pronounced suppression of fat oxidation than sucrose, with a concomitant reduction in endogenous carbohydrate oxidation. Lactose did not elicit gastrointestinal discomfort to a greater extent than sucrose or plain water ingestion, the potential for which had been a key concern. Following observations of endogenous carbohydrate sparing, Chapter 4 aimed to determine whether lactose ingestion resulted in hepatic, or muscular glycogen sparing. The execution of this study was limited by COVID-19 restrictions, meaning that it functioned as a pilot study to investigate an improved ^{13}C oral tracer methodology for assessing substrate utilisation. The limited data generated implied that the glycogen sparing observed was of hepatic origin. Chapter 5 investigated lactose in a post-exercise setting for the recovery of liver glycogen. Galactose and fructose, when ingested with glucose have been shown to accelerate liver glycogen repletion. Therefore lactose, as the major dietary source of galactose, was used to determine if galactose and fructose, alongside glucose, were better at

restoring liver glycogen than glucose and fructose. Although liver glycogen synthesis rates were high in both conditions, there was no clear benefit for ingesting the full spectrum of dietary monosaccharides above sucrose (glucose-fructose) ingestion on liver glycogen synthesis. In conclusion, the thesis presents novel insights into the metabolism of lactose in a variety of exercise contexts providing new possible applications for lactose in sports nutrition.

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List of abbreviations

ANOVA – analysis of variance

ATP – adenosine triphosphate

AUC – area under the curve

BBA – butaneboronic acid

CHO – carbohydrate

COVID-19 – Coronavirus disease 2019

CV – coefficient of variation

EDTA **–** Ethylenediaminetetraacetic acid

EE – energy expenditure

EI – energy intake

ELISA – enzyme linked immunosorbent assay

FO – fat oxidation

FODMAP – fermentable oligo-, di-, monosaccharides and polyols

FOS – fructooligosaccharides

FRU – fructose

G – gauge

G-1-P – glucose-1-phosphate

GI – gastrointestinal

GIQ – gastrointestinal questionnaire

GIS – gastrointestinal symptom

GLU – glucose

GLUT2 – glucose transporter 2

GLUT4 – glucose transporter 4

GLUT5 – glucose transporter 5

GOS – galactooligosaccharides

HRP – horseradish peroxidase

IC – indirect calorimetry

iCASE – industrial collaborative awards in science and engineering

IQR – interquartile range

IR – isotope ratio

LAC – lactose

LAC+FRU – lactose and fructose

LacHi – high ^{13}C lactose

LacLo – low ^{13}C lactose

LacLo+G – low ^{13}C lactose with a U-^{13}C glucose tracer added

LGO – liver glycogen oxidation

LI – lactose intolerance

LM – lactose maldigestion

MGO – muscle glycogen oxidation

MR – magnetic resonance

MRI – magnetic resonance imaging

MRS – magnetic resonance spectroscopy

MS – mass spectrometry

NEFA – non-esterified fatty acids

NMR – nuclear magnetic resonance

PG – plasma glucose

PGO – plasma glucose oxidation

PGR – postgraduate researcher

PhD – Doctor of Philosophy

RER – respiratory exchange ratio

RPE – rate of perceived exertion

RQ – respiratory quotient

SCFA – short chain fatty acid

SD – standard deviation

SGLT1 – sodium glucose transporter protein 1

SIBO – small intestinal bacterial overgrowth

SPSS – Statistical Package for the Social Sciences

SUC – sucrose

SucHi – high ^{13}C sucrose

SucLo – low ^{13}C sucrose

SucLo+G – low ^{13}C sucrose with a U-^{13}C glucose tracer added

TMB - 3,3',5,5'-Tetramethylbenzidine

TT – time trial

TTE – time to exhaustion

TTR – tracer/tracee ratio

UDP – uridine diphosphate

VAS – visual analogue scale

Thesis outline:

This thesis begins with Chapter 1; the General Introduction to the thesis. This took the form of a narrative review covering the potential applications for lactose in a sports nutrition context. These three applications were i) lactose as an energy source for exercise ii) lactose in a post-exercise setting, and iii) lactose as a prebiotic. The first two applications align broadly with the aims of the thesis, which are outlined in more detail in Chapter 1. The first aim was to investigate whether lactose represents a viable energy source for exercise. The second was to further understand effects of lactose on exercise metabolism. The third was to investigate whether lactose can play a role in post-exercise liver glycogen resynthesis.

Chapter 2 is the General Methods section which provides information on the techniques and methods used in the various experimental chapters of the thesis. Specific protocols used are detailed in the relevant experimental chapters, whilst Chapter 2 outlines the broad principles of the methods, as well as their advantages and disadvantages.

Chapters 3-5 are the experimental chapters, investigating i) the viability of lactose as a fuel source for exercise by determining its exogenous CHO oxidation rates during exercise, ii) the impacts of lactose on exercise metabolism in more detail using an improved oral ^{13}C tracer method, though data collection in this study was affected by COVID-19 restrictions, and iii) lactose for post-exercise recovery of liver glycogen alongside fructose, respectively.

Finally, Chapter 6 summarises the key findings from Chapters 3-5 in the context of existing literature on these topics. It also outlines the implications and applications of the research, as well as suggesting directions for future research.

Chapter 1: General Introduction

Work contained within this chapter has been published:

Odell OJ, Wallis GA. The application of lactose in sports nutrition. *International Dairy Journal.* 116; 104970, 2021.

1.1 Abstract**:**

Lactose is a disaccharide of glucose and galactose, found exclusively in milk. Carbohydrates represent an important fuel for endurance and prolonged exercise. Recommendations for athletes include high carbohydrate diets to maximise performance, especially before, during and after exercise. However, lactose does not feature in guidelines for carbohydrate intake for athletes, despite athletes likely consuming nutritionally relevant amounts. This review will explore possible applications for lactose in a sports nutrition context. These include lactose as a fuel source, for before and during exercise, where maximizing availability of readily oxidisable carbohydrate can optimise performance. Lactose could play a role in a post-exercise recovery setting, as a vehicle for the delivery of glucose and galactose, for the optimisation of muscle and liver glycogen. Lactose may also act as a prebiotic, possibly promoting beneficial changes to gut microbiota. A discussion of the possible risks associated with lactose over-consumption and intolerance will also be considered.

1.2 Introduction

Lactose is a disaccharide originating in mammalian milk, and is comprised of the monosaccharides glucose and galactose, bound by a β 1-4 glycosidic linkage (Mattar et al., 2012). Lactose within milk-derived dairy products is an extrinsic sugar, meaning it is not bound into the cellular structure of food (unlike intrinsic sugars such as glucose as starch in vegetables), and is free in the foods it is found in (Edgar, 1993). Milk-derived dairy products – containing variable amounts of lactose – have been a constituent of the human diet for approximately 8 000 years and feature prominently in the nutritional recommendations of most countries (Rozenberg et al., 2016). Governmental dietary recommendations encourage dairy intake in healthy individuals in the preponderance of countries, notably for their high calcium content, along with other micronutrients (Wang et al., 2016, U.S. Department of Health and Human Services and U.S. Department of Agriculture, 2015) demonstrating the extensive demand for milk and dairy products. Dietary sources of lactose are numerous, with bovine milk usually containing the greatest lactose content of common foods (See Table 1.1). Isolated lactose and whey permeate, (a high-lactose (76-85 %) dairy ingredient produced when proteins are removed from liquid whey in the manufacturing of whey protein isolate) are used extensively to improve technical aspects of product formulation in foodstuffs such as baked goods, confectionary and soups (U.S. Dairy Export Council, 2015). Such dairy ingredients are also widely used in non-specific roles, such as bulking agents, animal feed or as fillers in capsules. Thus, lactose may feature in the human diet from a variety of traditional dairy and non-dairy sources.

Table 1.1 Lactose content of a variety of dairy products

Food	Lactose content (g) per 100 g / 100 ml
Cow's Milk	5.0
Lactose-free milk	<1.0
Ice cream	3.3 - 6.0
Cottage cheese	0.3 - 2.4
Cream cheese	2.5 - 3.0
Hard cheeses	≤0.1
Yoghurt	4.5 – 6.0
Whey protein concentrate	3.5

(Misselwitz et al., 2013, Dieticians of Canada, 2013, Tetra Pak, 2018)

Despite the widespread potential presence of lactose, surprisingly limited information on the contribution of lactose to the total energy intake (EI) of different populations is available. Most investigations into the sugar content of individuals' diets have been primarily concerned with added sugars and therefore lactose predominantly originating from dairy-derived products has often been excluded, typically being classed as a non-added sugar. However, a limited number of more specific estimations of lactose intake have been made. In Swedish women average lactose intake was 12 ± 8 $g \cdot day^{-1}$, approximately 50 kcal or ~2% of EI (Larsson et al., 2004). Habitual lactose consumption in Canadian adults was reported to be similar, with milk sugar comprising ~11% of total sugar intake, estimated at approximately ~12 $g \cdot day^{-1}$ (Brisbois et al., 2014). Whilst lactose is present in the diet, it represents a relatively small amount, particularly in comparison to the other major dietary disaccharide sucrose. By comparison, mean added sugar intake (predominantly sucrose) totals ~58 $g \cdot day^{-1}$, far exceeding lactose intake (Brisbois et al., 2014) . Nonetheless, it is important to note

that the presence of lactose in the diet varies considerably, depending on lactose tolerance status, food preference, and numerous other factors (Keith et al., 2011).

Though it has not been directly investigated, it is possible that athletes and recreationally active individuals may have a higher lactose intake than the general population. Athletes have a high EI to permit high volumes and intensities of training, far exceeding the EI of non-athletic populations (Grandjean, 1997). Athletes also have specific macronutrient requirements to facilitate recovery (including carbohydrate and protein) which can be conveniently obtained from dairy foods such as milk (James et al., 2019), or dairy-derived products, such as whey or casein protein supplements, which can contain ~4% lactose (Tetra Pak, 2018). However, the specific role of lactose in the diets of athletes or recreationally active individuals has rarely been considered. Apart from guidance on the avoidance of lactose for those recommended low FODMAP (fermentable oligo-, di-, mono-saccharides and polyols) diets, in contrast to other carbohydrates lactose does not feature explicitly in sports nutrition guidelines, perhaps due to a paucity of research. Because athletes may be consuming nutritionally relevant quantities of lactose that exceed those consumed by the general population, it is important to elucidate its metabolic effects so that its specific application can be understood in the context of sports nutrition. Therefore, this review will briefly summarise lactose metabolism, critically review the literature relating to the utility of lactose for athletes, highlight potential risks of lactose consumption and suggest areas for future research.

1.3 Lactose digestion, absorption and metabolism

Lactose is digested exclusively by the disaccharidase lactase (lactase-phlorizin hydrolase), located on the brush border of the small intestine (See Figure 1.1). Lactose is hydrolysed at its β 1-4 glycosidic linkage, releasing the constituent monosaccharides; glucose and galactose (Mattar et al., 2012). Both glucose and galactose are then actively transported by sodium-glucose transporter proteins (SGLT1), followed by GLUT2 allowing entry to the hepatic portal vein (Thorens, 2014, Turk et al., 1994).

The glucose component of lactose reaches the liver, where first pass extraction occurs. Glucose is directed towards metabolic processes such as liver glycogen synthesis, or oxidised. However, much of the absorbed glucose will escape this fate and enter the systemic circulation. Divergence from blood glucose homeostasis results in insulin release (Koeslag et al., 2003). This leads to glucose uptake by tissues through GLUT4 transporter proteins, most notably in skeletal muscle leading to the formation of glycogen through the action of glycogen synthase (Boucher et al., 2014). Plasma glucose that appears after ingestion can also be oxidised directly by peripheral tissues, including skeletal muscle and the brain (Kelley et al., 1988). This is the typical metabolism of ingested glucose, including free glucose and that derived after digestion of disaccharides or polysaccharides (such as sucrose or starch respectively) containing glucose. Ingested glucose is metabolised efficiently in healthy, insulin sensitive individuals, which minimises prolonged elevations in blood glucose to ~60 minutes, depending on the quantity of glucose and the form of ingestion (Abdul-Ghani et al., 2010). Glucose is the comparator carbohydrate used in the glycaemic index, an

index of the 2-hr blood glucose response after carbohydrate ingestion *versus* the reference (100). Lactose has a lower glycaemic index of 46 due to the presence of a galactose molecule, and similarly the index of sucrose (61) is lowered by the presence of a fructose molecule (Wolever and Miller, 1995).

The metabolism of galactose is markedly different to glucose. Lactose is the primary dietary source of galactose, though it is also present in smaller amounts in legumes (e.g. garbanzo beans, lentils, soybean), and as bound galactose in other vegetables (Acosta and Gross, 1995). Like glucose, galactose is actively transported into the hepatic portal vein via SGLT1, and GLUT2 (Thorens, 2014, Turk et al., 1994). The hepatic portal vein transports galactose to the liver where first pass extraction occurs, and some galactose enters the Leloir pathway, the primary pathway for galactose metabolism in humans. The first step of the Leloir pathway involves the epimerisation of β-D-galactose to α-D-galactose by galactose mutarotase. In the second step, galactokinase phosphorylates α-D-galactose, yielding galactose 1-phosphate (Timson and Reece, 2003, Sørensen et al., 2011). The third step comprises a uridine monophosphate group being transferred from a uridine diphosphate-glucose to the galactose 1-phosphate by galactose-1-phosphate uridylyltransferase (GALT), forming glucose 1-phosphate and UDP-galactose. In the fourth step UDP-galactose is converted to UDP-glucose by UDP-galactose 4-epimerase. The fifth and final step involves UDP-glucose and diphosphate then forming glucose-1-phosphate and uridine triphosphate by UDP-glucose pyrophosphorylase (Holden et al., 2003, Williams, 1986).

The resulting glucose-1-phosphate is then available for glucose production or glycogen storage. However, the rate of gluconeogenesis from galactose is limited, which leads to the accumulation of galactose in the circulation in large amounts and in a sustained manner. Williams et al. (1983) demonstrated that ingestion of 0.5 $g \cdot kg^{-1}$ body mass of galactose caused substantial accumulation of plasma galactose, increasing from <0.1 $mmol \cdot L^{-1}$ at baseline to 1.70 ± 0.42 $mmol \cdot L^{-1}$ after 60 min. By feeding subjects ~22 $g \cdot h^{-1}$ of galactose, Sunehag and Haymond (2002) estimated the maximal rate of splanchnic uptake to be ~10 $g \cdot h^{-1}$. Because the liver is the primary site for galactose metabolism, and there is little evidence for extra-hepatic metabolism, circulating galactose is ultimately removed via hepatic metabolism or renal losses.

It has been observed that glucose can augment the metabolism of galactose when the two free monosaccharides are co-ingested (Sunehag and Haymond, 2002). Co-ingestion of galactose and glucose more than doubles the first pass splanchnic clearance of ingested galactose compared to galactose alone and can eliminate or drastically reduce the rise in plasma galactose after ingestion. Furthermore, glucose production was shown to be almost exclusively derived from galactose under these conditions, *via* the Leloir Pathway. However, the mechanisms explaining this increased splanchnic uptake have not been fully elucidated. It appears that intact lactose can exert a similar effect upon galactose metabolism to free glucose and galactose ingestion, both resulting in attenuated rises in plasma galactose (Williams et al., 1983). This occurs despite the requirement for digestion. In addition, Stellaard et al. (2000) demonstrated that the hydrolysis of lactose is not the rate-limiting step in its oxidation. Subjects ingested 40 g of naturally high ^{13}C lactose or pre-digested lactose (glucose

and galactose) and showed that breath $^{13}CO_2$ recovery over 4 hours was not significantly different indicating that both conditions oxidised the constituent monosaccharides of lactose at the same rate, even when delivered as the disaccharide. This may suggest that lactose and its constituent monosaccharides exert similar metabolic effects, without much impact of the digestion stage, at least at this dose. This augmentation of galactose metabolism by glucose has only been directly investigated under resting conditions.

The metabolic fate of lactose described previously assumes that lactose hydrolysis has occurred. It has been demonstrated that small amounts of lactose (0.02 mmol·L^{-1}) can appear in plasma following milk ingestion (~40g lactose; See Figure 1.1) in normal, non-pathological conditions (Pimentel et al., 2017). This was hypothesised to be due to passive diffusion through intercellular junctions in the intestinal cells at high lactose concentrations, which is subsequently excreted in the urine. If lactose is not digested in the small intestine (termed lactose maldigestion), it can pass from the small intestine into the colon (Misselwitz et al., 2013). Lactose may be fermented by a variety of local bacterial flora, which include *Bifidobacteria, Lactobacillus, Clostridium* and *Bacteroides* (Ito and Kimura, 2009). The fermentation of lactose by colonic bacteria can yield short-chain fatty acids (SCFA), lactic acid, methane (CH_4), hydrogen (H_2) and carbon dioxide (CO_2) in different quantities depending on the bacteria (Rangel et al., 2016, Yazawa and Tamura, 1982, Jiang and Savaiano, 1997b, Jiang and Savaiano, 1997a). Some of these products of fermentation can trigger gastrointestinal (GI) symptoms, which is termed lactose intolerance, and can depend on the quantity of lactose ingested. Section 4.1 discusses in greater detail these pathologies related to lactose ingestion.

The majority of research on lactose ingestion and metabolism has been performed under resting conditions, with limited studies directly investigating lactose metabolism in an exercise context. Nonetheless, studies performed at rest, and those limited studies involving exercise will be used in this narrative review to explore the possible impact of lactose on salient aspects of sports nutrition, with a particular focus on lactose as an energy source for exercise, recovery from exercise and lactose as a prebiotic. There will also be a discussion of the possible considerations and risks of over-consumption of lactose, which must be considered before practical application in a sporting context.

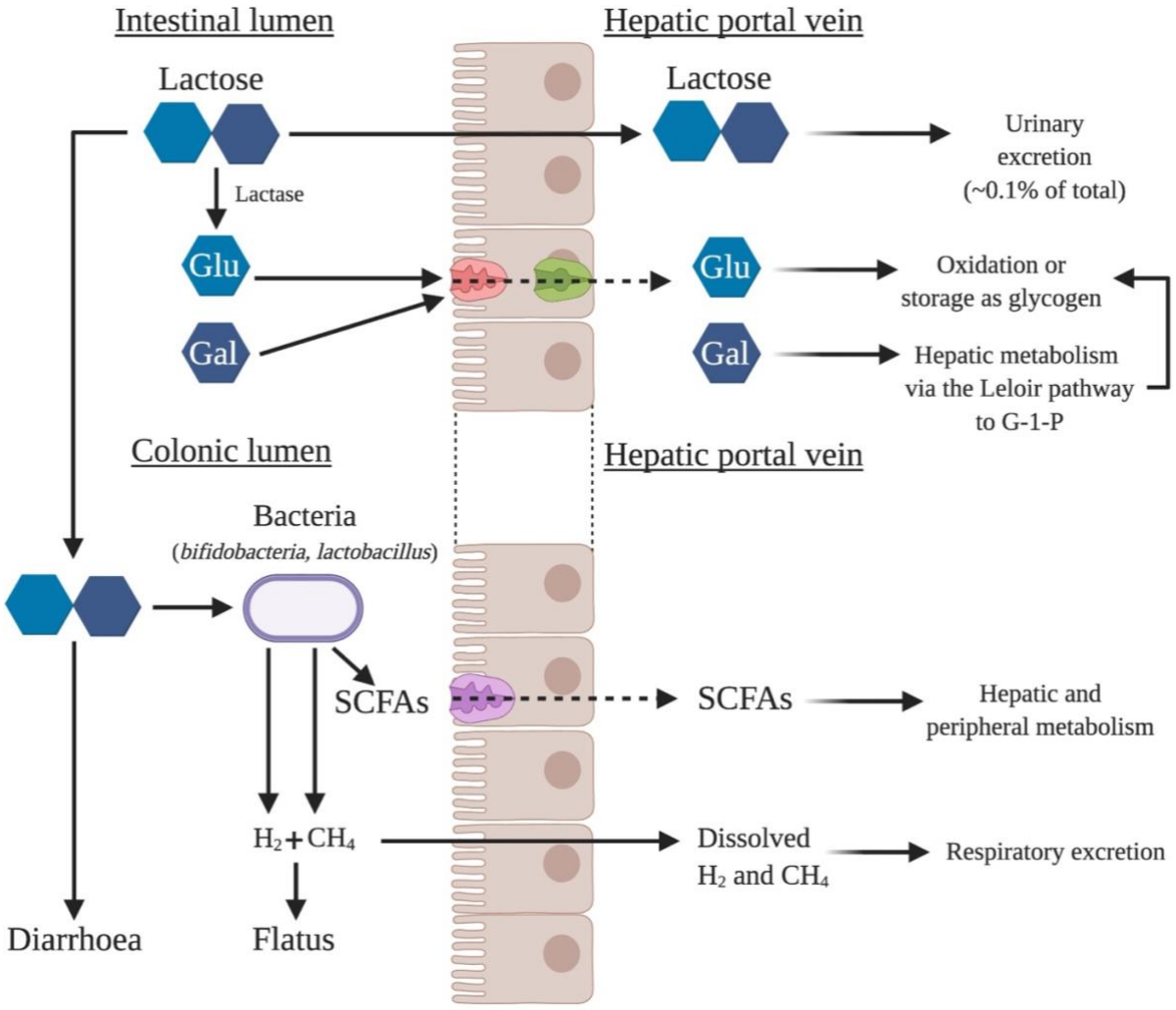

Figure 1.1 The digestion and absorption of lactose.

Glu – glucose. Gal – galactose. SCFAs – short chain fatty acids. CH4 – methane. H2 – hydrogen. G-1-P – glucose-1-phosphate. Created with BioRender.com

1.4 The role of carbohydrate in sports nutrition

During exercise the availability of endogenous carbohydrate (as plasma glucose, liver & muscle glycogen) may be reduced to due to the high energy cost of training or competition. At moderate to high exercise intensities (≥60-70% $\dot{V}O_{2max}$) carbohydrate is the predominant energy source for ATP resynthesis (Bergman and Brooks, 1999,

van Loon et al., 2001). Due to the limited quantities of stored carbohydrate, availability can limit performance in submaximal or intermittent high-intensity exercise of a duration >90 min, and is also required for short-lived, high intensity exercise performance (Burke et al., 2011). In addition to being an energy substrate, glycogen can also regulate adaptive signalling pathways. Therefore, carbohydrate availability can be positive or negative with regards to adaptation. Although adaptations to resistance-type exercise may be impacted positively by high glycogen availability, the converse is true for endurance-type exercise (Mata et al., 2019). However, carbohydrate availability is pivotal to sustaining high exercise intensities, therefore high-intensity training sessions, and competitions should be performed with high carbohydrate availability.

Recommendations for maximising carbohydrate availability for exercise are typically divided into pre-exercise and during exercise. Carbohydrate intake in the hours preceding endurance, or extended exercise is important to ensure high endogenous carbohydrate availability, as both muscle and liver glycogen. This is imperative when multiple endurance exercise sessions are completed in one day, such as stage races, or in tournament settings. It is also necessary to ensure high carbohydrate availability following an overnight fast, during which liver glycogen may be reduced (Papin et al., 2012). Ingesting carbohydrate during exercise (such as a drink or gel) is an effective strategy for improving performance in a variety of exercise scenarios, including endurance and intermittent exercise (Jeukendrup, 2011). Ingestion of additional carbohydrate provides an exogenous fuel source, which can reduce the reliance on

stored carbohydrate, permit glycogen sparing, prevent hypoglycaemia, and positively impact the central nervous system (Burke et al., 2011).

1.4.1 Lactose as an energy source for exercise

Expert recommendations for pre-exercise carbohydrate intake suggest 1-4 $g \cdot kg^{-1}$ body mass in the preceding 1-4 hours (Burke et al., 2011). The meal should contain little fibre, fat and protein, which all delay gastric emptying and could contribute to GI distress or discomfort (Nuttall and Gannon, 1991). Lactose ingestion pre-exercise may be advantageous due to its low glycaemic index of 46 (Wolever and Miller, 1995). Current guidelines recommend a low glycaemic index meal before exercise to prevent post-prandial hyperglycaemia, which may promote better maintenance of carbohydrate availability during exercise (Burke et al., 2011). However, there does not appear to be a benefit to exercise performance with low glycaemic index carbohydrate ingestion before exercise, compared to high glycaemic index carbohydrate ingestion (Burdon et al., 2017). Some athletes may avoid lactose-containing dairy foods in the meal before exercise due to concerns around GI discomfort. However, Haakonssen et al. (2014) demonstrated that a pre-exercise high carbohydrate dairy meal containing ~40g of lactose resulted in no differences in gut discomfort or performance during a cycling time-trial compared to a high carbohydrate dairy-free meal. Although there has been no direct investigation into lactose ingestion pre-exercise *per se*, there are no reasons to indicate that lactose could not form part of a pre-exercise carbohydrate ingestion regime.

Recommendations for carbohydrate intake during exercise, including type and amount, depend on intensity and duration. Endurance exercise lasting <30 min is not thought to be limited by glycogen availability, though there may be a performance benefit to carbohydrate intake, however, exercise lasting 30-75 min may require small amounts of carbohydrate to offset what may be substantial glycogen oxidation (Jeukendrup, 2011). Exercise lasting 60-120 min requires greater intake of 30-60 $g \cdot h^{-1}$ of most carbohydrate forms, whilst durations of 120-180 min require up to 60 $g \cdot h^{-1}$ of rapidly oxidizable carbohydrate (glucose, glucose polymers). Exercise lasting >150 min may require up to 90 $g \cdot h^{-1}$ and necessitates the use of multiple transportable carbohydrates (glucose and fructose). Carbohydrate may be consumed in a liquid, gel or solid form, as all are oxidised at comparable rates (Pfeiffer et al., 2010b, Pfeiffer et al., 2010a). The guidelines on carbohydrate ingestion during exercise do not mention lactose, and focus on ingestion of glucose, glucose polymers, sucrose and fructose. This is likely due to a paucity of research into lactose and the limited data on its metabolism in an exercise context. Though the main requirement for a carbohydrate ingested during exercise is that it is readily oxidised, there are other considerations that may impact the appropriateness of a carbohydrate. For example, the cariogenicity of a carbohydrate is important for dental health, which is relevant for athletes who generally display poor dental health, including high rates of dental caries (Ashley et al., 2015). Therefore, ingestion of carbohydrates that are less cariogenic, such as lactose, may offer specific benefits over other carbohydrates for athletes, beyond the confines of exercise metabolism (Koulourides et al., 1976).

There are limited data on the metabolism of lactose and its monosaccharide constituents during exercise. However, pre- and during-exercise ingestion of free glucose and galactose in a 1:1 ratio has been investigated as a method to improve time trial (TT) performance (Stannard et al., 2009). Subjects consumed galactose, galactose/glucose (1:1 ratio) or glucose/fructose (4:1 ratio) 45 min before beginning cycling for 120 min, while consuming 1 $g \cdot min^{-1}$, followed by a TT. TT performance was poorest in the galactose-only condition, and the glucose/galactose and glucose/fructose conditions were not different. Ingested galactose is oxidised ~50-60 % slower than glucose, due to the requirement for hepatic metabolism (Leijssen et al., 1995, Burelle et al., 2006), which may explain the poorer TT performance with galactose ingestion. This demonstrates the potential for a blend of the free monosaccharides constituting lactose, both for improving TT performance, and for the maintenance of blood glucose concentration during exercise. Glucose production is almost exclusively derived from galactose when it is co-ingested with glucose at rest (Sunehag and Haymond, 2002), though it is not clear whether this is the case under exercise conditions when hepatic and GI metabolism are altered.

Lee et al. (2008) investigated the effects of ingestion of low-fat milk during exercise. Subjects exercised at 70% $\dot{V}O_{2peak}$ to exhaustion, whilst ingesting water, a glucose drink (~36 $g \cdot h^{-1}$ of carbohydrate), low-fat milk (~30 $g \cdot h^{-1}$ of carbohydrate) or low-fat milk with added glucose (~36 $g \cdot h^{-1}$ of carbohydrate). Whilst time to exhaustion (TTE) was not different between any conditions, when considered as percentage change *versus* water, the carbohydrate drink and milk ingestion resulted in an extended TTE. Although milk ingestion does not isolate the lactose component and the confounding

effects of other macronutrients in milk are not accounted for, this suggests that lactose could be a viable exogenous energy source during exercise. Additionally, Stellaard et al. (2000) compared ingestion of naturally high abundance ^{13}C glucose and lactose before 4 hours of light physical activity, and showed $^{13}CO_2$ recovery was comparable between carbohydrates. This implies that the oxidation of lactose may be equal to glucose alone and also suggests that oxidation of galactose is likely to have been augmented by the presence of glucose. However, interpretation of this study is hampered by its small sample size (*n*=5) and low exercise intensity (50 W).

Lactose has the potential to act as a viable exogenous energy source during, and potentially before exercise. It performs comparably to other carbohydrates in isolation and within milk, in terms of positive metabolic outcomes for exercise. Consideration of individual differences and lactose tolerance status should be made.

1.4.2 Lactose in a post-exercise setting

Prolonged endurance or intermittent exercise can deplete or reduce glycogen, stored in the liver and the muscle. Whilst muscle glycogen is hydrolysed and oxidised locally by the muscle, liver glycogen is utilised in the maintenance of blood glucose homeostasis (Taylor et al., 1996). The impact of pre-exercise muscle glycogen content on exercise performance is well established. Low muscle glycogen content causes reduced endurance performance and capacity, whilst high muscle glycogen content predicts better performance and capacity (Bergström et al., 1967, Widrick et al., 1993). Therefore, post-exercise replenishment of muscle and liver glycogen is a priority to ensure adequate endogenous carbohydrate for later exercise. This is particularly

relevant for athletes in sports with multiple events in a day, such as in tournaments, events over multiple days or stage races such as the Tour de France, or even when multiple training sessions per day are performed.

Current recommendations for post-exercise nutrition advocate carbohydrate ingestion as soon as is practical to maximise preparedness for the next exercise bout. Current guidelines recommend ingestion of carbohydrate at a rate of 1.2 $g \cdot kg^{-1} \cdot h^{-1}$ in the initial hours after exercise, which is sufficient to maximise muscle glycogen replenishment in a post-exercise state (Burke et al., 2011). It has been extensively demonstrated that if sufficient quantities of carbohydrates of moderate to high glycaemic index are provided in the post-exercise period, muscle glycogen can be replenished optimally, though it has been observed that some carbohydrates perform poorly in the restoration of muscle glycogen, such as fructose (Blom et al., 1987). However, more recently there has been an interest in carbohydrates with divergent paths for endogenous storage, namely fructose and galactose.

It has been observed that post-exercise glucose ingestion is a relatively poor strategy for optimising the resynthesis of liver glycogen, despite its positive effects on muscle glycogen replenishment (Décombaz et al., 2011). Both fructose and galactose have been demonstrated to be superior substrates for liver glycogen replenishment when co-ingested with glucose, which is likely due to their requirement for hepatic metabolism. Décombaz et al. (2011) compared combined maltodextrin and galactose, fructose or glucose ingestion, in total doses of 450 g (~69 $g \cdot h^{-1}$) after glycogen depleting exercise. Liver glycogen content was assessed via ^{13}C magnetic resonance

imaging (MRI) and spectroscopy (MRS) over a 6.5h recovery period. Both galactose (8.6 ± 0.9 $g \cdot h^{-1}$) and fructose (8.1 ± 0.6 $g \cdot h^{-1}$) ingestion effectively doubled the liver glycogen deposition compared with glucose-only (3.7 ± 0.5 $g \cdot h^{-1}$). This increase in liver glycogen resynthesis can also be observed with sucrose ingestion compared to glucose (Fuchs et al., 2016). This demonstrates the requirement for carbohydrate types that require hepatic metabolism, in addition to glucose. To the author's knowledge, Décombaz et al. (2011) performed the only investigation into liver glycogen replenishment post-exercise comparing galactose to other carbohydrates.

To promote optimal combined liver and muscle glycogen replenishment post-exercise, it is likely that glucose must be combined with a carbohydrate source that undergoes predominant hepatic metabolism (i.e. galactose, fructose). It is possible that lactose, as a disaccharide of glucose and galactose, may be sufficient to promote combined muscle and liver glycogen resynthesis, effectively acting as a blend of glucose and galactose. However, it remains to be investigated whether combined glucose and galactose feeding is as effective at replenishing muscle glycogen as glucose alone, which has been demonstrated for combined glucose and fructose ingestion (Wallis et al., 2008, Fuchs et al., 2016, Trommelen et al., 2016). This is an important factor determining whether glucose and galactose or lactose may be advantageous compared to glucose alone in the restoration of glycogen stores.

Whilst no studies have investigated muscle or liver glycogen replenishment and/or recovery of exercise performance or capacity with lactose ingestion, milk and chocolate milk have been extensively studied in recovery from exercise (Alcantara et

al., 2019, Amiri et al., 2018). Milk ingestion has been shown to be equally effective at improving exercise capacity after an initial bout of exercise as carbohydrate-electrolyte beverages delivering similar amounts of carbohydrate in a number of investigations (Lee et al., 2008, Watson et al., 2008). Indeed, Loureiro et al. (2020) in a systematic review concluded that milk-based beverages result in similar effects on glycogen resynthesis and restoration of exercise performance to carbohydrate beverages. Chocolate milk has also been investigated for post-exercise recovery, as a convenient, carbohydrate and protein rich recovery beverage. A systematic review and meta-analysis by Amiri et al. (2018) showed that chocolate milk ingestion after a variety of exercise types (including running and cycling) increased time-to-exhaustion (TTE) in a subsequent exercise bout, compared to placebos and mixed macronutrient beverages (containing carbohydrate, fat and protein). Furthermore, an investigation into chocolate milk ingestion and glycogen replenishment demonstrated that chocolate milk resulted in higher muscle glycogen restoration than an isocaloric carbohydrate beverage after an exercise bout, despite lower total carbohydrate content (0.84 $g \cdot kg^{-1} \cdot h^{-1}$ *versus* 1.2 $g \cdot kg^{-1} \cdot h^{-1}$) in the chocolate milk condition, which may be attributable to its protein content (Karfonta et al., 2010). Ferguson-Stegall et al. (2011) showed that after an intense cycling exercise bout, chocolate milk was equally effective at replenishing muscle glycogen as an isocaloric carbohydrate beverage but resulted in improved TT performance.

One potential benefit to chocolate milk ingestion is that it contains a full spectrum of monosaccharides, including galactose (as lactose), fructose (as sucrose) and glucose from both disaccharides. Although it has not been directly investigated, the

combination of two carbohydrates known to increase liver glycogen replenishment, as well as glucose, could have an additive effect over and above just one of these carbohydrates. This could explain the superior effects of chocolate milk ingestion compared to the carbohydrate (glucose) beverage on TT performance (Ferguson-Stegall et al., 2011). However, it is not possible to isolate the effects of the lactose component of milk or chocolate milk, compared to the other nutrients in milk such as protein, which can amplify insulin secretion and glycogen replenishment when carbohydrate intake is insufficient (Burke et al., 2011) as well as fat and electrolytes. A further benefit to chocolate milk in the post-exercise setting is its high protein content (~16 g per 500 ml), and high protein quality, containing whey and casein (Amiri et al., 2018). Indeed, Phillips et al. (2009) note that milk proteins are superior to carbohydrate alone for post-exercise nutrition in the promotion of skeletal muscle hypertrophy. Therefore, chocolate milk and other flavoured dairy beverages can be considered multifunctional foods in a post-exercise setting.

1.4.3 Lactose as a prebiotic

There is potential for lactose, in some scenarios to act as a prebiotic, which represents an additional application in sports nutrition. Prebiotics are indigestible compounds, that are metabolised by gut microbiota, which affect the microbiota composition or activity (Bindels et al., 2015) in a manner beneficial to the host (Carlson et al., 2018). This potential aspect of lactose is supported by only limited research, and so the potential for lactose to act as a prebiotic requires more thorough investigation to further understand any possible benefits that could be conferred. Lactose can be considered a prebiotic, as in some individuals, undigested lactose may pass from the small

intestine to the colon, where it is available for fermentation by local bacteria, much like established prebiotics such as galactooligosaccharides (GOS) and fructooligosaccharides (FOS) (Carlson et al., 2018). This is likely most common in individuals with a limited ability to digest lactose, though undigested lactose could also appear in the colon of individuals with a normal ability to digest lactose, thereby also conferring a prebiotic effect. However, in both groups this is likely dependent on the amount of lactose ingested and the individual's lactose digestion capacity.

The mechanisms explaining the positive impacts of prebiotics are not fully understood but may be linked to the cultivation of beneficial colonic bacteria, capable of producing short chain fatty acids (SCFAs) which are favourable for bowel health and immune function (Parada Venegas et al., 2019). Prebiotics could offer athletes and recreationally active individuals a performance benefit through a number of indirect mechanisms, though to the authors knowledge, the impact of prebiotic supplementation on athletic performance has not been assessed. However, some benefits have been observed in healthy individuals, which may be applicable to athletes. Supplementation with GOS is associated with reduced GI illness, as well as reduced cold and flu symptom severity (Hughes et al., 2011). Preventing illness in athletes may reduce interruption to training or attenuate the direct impact of illness upon exercise performance in competition. Another potential benefit is the suppression of appetite by FOS supplementation that has been observed, which may benefit energy-restricted athletes (Cani et al., 2009). Although different prebiotics exert their effects through similar mechanisms, it is not known whether certain effects are linked

to specific prebiotics. Therefore, the benefits of certain prebiotics should not be ascribed to others without direct investigation.

Whether lactose exerts a prebiotic effect has not been investigated, and it is not clear whether any prebiotic effect might occur alongside GI symptoms due to the presence of lactose in the colon. However, ingestion of any prebiotic can lead to GI symptoms, including flatulence and osmotic diarrhoea, which can be modulated by individual factors, as well as the total dose (Livesey, 2001). It is possible that regular consumption of dairy products containing lactose may confer some prebiotic effect in both lactose digesters and maldigesters, though the quantity of lactose required to achieve this is not known. Although not directly related to sports nutrition, prebiotics may also exert a number of health benefits which include a protective effect on colon cancer and the reduction of certain risk factors for cardiovascular disease (Slavin, 2013). These health benefits are thought to be conferred by SCFA production, of which butyrate is likely the most beneficial for colonic health.

1.5 Considerations for the application of lactose in sports nutrition

1.5.1 Relevant conditions and pathologies, relating to lactose

The application of lactose in sports nutrition may be confounded due to the presence of a variety of pathologies related to lactose digestion and absorption, including lactose maldigestion and subsequent intolerance. These conditions are a result of lactase non-persistence, the genetically determined, gradual decline in lactase activity to ~10 % of previous activity, after 2 – 3 years of age (Misselwitz et al., 2013). This condition is

present in >90% of East Asians and ~70% of Mexicans and African Americans (Itan et al., 2010, Scrimshaw and Murray, 1988). However, in some individuals the activity of lactase does not reduce, remaining high into adulthood, which is termed lactase persistence, which is the normal condition in 80-95% of adults in Britain, Scandinavia and Germany (Itan et al., 2010). The development of lactase persistence is thought to be linked to the development of dairy farming over the past ~10 000 years (Ingram et al., 2009).

Lactose maldigestion refers to the inefficient or incomplete digestion of lactose, generally due to lactase non-persistence (Misselwitz et al., 2013). Lactose maldigestion may also be referred to as lactose malabsorption. However, this term does not accurately describe the condition, as the absorption of lactose is not impaired, whereas the digestion stage is limited. Therefore, the term maldigestion should be used when referring to the ineffective digestion of lactose and use of the term malabsorption should be avoided unless specifically describing reduced absorption of lactose (or its constituent's glucose and galactose) into the splanchnic circulation.

Lactose intolerance (LI) refers to the GI symptoms that result from lactose maldigestion, which are a result of unhydrolyzed lactose passing from the small intestine into the colon (Misselwitz et al., 2013). These can include diarrhoea due to the lactose increasing the osmotic load, which draws water into the intestines, and can lead to abdominal cramping or pain (Rangel et al., 2016). Flatulence and bloating can also result from the fermentation of the lactose by colonic bacteria which may produce gases including CH_4, H_2 and CO_2 (Rangel et al., 2016, Yazawa and Tamura, 1982,

Jiang and Savaiano, 1997b, Jiang and Savaiano, 1997a). Certain bacteria favour reduced gas production (such as *Bifidobacteria* and *Lactobacillus*) compared to other colonic bacteria (Tsukahara et al., 2009).

The symptoms of LI, and their severity vary widely between individuals, depending on the individual's lactase activity and bacterial flora. There may also be variation between LI symptoms in the same individual in response to different foods or meals. For example, high fat or high osmolality foods can slow gastric emptying, which can reduce symptoms of intolerance (Jellema et al., 2010). Ingesting yoghurt can improve GI symptoms and breath H_2 response compared to milk, which is likely due to the presence of bacteria capable of hydrolyzing the lactose (Pelletier et al., 2001). Consideration of the dairy 'matrix' is important when discussing LI and other issues surrounding dairy and health, rather a reductionist single-ingredient approach (Szilagyi and Ishayek, 2018).

It is important to note that many individuals with lactose maldigestion can tolerate small amounts of lactose without GI symptoms. Indeed 0.4 g of lactose ingested as a pill did not alter GI symptoms significantly compared to a placebo, nor did it trigger a positive (>20 ppm) breath H_2 test (Montalto et al., 2008). Shaukat et al. (2010) reported that dairy products containing up to 12-15g of lactose are well tolerated by most adults with lactose maldigestion, equivalent to 250-300 mL of milk. Therefore, consumption of dairy products, as well as dietary supplements or medication capsules that use lactose as a filler should not necessarily be avoided by lactose intolerant individuals, and the

avoidance of all dairy products is no longer recommended (Szilagyi and Ishayek, 2018).

1.5.2 Diagnosis of lactase-related pathologies

There are numerous tests to diagnose lactose maldigestion or intolerance, though none is considered a 'gold standard' test. The lactose tolerance test involves ingesting a bolus of 25-50g lactose, measuring blood glucose, and reporting acute GI symptoms (Hovde and Farup, 2009). If the lactose is hydrolysed, serum glucose is expected to rise, whilst no change, and/or the presence of GI symptoms suggests LI. However, the test has very low sensitivity and specificity (Misselwitz et al., 2013). The National Institute of Health requires the diagnosis of LI to involve comparison to an inert placebo (Suchy et al., 2010), which can reduce the impact of self-reported food intolerances which are often unsubstantiated (Misselwitz et al., 2013).

The H_2 breath test is widely used to assess lactose maldigestion, as GI symptoms after a lactose challenge are better correlated with breath H_2 production than with changes in blood glucose (Hermans et al., 1997). Expired breath is collected before and after ingestion of 25 g of lactose, and breath [H_2] >20 ppm implies that the lactose has not been completely hydrolysed and has entered the colon where it is fermented by bacteria. The measurement of CH_4 alongside H_2 has been shown to improve the prognostic capability of the breath test (Hovde and Farup, 2009). However, false positives can be caused by small intestine bacterial overgrowth, whilst false negatives may be caused by H_2 or CH_4 non-producing subjects (between 2-43% of subjects; Gasbarrini et al. (2009)). A process known as 'full colonic adaptation' can also occur,

where repeated lactose intake selectively favours flora that ferment lactose without the production of H_2 or CH_4 (Misselwitz et al., 2013). Inadequate dietary control in the day(s) preceding the test may artificially inflate pre-test values, due to presence of fermentable substrates such as fibre in the colon. Furthermore, the boundary of breath [H_2] of >20 ppm is arbitrary and does not relate to a threshold of lactase expression or the presence of GI discomfort.

Genetic testing can also be employed to diagnose of lactase non-persistence. Presence of the genetic polymorphism 13910C/C indicates lactase non-persistence. This test has a low false positive rate (<5 %) but generates false negatives if LI is caused by secondary factors. Testing for this polymorphism is only appropriate in Caucasian subjects (Misselwitz et al., 2013). Lactase activity in the jejunum can be assessed via biopsy, which is considered a 'reference standard' for the assessment of lactase deficiency. However, this method is extremely invasive and, lactase expression in enterocytes forms a 'mosaic' pattern meaning not all areas of the brush border express lactase strongly (Maiuri et al., 1991, Misselwitz et al., 2013). Therefore, biopsies can sample areas without lactase even in lactase persistent individuals, generating a false positive result.

It has been noted that it is possible for individuals to 'adapt' to lactose ingestion, showing decreased breath H_2 responses and GI symptoms in response to lactose feeding after chronic ingestion. Lactase activity does not increase in response to lactose exposure in humans (Gilat et al., 1972). However, adaptation of colonic bacteria in response to lactose ingestion is known to occur (for a review, see Szilagyi

(2015)). ‘Full colonic adaptation’ is defined by Misselwitz et al. (2013) as a process in which “repeated intake of lactose selectively favours the growth of colonic flora that rapidly ferment lactose without producing hydrogen”. Colonic bacterial adaptation may also occur in such a manner that hydrogen-producing bacteria remain, but that lactose that reaches the colon is still fermented, without the presence of symptoms (Johnson et al., 1993b, Di Stefano et al., 2007). Indeed, only approximately one third of individuals with positive H_2 breath tests experience GI symptoms (Di Stefano et al., 2007), highlighting the disconnect between breath H_2 responses and LI symptoms.

It has been demonstrated that lactose intolerant individuals can adapt to increasing lactose intake without intolerance symptoms. Johnson et al. (1993a) provided 22 lactose intolerant maldigesters with progressively increasing daily doses of lactose. All subjects increased the threshold at which symptoms were induced, to at least 7 g lactose, and 17 subjects increased to ≥12 g. Lactose maldigesters regularly consuming dairy foods may not suffer from LI, except at high doses where the ability of colonic bacteria (and limited lactase) to metabolise lactose is exceeded, whilst maldigesters with low lactose intake may suffer intolerance even at very low doses. Most individuals with lactose maldigestion can tolerate ingestion of ~12-15 g of lactose (~250-300 mL of milk) without symptoms (Shaukat et al., 2010).

1.6 Potential limitations of lactose in sports nutrition

It is not entirely clear whether lactose can be a viable carbohydrate for exercise settings in light of the issues surrounding LI and maldigestion. A known or diagnosed intolerance generally precludes consumption of lactose, and although many

maldigesters can tolerate small amounts of lactose without symptoms (Shaukat et al., 2010), these amounts are far below the quantities required for carbohydrate ingestion before (1-4 $g \cdot kg^{-1}$), during (30-90 $g \cdot h^{-1}$) or after (1.2 $g \cdot kg^{-1} \cdot h^{-1}$) exercise (Burke et al., 2011). It is also not clear what the upper limit for lactose digestion is even in healthy individuals, though absorption of glucose and galactose via SGLT1 is limited to ~60 $g \cdot h^{-1}$, therefore at such ingestion rates the absorption of lactose-derived glucose and galactose may be limited (Jeukendrup, 2010). Additionally, lactose should not form the major part of a carbohydrate ingestion regimen for exercise in individuals with diagnosed LI, except up to quantities of ~12-15 g, if tolerable. Furthermore, individuals following FODMAP exclusion diets should also avoid lactose as required.

Individuals may have concerns about ingesting large quantities of lactose-containing dairy foods in the post-exercise period to achieve the required carbohydrate intake. It is unclear whether the hydrolysis of lactose in a post-exercise setting might limit the delivery of its constituent monosaccharides. However, ingestion of a large quantity of lactose (80 g) before exercise, did not result in side effects of maldigestion in the majority of lactose-tolerant subjects (Stellaard et al., 2000). Recommendations for post-exercise carbohydrate intake are higher than before or during exercise, but an investigation by Costa et al. (2020) involving participants ingesting 1.2 $g \cdot kg^{-1}$ of carbohydrate from chocolate milk showed that neither GI symptoms nor breath H_2 were elevated with a high carbohydrate post-exercise dairy meal compared to water ingestion. Therefore, lactose could be an appreciable contributor to post-exercise carbohydrate requirements. Dairy foods such as milk or yoghurt may offer an additional benefit over isolated lactose, as the dairy matrix (as seen in yoghurt) is known to

improve GI comfort and deliver relevant nutrients such as protein and electrolytes (Pelletier et al., 2001, Szilagyi and Ishayek, 2018, Thorning et al., 2017). Individuals should determine which carbohydrates and foods, and in what quantities, are appropriate for them to use pre-, during and post-exercise, based on their personal preference and responses. This is the case for all carbohydrates, including fructose in light of the presence of fructose malabsorption in some individuals (Putkonen et al., 2013).

An impediment to the application of lactose in sports nutrition is the unfavourable perception of dairy and lactose. Dairy products are commonly included in a description of healthy eating by children and adolescents, but to a lesser extent by adults (Paquette, 2005). The potential of milk to cause allergic symptoms is considered to be high (*versus* soy milk), and it is thought to cause serious disease (including heart disease, high cholesterol and obesity) to a greater extent than soy milk (Bus and Worsley, 2003). Self-diagnosis of LI is also common, with 11-16% of North Americans self-diagnosing LI, in most cases without clinical diagnosis of intolerance or maldigestion (Barr, 2013, Nicklas et al., 2011). This leads to inappropriate avoidance and reduced consumption of dairy foods (Keith et al., 2011, Barr, 2013).

Table 1.2 Summary of applications & considerations for lactose in sports nutrition

Potential application	Comments
Lactose as an energy source for exercise	• Ingested lactose may be oxidised effectively during exercise (Stellaard et al., 2000). • Lactose can form part of a pre- or during exercise carbohydrate feeding regimen for lactose tolerant individuals.
Lactose for glycogen restoration	• Lactose is the primary source of dietary galactose, which has been shown to increase post-exercise liver glycogen resynthesis (Décombaz et al., 2011) • Studies on milk and chocolate milk suggest that lactose can be a substrate for muscle glycogen resynthesis, resulting in similar subsequent exercise performance to carbohydrate beverages (Amiri et al., 2018)
Lactose as a prebiotic	• Lactose can exert a prebiotic effect on the purportedly beneficial bacterial flora *Bifidobacteria* and *Lactobacilli* (Ito and Kimura, 2009) • It is not clear whether this may be concomitant with GI symptoms in lactose intolerant individuals
Considerations	
Lactose intolerance	• Lactose intolerant individuals should avoid large quantities of lactose, but 12-15 g is generally well tolerated (Shaukat et al., 2010)
Amount of lactose required	• Nutritional recommendations for exercise settings involve large quantities of carbohydrate (Burke et al., 2011) • Lactose and milk beverages do not trigger GI symptoms in most lactose tolerant individuals during, before or after exercise, even when ingested in large amounts (Alcantara et al., 2019)

GI - gastrointestinal

1.7 Summary

Although lactose does not feature in guidelines for carbohydrate intake for sports nutrition, it is apparent that the dairy carbohydrate could play a role in the diets of athletes and recreationally active individuals. Lactose from milk or its free monosaccharide constituents represents a possible fuel source for endurance exercise and can be incorporated into carbohydrate feeding regimens for during, and possibly pre- and post-exercise. Lactose may be a good choice for combined replenishment of muscle and liver glycogen after exercise due to its galactose component. Lactose could also be considered a prebiotic in some scenarios, and future research should aim to determine whether it can engender similar benefits to established prebiotics.

1.8 Aims of this thesis

The specific aims of this thesis, which correspond to the three experimental chapters are:

1. To investigate whether lactose represents a viable energy source for exercise
2. To further understand the metabolism of lactose, and its effects on exercise metabolism
3. To investigate whether lactose can play a role in post-exercise liver glycogen resynthesis, specifically as a delivery vehicle for galactose, alongside fructose

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Chapter 2. General Methods

This section will describe the common methods used in the chapters of this thesis, their rationale, and where appropriate justifying their use and discussing their limitations. However, it will not repeat information relating to specific protocols described in later chapters, unless pertinent to discussing the principles of the method.

2.1 Indirect calorimetry

2.1.1 Principles and Stoichiometry

The measurement of energy expenditure (EE) and the substrate oxidation that comprises it is an essential method for research in sports nutrition and exercise metabolism. Direct calorimetry is the gold standard for measuring EE, as it directly quantifies energy expenditure (as heat), and is thus termed ‘direct’ (Kenny et al., 2017). However, a number of practical limitations limit its utility in human exercise metabolism research, including its lack of ability to differentiate oxidation of different substrates and the requirement for whole-room calorimeters, which are uncommon. More widely used in the measurement of EE and substrate utilisation is indirect calorimetry (IC), which measures O_2 consumption and CO_2 production (Kenny et al., 2017).

By measuring O_2 consumption and CO_2 production at the mouth, a proxy for respiratory quotient (RQ) can be obtained. RQ represents the quantity of O_2 consumption compared to CO_2 production at the tissue level, whilst respiratory exchange ratio (RER)

represents pulmonary gas exchange. Different substrates require and produce varying quantities of O_2 and CO_2 respectively, in their oxidation, and by measuring these ratios it is possible to determine substrate utilisation. The stoichiometric equations below show the O_2 consumption and CO_2 production resulting from glucose and palmitic acid oxidation.

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

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

To oxidise 1 mole of glucose, 6 moles of O_2 (134 L) is required, which generates 6 moles (134 L) of CO_2, therefore generating a RQ (and RER) of 1.0. By contrast, oxidation of 1 mole of palmitic acid requires 23 moles of O_2 (515 L) and produces 16 moles of CO_2 (358 L), generating an RQ (and RER) of 0.7.

Although carbohydrates may differ stoichiometrically to a small degree, these differences are considered to be inconsequential in the calculation of carbohydrate oxidation (Frayn, 1983). However, many stoichiometric equations were designed for use under resting conditions, during which carbohydrate oxidation is derived primarily from liver glycogen derived glucose, however during exercise muscle glycogen oxidation comprises a large proportion of oxidised carbohydrate. Some equations for the calculation of carbohydrate oxidation attempt to correct for this by apportioning a percentage of the carbohydrates oxidised to be glycogen (Jeukendrup and Wallis, 2005), but Ferrannini (1988) suggests that assuming exclusive glucose oxidation is unlikely to introduce a major error. By contrast, fatty acid chain lengths vary which can

affect the stoichiometric equations considerably. Therefore, there are numerous iterations of such equations, each with different assumptions. Frayn (1983) assumed the oxidation palmytoyl-stearoyl-oleoyl glycerol, which is similar to the average triacylglycerol in human adipose tissue. Similarly, Ferrannini (1988) assumed the oxidation of tripalmitoylglycerol because palmitic acid is the most abundant fatty acid in the body (and it is comparable to the triacylglycerol used by Frayn (1983)). Péronnet and Massicotte (1991) used a weighted average of 13 fatty acids in their equation, which account for 99 % of the fatty acids found in the body, which was later suggested for use by Jeukendrup and Wallis (2005).

Oxidation of amino acids occurs both at rest and during exercise. To oxidise 1 mole of an average amino acid, 5.1 moles (114 L) of O_2 is required, which generates 4.1 moles (92 L) of CO_2, therefore generating a RQ (and RER) of 0.81 (Jeukendrup and Wallis, 2005). To calculate oxidation of amino acids, nitrogen excretion (as urea) in urine must be measured, and 1 g of nitrogen equates to approximately 5.57 g of protein oxidation (Jungas et al., 1992). Amino acid oxidation is often calculated when IC is used at rest, but only rarely during exercise. This is the case despite amino acid oxidation increasing during exercise, and increased activity of catabolic enzymes such as branched-chain 2-oxoacid dehydrogenase (Wagenmakers et al., 1991). However, even in situations of low energy availability, such as in a fasted or glycogen depleted state, or during extended exercise, amino acid oxidation reaches a maximum of ~10 % of EE (Wagenmakers, 1998). Due to the intermediate RER resulting from amino acid oxidation (0.81), inaccuracies in the calculation of substrate utilisation are minimised. Therefore, it is assumed that protein oxidation is minimal for the purposes of using IC

during most exercise settings. Furthermore, amino acid oxidation is reduced (by ~25 %) by endurance training (McKenzie et al., 2000), lessening the rationale for calculating amino acid oxidation in endurance trained individuals.

2.1.2 Methodologies

Indirect calorimetry requires measurement of O_2 consumption and CO_2 production, which can be achieved using a number of different pieces of equipment. A variety of methods are used for IC, including the Douglas bag and metabolic cart, which are commonly utilised in exercise settings, and metabolic chambers, which are useful for taking measurements over longer time periods. The Douglas bag is often considered the 'gold standard' measurement for IC due to its excellent reliability (Hopker et al., 2012), despite the development of alternative equipment and methods such as metabolic carts and metabolic chambers. In short, Douglas bags collect expired air for a defined time period through a mouthpiece into a plastic bag. At the end of the measurement period, the bag is sealed, a sample of air taken for analysis of O_2 and CO_2 content, and the volume of air in the bag evacuated and measured. In more recent years, computerised metabolic carts have become widespread in exercise metabolism research. Many such systems deliver good agreement with Douglas bags (Carter and Jeukendrup, 2002), although some systems fail to produce accurate and reliable results, therefore their use is dependent on effective and regular calibration, validation and maintenance (Gore et al., 1997). The proper use of Douglas bags should also include measurement of atmospheric O_2 and CO_2. Although assumption of atmospheric constants for these gases is common (at the values of 20.93% and 0.03% respectively), these are not universally accurate in part due to climate change, resulting

in current values of 20.95% and 0.04% respectively (Scripps O2 Program, 2022, National Oceanic and Atmospheric Administration (NOAA), 2022). Furthermore, when IC is used during exercise, even in well-ventilated rooms expired gases can 'contaminate' the ambient air, resulting in systematic inaccuracies in calculation of substrate utilisation and energy expenditure (Betts and Thompson, 2012).

Metabolic carts have a number of integrated measurement systems, including those to measure flow rate (used to calculate minute ventilation; $\dot{V}E$) as well as separate gas (O_2 and CO_2) analysers. Turbines containing an impellor are commonly used to measure flow rate, the rotation of which is detected by a photocell-device, which may be infrared (Macfarlane, 2012). Turbines are generally insensitive to changes in gas composition, temperature, and humidity, but may be less accurate at low and high flow rates, especially in the determination of the start and end of breaths. CO_2 analysers typically utilise the ability of CO_2 gas to absorb infra-red radiation. In non-dispersive infra-red (NDIR) systems, dual beams of infra-red are sent through a sample and reference cells, which are mechanically interrupted, providing an oscillating signal to the sensor. The magnitude of the oscillations is proportional to the differences in concentration of CO_2 between the two gases. There are a variety of methods for analysing expired O_2, but amongst the most common (and the method used in the experimental chapters of this thesis) is an electrochemical system (Macfarlane, 2012). These systems have a fuel cell, which generates a stable current that is proportional to the partial pressure of O_2.

Metabolic carts are widely used, despite being more expensive than the 'gold standard' Douglas bags. They provide immediate data and are less time consuming to use. Furthermore, the data is often on a breath-by-breath basis, which permits greater resolution and density of measurements than Douglas bags. However, metabolic carts vary from system to system, each with their own assumptions and calculations inherent in the software, which makes direct comparisons between systems potentially unreliable. Many of the results from metabolic carts are not directly measured (such as VE) but are calculated based on surrogate measurements (such as flow rate). There is a high possibility of error, and of poor reliability if the metabolic cart is not calibrated, validated and maintained properly.

Metabolic carts (Vyntus, Vyaire Medical, Mettawa, IL, US) were used in the experiments described in Chapters 3-5. This metabolic cart used an electrochemical system for its O_2 analyser, and an infra-red CO_2 analyser. Vyaire Medical state the reliability of key physiological measurements, including Ventilation (2%), VO_2 (3%), VCO_2 (3%) and RER (4%), but there are no independent verifications compared to the gold standard Douglas bag system. However, it was possible to assess the reliability of this system using the combined data from Chapters 3 and 4, using data from repeat trials (see Table 2.1). These data were measured in replicate trials from Chapters 3 and 4, using a Vyntus automated online gas analyser for combined carbohydrate conditions between 30-150 minutes of steady state cycling exercise at 50% W_{max}. These data support the manufacturers estimates of reliability, though it should be noted that the trials are not exact replicates, in that in one condition for each CHO blood sampling via a cannula was performed.

Table 2.1 Coefficient of variation (%) of VO2 and VCO2.

	VO_2	VCO_2
CV	3.0	2.8

2.1.3 Assumptions

IC assumes that gas exchange at the lungs, and the mouth reflect cellular gas exchange, (that RQ is equal to RER), which is the case in the presence of a stable bicarbonate pool, up to intensities of 75-85 % $\dot{V}O_{2max}$ (Romijn et al., 1992, Jeukendrup and Wallis, 2005), or more specifically until maximum lactate steady state (MLSS). The MLSS is defined as the highest speed or power output that does not result in a rise of blood lactate concentration of greater than 1 $mmol.L^{-1}$ between 10 and 30 min (Jones et al., 2019). Beyond this intensity H^+ ions from the working muscle will be produced at a rate leading to H^+ ion movement into extracellular fluid and plasma, which is buffered by HCO_3^-. Excess CO_2 produced as a result of this buffering is then expired through hyperpnoea, leading to contamination of VCO_2 by non-oxidative CO_2. This leads to overestimation of CHO oxidation rates, and underestimation of fat oxidation rates. This is most notable when the RER value is >1.0, whilst the RQ remains <1.0, which can lead to negative fat oxidation rates when calculated. Therefore, at exercise intensities above MLSS calculations of fat oxidation are incorrect.

Therefore, in investigations into substrate utilisation exercise intensities in the moderate or heavy exercise intensity domains should be used. Indeed, in Chapters 3 and 4, where the calculation of substrate utilisation rates were the main outcomes, an exercise intensity of 55% W_{max} (~65% $\dot{V}O_{2max}$) was chosen to permit accurate CHO and fat oxidation rates. This also permitted accurate estimation of exogenous CHO oxidation, which requires prolonged, steady state exercise for accurate estimation, which is discussed in the next section. Comparable exercise intensities have been used extensively (Jeukendrup et al., 1999b, Wallis et al., 2007b, Rowlands et al., 2005).

IC also assumes that metabolic processes that utilise O_2 or produce CO_2 are negligible, which include gluconeogenesis, lipogenesis and ketogenesis. Gluconeogenesis contributes to glucose production to small extent during exercise, and gluconeogenesis from alanine is the predominant gluconeogenic precursor which requires gas exchange and could therefore influence RER. However, Ahlborg et al. (1974) showed that splanchnic alanine extraction increased during 4 h of exercise at 30% $\dot{V}O_{2max}$, but that this contributed to just 8 % of the total glucose production, and therefore <1 % of total EE. Therefore, not accounting for gluconeogenesis is unlikely to influence calculation of substrate utilisation to a great extent. This is the case for most exercise scenarios, though gluconeogenesis from alanine is higher under fasted conditions and with longer exercise duration (Jeukendrup and Wallis, 2005). However, during exercise involving CHO ingestion gluconeogenesis has been shown to be very low (Howlett et al., 1998, Jeukendrup et al., 1999a) and the major comparisons between conditions in Chapters

3 and 4 are between different CHO conditions. Therefore, the impact of gluconeogenesis is likely not impactful in the subsequent chapters of this thesis.

Lipogenesis (formation of lipids from carbohydrate and protein) is also an oxidative process that could contribute to inflation of RER above 1.0 (Jeukendrup and Wallis, 2005). However, lipogenesis is characteristic of the postprandial state, in situations of high energy availability and positive energy balance, as opposed to exercise (Acheson et al., 1988). Therefore, contribution of lipogenesis to O_2 consumption and CO_2 production can be considered to be negligible. Ketogenesis can also occur during exercise, especially when rates of fat oxidation are high. Ketone bodies can be measured in the breath, and used to correct calculations of substrate utilisation, but the effect of this is minimal (Frayn, 1983).

As previously mentioned, amino acid oxidation is assumed to be negligible when calculating substrate oxidation. This is because the measurement of urea and calculation of nitrogen excretion to ascertain amino acid oxidation is a burdensome method, which adds a level of complexity to the relatively straightforward assessment of carbohydrate and fat oxidation through IC, which is likely only justifiable in a few scenarios. Although not quantifying amino acid oxidation introduces a level of theoretical error, the impact upon calculation of substrate utilisation is likely small.

2.2 Stable isotope (^{13}C) tracer methodology

Stable isotopes are molecules of an element with an additional neutron in the atomic nucleus, which results in an increase in the atomic mass, without altering the reactive properties of the element. This permits the tracing of metabolic processes, allowing more detailed information to be obtained on metabolic flux, beyond measures of IC or blood metabolite concentrations. Stable isotope tracers have been used extensively in exercise metabolism research, using isotopes such as deuterium oxide (D_2O), N^{15} labelled amino acids, and ^{13}C labelled CHOs. This thesis has used ^{13}C stable isotopes extensively to estimate ingested CHO oxidation during exercise, whilst other methods allow the estimation of plasma glucose oxidation, using established formulae.

In short, the methods rely on low abundance of ^{13}C CHO in the body, and the measurement of change in the ^{12}C:^{13}C ratio of the analyte of interest (e.g. expired CO_2, plasma glucose). Abundance of ^{13}C is measured using mass spectrometry (MS), with the type (e.g. isotope-ratio, gas chromatography) depending on the vector of analysis (e.g. breath, liquid). ^{12}C:^{13}C ratio is compared to the international standard Pee Dee Belemnitella (PDB), a Cretaceous marine fossil (*Belemnitella americana*) with a naturally high abundance of ^{13}C, found in the Peedee formation in South Carolina, USA (Brand et al., 2014). The unit of measurement is $\delta^{13}C$ in parts per thousand (or per mil, ‰), and PDB is set as the ‘zero’ standard.

2.2.1 ^{13}C tracers of CHO metabolism

Synthetic tracers refer to man-made molecules, with very high presence of the stable isotope. For example, $U^{13}C_6$ glucose tracer has the ^{13}C in all 6 carbons of its structure,

though tracers with less abundance of ^{13}C can also be produced, with between 1-6 ^{13}C atoms per glucose molecule. However, even these tracers with lower presence of ^{13}C molecules result in enrichments of nutritional interventions higher than can be found naturally. Although this increases the measurement signal, the cost is comparatively high, hence why many investigations utilise high natural abundance ^{13}C carbohydrates.

2.2.2 Naturally high abundance ^{13}C carbohydrates

Synthetic tracers are the only way to acquire CHOs with very high ^{13}C enrichments and are expensive to manufacture. However, the presence of ^{13}C through natural means in organically derived compounds provides a lower-cost option for using tracer-methodologies. Photosynthesis is defined as the synthesis of organic compounds (primarily sugars) from CO_2 and water, using light energy (Lopez and Barclay, 2017). There are three primary methods of photosynthesis; C3, C4 and Crassulacean acid metabolism (CAM), and each has specific evolutionary benefits, though only C3 and C4 are relevant to this section. C3 plants represent the majority of plants, and undergo the wasteful metabolic process of photorespiration, which occurs in high temperatures when O_2 presence increases and Rubisco binds to O_2, rather than CO_2 (O'Leary, 1988). C4 plants have evolved to reduce photorespiration, and physically separate Rubisco (and the Calvin cycle) from the stomata, and therefore O_2 and CO_2 diffusion into the mesophyll. Fractionation of carbon isotopes occurs with CO_2 uptake and metabolism in plants, and because ^{13}C molecules are heavier than ^{12}C, they diffuse more slowly (by ~4 %), and also tend to form slightly stronger chemical bonds (Risk et

al., 2008). CHOs derived from C3 plants have low $\delta^{13}C$ values (~-25-29‰) and enrichment because Rubisco discriminates strongly against ^{13}C molecules in mesophyll cells (Farquhar et al., 1989). By contrast, C4 plants have higher $\delta^{13}C$ values (~-12-16‰) and enrichment, which occurs because phosphoenolpyruvate carboxylase does not discriminate to the same extent as Rubisco (O'Leary, 1988).

C3 plants make up the vast majority of plants, including many of the most popular food crops (including rice, wheat and soybeans) and all trees, whilst examples of C4 plants include corn and sugar cane (Wang et al., 2012). High ^{13}C abundance of carbohydrates can manifest downstream in the food chain, for example when C4 plants are used as feed for animals. For example, by purposefully feeding cows exclusively on maize (naturally high abundance ^{13}C), it is possible to obtain milk with high ^{13}C enrichment (-14‰) with similarly enriched constituents, including lactose and protein fractions (Reckman et al., 2016). Similarly, infusion of ^{13}C amino acid tracers into cows can result in very highly enriched (10.5-19.4% [^{13}C] leucine atom percent excess) amino acids (Boirie et al., 1995). However, these methods are difficult and require animal husbandry expertise and access to dairy cows. Therefore, by investigating the ^{13}C enrichment of commercially available lactose from different locations, it is possible to find lactose with both high and low ^{13}C enrichment, depending on the habitual diet of the cows producing the milk. For example, cows on a high corn diet may have higher ^{13}C abundance in lactose, whilst those on a high grass diet may have lower ^{13}C abundance in lactose. This is the origin of the high and low ^{13}C lactose used in Chapters 3 and 4. The ^{13}C enrichment of the lactose used in Chapters 3 and 4 (Milk Specialities Global, Minnesota, United States) was -16.29 ‰, which is lower than the

enrichment of the milk used by Reckman et al. (2016), suggesting the cows were not fed solely on a corn-derived diet. A fully labelled U^{13}C lactose tracer is not currently commercially available, but can be made bespoke, though at a significant cost.

2.2.3 Exogenous CHO oxidation

One of the primary outcomes for Chapters 3 and 4 is the estimation of exogenous carbohydrate oxidation during steady state endurance exercise. As shown in the equation in the Indirect Calorimetry section, in the oxidation of CHO, the CHO is the exclusive source of carbon atoms, and all of these carbon atoms are metabolised to CO_2. Therefore, by ensuring that any CHOs to be traced are high in ^{13}C and other ingested CHOs or endogenous CHOs are low in ^{13}C, it is possible to estimate (using the equation below) the rate of oxidation of the ingested CHO. Exogenous CHO oxidation can be calculated using the established formula below (Mosora et al., 1976), based on the principles of Craig (1957).

$$Exogenous\ CHO\ oxidation = \dot{V}CO_2 \times \left(\frac{\delta Exp - \delta Exp_{bkg}}{\delta Ing - \delta Exp_{bkg}}\right) \times \left(\frac{1}{k}\right)$$

In which δExp is the ^{13}C enrichment of the expired air, δIng is the enrichment of the ingested CHO, δExp_{bkg} is the background ^{13}C enrichment of expired air and k is the amount of CO_2 (in litres) produced by the complete oxidation of 1 g of glucose (k = 0.7467 L).

A sample of expired breath must be taken before ingestion of CHO, to provide a background level of $^{13}CO_2$ enrichment. This breath sample is then analysed for the $^{13}CO_2$:$^{12}CO_2$ ratio by isotope ratio mass spectrometry (IR-MS). Similarly, exogenous CHO oxidation at a specific time point can be determined by measuring $^{13}CO_2$:$^{12}CO_2$ in an expired breath sample from that time point. The ^{13}C enrichment of the ingested CHO is also required to calculate exogenous CHO oxidation, as a more highly enriched CHO will lead to a higher breath $^{13}CO_2$ enrichment when oxidised. Ingested CHOs can be tracers or of natural high abundance ^{13}C. If a tracer is used, usually only a small proportion of the total ingested CHO is tracer, whilst the remainder is the same CHO (which can be high or low natural abundance ^{13}C). Measurement of $\dot{V}CO_2$ must also be made, usually by IC, which is required in the equation to indicate the total production of carbon atoms, and then calculate the proportion arising from exogenous CHO oxidation.

2.2.4 Corrections

The equation of Mosora et al. (1976) requires measurement background breath $^{13}CO_2$ enrichment to determine the change from baseline which occurs as the enriched CHO is oxidised. A pre-exercise, resting measurement of breath $^{13}CO_2$ can be used, as this should reflect the enrichment of endogenous fuel sources before supplementation. However, during exercise breath $^{13}CO_2$ enrichment increases above pre-exercise resting levels, which is observed in exercise conditions even where no CHO is ingested. This increase in breath $^{13}CO_2$ is termed the ‘background shift’ and occurs due to oxidation of endogenous CHO (plasma glucose, muscle and liver glycogen).

This endogenous CHO is enriched with ^{13}C from ingestion of naturally high ^{13}C CHOs, such as corn or sugar cane derived sucrose within the normal diet. This background shift can be minimised by avoiding such foods for 5-7 days before measurements are made, and perhaps also by performing a bout of exercise to reduce endogenous CHO stores in the days preceding measurement.

To address this issue, a background correction can be made. This is generally done by adding a study arm involving a fasted exercise trial (with an otherwise identical protocol to trials involving CHO ingestion). Breath $^{13}CO_2$ enrichment from this trial at corresponding time-points can then be used in the numerator of the equation (δExp_{bkg}) when calculating exogenous CHO oxidation at specific time-points. This method of correction has been extensively used and is widely thought to provide accurate estimation of exogenous CHO oxidation.

In Chapter 3, due to the relatively low enrichment of the high natural abundance ^{13}C lactose (-16.29 δ‰ versus PDB), it was possible that the background shift may contaminate the signal to an unacceptable extent and so an 'authentic' background correction was used, as well as a water-correction. This is attributable to the different metabolic impacts of fasted exercise (water ingestion) compared to exercise involving CHO ingestion, and the application of a background correction that does not reflect exercise metabolism in a fed state. A comparison of these methods demonstrated the potential for inaccuracies using a water-correction in the calculation of exogenous CHO oxidation, which is discussed in Chapter 3 and in the General Discussion.

2.2.5 Plasma glucose oxidation

Plasma glucose oxidation can be estimated by utilising an ingested ^{13}C tracer. High natural abundance ^{13}C CHOs cannot be used to assess plasma glucose oxidation, as the change in enrichment of plasma glucose is not great enough to be detected. Therefore, a ^{13}C glucose tracer must be used to result in sufficiently high plasma glucose enrichment. Plasma glucose oxidation is calculated using the equation below, in which δExp is the ^{13}C enrichment of the expired breath, δExp_{bkg} is the ^{13}C enrichment of the expired breath at rest (before tracer ingestion), δPG is the ^{13}C enrichment of plasma glucose, δPG_{bkg} is the ^{13}C enrichment of plasma glucose at rest (before tracer ingestion), and k is the volume of CO_2 produced by the oxidation of 1 g of glucose (0.7467 L). This method was used in Chapter 4.

$$\text{Plasma glucose oxidation} \quad = \dot{V}CO_2 \times \left(\frac{\delta\text{Exp} - \delta\text{Exp}_{\text{bkg}}}{\delta\text{PG} - \delta\text{PG}_{\text{bkg}}}\right) \times \left(\frac{1}{k}\right)$$

Estimation of plasma glucose oxidation permits non-invasive estimation of muscle glycogen oxidation (which removes the need for muscle biopsy), and liver glycogen oxidation (which cannot be assessed directly using a biopsy in most non-medical scenarios). The following formulae describe their calculation.

$$Muscle\ glycogen\ oxidation = Total\ CHO\ oxidation - Plasma\ glucose\ oxidation$$

$$Liver\ glycogen\ oxidation = Plasma\ glucose\ oxidation - Exogenous\ CHO\ oxidation$$

Whilst this method permits estimation of plasma glucose oxidation, it relies on a number of assumptions. In order to calculate plasma glucose oxidation, a steady state in plasma glucose ^{13}C enrichment must be attained. This cannot be guaranteed in all subjects during exercise, and usually does not occur for the first 45-60 min during steady state endurance exercise (Rowlands et al., 2005, Jeukendrup et al., 1999b), which makes data from the early stages of exercise difficult or impossible to interpret. The equations have not, to the authors knowledge, been validated against other methods such as muscle biopsy or ^{13}C magnetic resonance spectroscopy (MRS) of liver or muscle.

2.2.6 Limitations of stable isotope tracer methodologies

The equations presented above are not direct measurements of the processes of interest, and only represent estimations. Therefore, there is a potential for systematic error resulting from their use. One error, mentioned above, is the lack of an appropriate background correction in the calculation of exogenous CHO oxidation. The majority of investigations into exogenous CHO oxidation have used a water-correction, whilst some have used no correction at all. To the authors knowledge, no studies have used low ^{13}C corrections. Therefore, when comparing results to previous findings, care must be taken in interpreting results from past studies, which may have overestimated exogenous CHO oxidation rates.

The presence of the bicarbonate pool represents a potential limitation to the method, because it has the potential to sequester $^{13}CO_2$ molecules arising from the oxidation

of high ^{13}C CHOs. In the early stages of exercise, the expiration of CO_2 does not reflect the flux of CO_2 from the muscle, and there is a delay in the bicarbonate pool turnover reaching equilibrium. Therefore, to prevent underestimation of exogenous CHO oxidation rates, only once equilibrium is reached should calculations be considered accurate. This has been thought to occur only by ~60 min after ingestion of ^{13}C CHOs during exercise, when recovery of $^{13}CO_2$ from oxidation reaches 100% (Pallikarakis et al., 1991, Robert et al., 1987). However, Podlogar and Wallis (2020) showed that this may occur after as little as 21 min of exercise. Therefore, exogenous CHO oxidation rates should be considered most accurate and representative from ~60 min, though tentative interpretation of data from as early as 30 min is feasible. This has generally necessitated exercise lasting a minimum of 60 min, though 120-180 min is common.

One further limitation of these ^{13}C tracer methodologies is the requirement to minimise background ^{13}C enrichment of endogenous CHO, though this is most relevant when using high natural abundance ^{13}C CHOs due to the relatively small shift in breath $^{13}CO_2$ enrichment compared to tracers. As described earlier, this is usually achieved by avoidance of foods with naturally high abundance of ^{13}C (C4 plants and meat or animal products from animals fed diets high in C4 plants). This requirement for dietary adherence is an additional burden for participants in research using this method, and adherence to this dietary intervention may not be adequate. Poor adherence can often be detected by high background breath $^{13}CO_2$ enrichment, or by large background shifts during background correction trials (involving water or low ^{13}C CHO ingestion). However, this potential issue may not be impactful if motivated participants are recruited. Some studies also include a bout of exercise in the day(s) preceding

experimental trials in order to deplete or reduce muscle and liver glycogen, which may be comprised of ^{13}C CHOs. However, this increases participant and researcher burden, especially if these exercise sessions are supervised.

2.3 Breath H_2 measurement

Measurement of breath H_2 concentration allows assessment of the gas' production by bacteria in the GI tract, which are the exclusive source of H_2 gas in the human body when in the presence of a fermentable substrate. Breath H_2 measurement can be used as a diagnostic tool for a number of GI disease and conditions, most notably lactose maldigestion, but also fructose malabsorption and small intestinal bacterial overgrowth (SIBO) (Misselwitz et al., 2013). Alongside specific diagnostic tests for disease, H_2 breath testing can also be used more generally to detect whether a substrate is being fermented by bacteria, described in Chapters 3-5, where the specific criterion values used for diagnostic tests are not used, though the principles remain the same.

H_2 is produced by bacterial flora in the colon (and the small intestine in the case of SIBO) when fermentable substrates are present. This may occur in the normal digestive process of foods containing indigestible compounds such as fibre (both soluble and insoluble), which resist digestion and absorption in the stomach and small intestine but can be fermented by bacteria in the colon (Williams et al., 2017). However, other substrates in some individuals may also escape the small intestine, through either inadequate digestion or absorption (such as lactose or fructose respectively) and can enter the colon where it is possible for them to be fermented by bacteria. The

presence of such CHOs in the colon is associated with GI symptoms, which are characteristic of lactose intolerance (LI) or fructose malabsorption. The presence of CHO in the colon increases the osmotic load, which causes water to be absorbed into the colon, which can cause diarrhoea (Rangel et al., 2016). The osmotic load can be further increased by the production of lactic acid, by bacteria (such as *Bifidobacteria* and *Lactobacillus*) (Jiang and Savaiano, 1997b, Jiang and Savaiano, 1997a). These bacteria also produce other metabolites in the fermentation process, including H_2, CH_4 and CO_2, which contribute to bloating, abdominal pain, and flatulence. H_2 (and CH_4) are absorbed into the circulation, diffuse into the alveoli, and are expired. Therefore, presence of H_2 or CH_4 in the breath indicates fermentation of ingested substrates.

2.3.1 H_2 breath tests in the diagnosis of lactose maldigestion

H_2 breath tests are used in clinical settings to diagnose lactose maldigestion and as a proxy for lactose intolerance, which maldigestion is the primary cause of. There are a variety of other diagnostic tools for the assessment of lactose intolerance, maldigestion and lactase non-persistence, which have been described in Chapter 1. Briefly, a clinical H_2 breath test involves the ingestion of 25 g of lactose in a beverage. Breath H_2 is usually measured before ingestion, and over a time-course of 2-5 h, with measurements made every 15, 30 or 60 min (Gasbarrini et al., 2009). An increase to ≥20 ppm indicates lactose maldigestion. Measurements of GI symptoms may also be made, though only approximately one third of those lactose maldigesters with positive H_2 breath tests experience symptoms (Di Stefano et al., 2007). Breath H_2 concentration is usually measured using a tabletop or handheld device with a mouthpiece, into which

subjects exhale, with an electrochemical sensor that calculates H_2 concentration in ppm. A handheld breath H_2 device was used in Chapters 3-5 (Hydrogenius, Bedfont Scientific Ltd, Maidstone, England).

2.3.2 Limitations

The H_2 breath test for the diagnosis of lactose maldigestion has a number of limitations. The breath H_2 threshold of ≥20 ppm after ingestion of lactose is arbitrary and does not correspond directly to a threshold of lactase expression or GI symptoms. Some clinicians and researchers have suggested a cut-off of ≥10 ppm, and though this would increase the sensitivity of the test, its specificity would be affected (Gasbarrini et al., 2009), whilst others have suggested a positive test being defined as an increase of 10 or 20 ppm over basal values. An additional problem with a specific cut-off is that breath H_2 increases with the dose ingested (Yang et al., 2013), and lactose maldigestion tests in clinical settings use varying doses. Because only approximately one third of lactose maldigesters with positive H_2 breath tests experience symptoms at that dose (Di Stefano et al., 2007) this suggests that the relationship between lactose intolerance (presence of symptoms) and maldigestion (indicated by positive H_2 breath test) may not be sufficiently predictive. Furthermore, if 25 g of lactose (the standard test dose) can be ingested without symptoms but elicits a rise in breath H_2, the ecological validity of a positive H_2 breath test is questionable.

The method also has more general limitations, which are also relevant for the methods used in Chapters 3-5. It is important for subjects to avoid a number of foods in the 24

h preceding the test, which contain fermentable substrates that may be present in the colon in the 24 h after ingestion (examples described in Chapter 3). Implementing dietary controls in advance of H_2 breath testing can help to ensure a low baseline breath H_2 result, whilst the other controls reduce the possibility of a lack of a H_2 response to substrate fermentation. Despite dietary control, it is common for individuals to present with baseline fasted breath H_2 concentrations of >20 ppm (see Chapters 3 and 4). It has been suggested that a resting breath H_2 concentration of >16 ppm is considered 'high' (Kumar et al., 2010) and may indicate SIBO (Pande et al., 2009). However, SIBO is unlikely to account for all cases of high fasted breath H_2 concentrations and is usually accompanied by symptoms. However, that SIBO can in some cases influence the breath H_2 concentration even without ingestion of a substrate, demonstrates a further flaw in the methodology of measuring breath H_2 concentration for maldigestion or malabsorption.

Although in some cases, breath H_2 concentration increases without the presence of GI symptoms, the inverse has also been observed; some individuals experience GI symptoms despite a negative H_2 breath test (Johnson et al., 1993, Hertzler and Savaiano, 1996). This is thought to occur due to 'full colonic adaptation', where repeated ingestion of lactose selectively favours bacterial flora that do not produce H_2 (Szilagyi et al., 2004, Hertzler and Savaiano, 1996). Repeated ingestion of lactose in lactose intolerant maldigesters has been shown to reduce GI symptoms in response to a 'challenge dose' of lactose. It is possible to improve the ability to detect fermentation of lactose by bacteria using combined H_2 and CH_4 breath testing. The proportion of individuals who do not produce H_2 during fermentation of lactose is

estimated to be 2 – 43 % (Gasbarrini et al., 2009), meaning the potential for a false negative result is high. However, it is unclear whether full colonic adaptation may also lead to reduced production of CH_4, as well as H_2. Although CH_4 measurement may improve the prognostic capability of the breath test, it does not resolve all of the issues, and measuring CH_4 in addition to H_2 increases the cost of testing, due to the need for double the analysis.

The pH of the colon can also impact breath H_2 responses. If colonic pH is <5.5 the production of H_2 by colonic bacteria in response to fermentable substrates is reduced by 75% (Perman et al., 1981). If an individual is a lactose maldigester, but also has a low colonic pH, a breath H_2 test may not detect maldigestion if the lactose in the colon is not being fermented. These individuals would still likely experience symptoms including loose stools and diarrhoea due to the undigested, non-fermented lactose presence in the colon (Rangel et al., 2016). The colonic pH in a healthy individual is ~5.7, so only small deviation could affect H_2 production (Fallingborg, 1999).

Although there are clearly numerous limitations in the use of H_2 breath testing, it still remains the first line clinical tool in the diagnosis of lactose maldigestion and is often used in studies in humans at rest. It has also been used in a number of studies involving exercise, as a marker of malabsorption of ingested carbohydrates (Costa et al., 2017). Exercise appears to result in a transient reduction in breath H_2 that then rapidly returns to basal levels (Payne et al., 1983). However, ingestion of some carbohydrates during exercise can increase breath H_2, which correlates well with the incidence and severity of GI symptoms during exercise (Buckley et al., 2009). Therefore, there is value in

using breath H_2 testing in an exercise context. In Chapters 3-5, breath H_2 testing was not used as a diagnostic tool, but simply to indicate if any fermentation was occurring after the ingestion of test beverages. Therefore, specific diagnostic thresholds were not relevant, and individual responses were less relevant than they would be for diagnostic purposes, whilst group means were important to indicate the occurrence or absence of fermentation. Therefore, provided the interpretation of the data was cautious and took into account the large doses of ingested CHOs and the differences in procedures compared to clinical tests, this method had the potential to add valuable insight into understanding of the digestion and metabolism of CHOs, and GI symptoms.

2.4 Blood sampling and analysis

2.4.1 Blood sampling

Blood sampling is a valuable tool in sports nutrition and exercise metabolism research. Measuring circulating metabolites and substrates provides an indication of the metabolic processes and substrate utilisation occurring at that specific time-point. However, it is important to consider that concentration is not an indication of flux of a metabolite or substrate and may remain constant even with high rates of flux. Only with measurement of the rate of appearance (Ra) or rate of disappearance (Rd) can this be ascertained. Venous blood samples were obtained in Chapters 3-5 through venepuncture and cannulation. Descriptions of the specific protocols used to obtain a blood sample can be found in the relevant chapters. Although cannulation was used for the majority of blood samples, venepuncture was used in situations such as loss of

cannula patency if few samples remained to be taken. 10 ml blood samples were taken, and aliquoted into vacutainers (containing ethylenediaminetetraacetic acid, or fluoride oxalate as appropriate), stored on ice, centrifuged and separated for plasma, which was stored for later analysis at –70 °C.

All blood samples taken in Chapters 3-5 were venous blood samples, which is common practice in the discipline. Although, arterial blood samples provide an indication of the circulating substrates, metabolites and hormones the muscle is exposed to, whereas concentrations in venous blood samples are impacted by extraction or addition by the working muscle. However, arterial blood samples are difficult to obtain and require arterial cannulation. Compared to venepuncture or venous cannulation the risk of haemorrhage is comparatively high. It is also possible to obtain 'arterialized' venous blood samples from the hand or forearm using the 'heated-hand technique', which utilises the ability of these body parts to promote heat loss through radiation by allowing rapid perfusion by arterial blood into the capillary beds (Nauck et al., 1992). However, there is relatively good agreement between venous and arterial blood samples for a number of metabolites such as lactate (Felippe et al., 2017) and at high flow rates, such as during exercise, the differences between arterial and venous blood are minimised (Wallis et al., 2007a).

2.4.2 Photometric and colourimetric assays

2.4.2.1 Photometry

Plasma samples were analysed by enzymatic photometric assays for glucose concentrations in Chapters 3 and 5. Photometry involves the measurement of absorption of light by molecules in a solution. When a light at a particular wavelength is passed through a solution, some is absorbed, which can be measured as a reduction in the intensity of the light leaving the solution. Photometry relies on the Beer-Lambert law, which states that the quantity of light that is absorbed by a solute is directly proportional to the concentration of the absorbing solute (Beer, 1852). Lamberts law states that the thickness of an absorbing medium causes an exponential reduction in the amount of light that can be transmitted (Kumar and Gill, 2018).

Plasma glucose was analysed using the UV method (Schmidt, 1961), utilising hexokinase (reaction 1) and glucose-6-phosphate dehydrogenase (reaction 2).

$$\boldsymbol{Glucose} + \boldsymbol{ATP} \rightarrow \boldsymbol{Glucose}\text{-6-}\boldsymbol{phostphate} + \boldsymbol{ADP}$$

$$\boldsymbol{Glucose}\text{-6-}\boldsymbol{phostphate} + \boldsymbol{NAD}^{+} \rightarrow \boldsymbol{Gluconate}\text{-6-}\boldsymbol{phosphate} + \boldsymbol{NADH} + \boldsymbol{H}^{+}$$

Absorbance of the generated light is measured and related to a standard curve, which is used to calculate glucose concentration.

2.4.2.2 Colourimetry

Colourimetry allows the quantitation of the concentration of a solute by measuring the transmittance or absorbance of light at a specific wavelength. The production of colour in a solution from a colourless solute is achieved by the addition of a reagent, which may produce a coloured compound, or a compound which triggers the activity of a colour-producing enzyme.

2.4.2.2.1 Galactose

Plasma galactose concentrations were quantified in Chapter 3 using a colourimetric plate-based assay (Sigma Aldrich, St Louis. MO, USA). In this assay, galactose in the sample is oxidised by galactose oxidase which generates a product which reacts with a galactose probe, resulting in hydrogen peroxide production. This reacts with horseradish peroxidase (HRP), producing colourimetric output (570 nm) at an intensity proportional to the concentration of galactose in the sample. The absorbance is then measured by a microplate reader, which is used to generate a standard curve, allowing the conversion of absorbance values to concentration.

2.4.2.2.2 Lactate

Plasma lactate concentrations were quantified in Chapters 3 and 5 using an automated photometric clinical chemistry analyser. The following equations are catalysed by lactate oxidase (reaction 1) and peroxidase (reaction 2) and result in a purple product; the intensity of which was measured and used to calculate lactate concentration.

$$L\text{-}Lactate + O_2 \rightarrow pyruvate + H_2O_2$$

$$H_2O_2 + 4\text{-}aminoantipyrine + N\text{-}ethyl\text{-}N\text{-}(2\ hydroxy\text{-}3\text{-}sulphopropyl)\ m\text{-}toluidine \rightarrow purple\ product + 4H_2O$$

2.4.2.2.3 NEFA

Plasma NEFA concentrations were quantified in Chapter 3 using an automated photometric clinical chemistry analyser. The following equation, catalysed by Acyl CoA Synthetase (reaction 1), Acyl CoA Oxidase (reaction 2) and peroxidase (reaction 3) produced a purple product; the intensity of which was measured spectrophotometrically and used to calculate NEFA concentration.

$$NEFA + ATP + CoA \rightarrow Acyl\ CoA + AMP + PPi$$

$$Acyl\ CoA + AMP + PPi \rightarrow 2{,}3\ trans\text{-}Enoyl\text{-}CoA + H_2O_2$$

$$2H_2O_2 + N\text{-}ethyl\text{-}N\text{-}(2\ hydroxy\text{-}3\text{-}sulphopropyl)\ m\text{-}toluidine + 4\text{-}aminoantipyrine \rightarrow purple\ product + 4H_2O$$

2.4.3 Enzyme immunoassays

Plasma insulin concentrations in Chapter 3 were measured using an enzyme-linked immunosorbent assay (Ultrasensitive Insulin ELISA, Mercodia, Uppsala, Sweden). This ELISA method is a solid phase two-site enzyme immunoassay, which utilises the direct sandwich technique, which involves two monoclonal antibodies directed against the insulin molecule, at different sites. The sample is added to the wells of a 96-well microplate, at the bottom of which the first of the two antibodies is bound, which is specific to one site of the insulin molecule. Anti-insulin antibodies are also added, which are conjugated with peroxidase, which bind to the second site of the insulin molecule.

During the incubation stage of this assay, all of the insulin in the sample binds to both antibodies and is thus bound to the microplate and to the peroxidase. A washing stage removes any unbound peroxidase-conjugated antibody. The bound insulin is detected by a reaction with 3,3',5,5'-tetramethylbenzidine (TMB), and the reaction stopped by adding sulphuric acid. This generates a colorimetric endpoint, which is read spectrophotometrically at 450 nm using an automatic plate reader. As with other colourimetric assays, the intensity of the colour is proportional to the concentration of insulin. Therefore, for each assay a standard curve must be generated to allow calculation of concentration from absorbance.

Table 2.2 Analytes and measurement variation.

	Coefficient of variation (%)	
Analyte	Intra-assay	Inter-assay
Plasma glucose	1.0	3.5
Plasma lactate	1.5	3.4
Plasma NEFA	0.4	8.5
Plasma insulin	7.6	14.8
Plasma galactose	7.3	6.4

All analytes intra-assay CVs were calculated from technical repeats from the same analysis or plate. Inter-assay CVs for plasma glucose, lactate and NEFA were calculated from repeats of control solutions, whilst plasma insulin and galactose inter-assay CVs were calculated from repeated standards and control solutions.

2.5 Mass spectrometry

2.5.1 Gas chromatography mass spectrometry

Gas chromatography mass spectrometry (GC-MS) was used in Chapter 4 for the measurement of the ^{13}C enrichment of plasma glucose to permit calculation of plasma glucose oxidation. MS is used to calculate the mass-to-charge ratio (*m/z*) of compounds that are present in a sample, allowing assessment of their exact molecular weight and abundance. Studies involving stable isotope tracers make use of MS to differentiate between different isotopes of the same compound (e.g. ^{13}C and ^{12}C or ^{15}N and ^{14}N). GC-MS was performed using an Agilent 6890N GC with 5973N MS (Agilent Technologies, Stockport, UK). Butaneboronic acid (BBA) derivatisation was performed after deproteinization of samples as previously described (Newell et al., 2018) and a detailed description of the protocol can be found in Chapter 4, whilst a summary of the general principles is here.

The sample is injected into the front inlet and vaporised into a gas (Agilent Technologies, 2001). The sample is carried by an inert gas (helium in this case) through the column where compounds elute after different lengths of time, known as the retention time. The retention time of a compound is affected by its boiling point and its interaction with the stationary phase. Once the sample elutes from the GC, it enters the MS. The MS breaks the compounds into ionised fragments using a high energy beam of electrons (Agilent Technologies, 1999). The fragments travel through a short tunnel, undergoing a process of acceleration and deflection, through exposure to a magnetic field. The fragments finally interact with a detector plate at the end of the tunnel where the *m/z* and relative abundance of ^{12}C and ^{13}C glucose are measured,

allowing tracer:tracee ratio (TTR) to be calculated. Samples were measured in biological duplicate and the intra-assay CV of TTR was 4.3%, after samples with a CV of >10% were repeated, and the inter-assay CV was 10.8%.

GC-MS is a common technique for this differentiation, though liquid chromatography (LC) MS can also be used. Both instruments were available for use in Chapter 3, but a method for the derivatisation of glucose and subsequent analysis of ^{13}C enrichment was already established for the GC-MS in this laboratory. GC-MS is a less sensitive technique than LC-MS (Lee et al., 2012), necessitating the use of larger quantities of ingested ^{13}C glucose tracer to achieve enrichment within the detection limit. These two techniques are similar, with the main difference being that the mobile phase of GC-MS is gaseous, using an inert gas to carry the sample through the column, whilst the LC-MS uses a solvent to carry the sample.

2.5.2 Isotope ratio MS

Isotope ratio (IR) MS was performed by third party laboratory, Iso-Analytical, on expired breath samples from Chapters 3 and 4 to determine $^{12}CO_2$:$^{13}CO_2$ ratio using GC-IR-MS (Europa Scientific, Hydra 20-20, Crewe, UK). In this technique, breath samples in Exetainers were flushed via a double holed needle through a packed GC column at a temperature of 60 °C and the isopotomers at *m/z* 44, 45 and 46 for CO_2 were measured. The δ ^{13}C value was then calculated from TTR. GC-IR-MS is a more sensitive method than GC-MS (Croyal et al., 2016), making it preferable for samples

with low ^{13}C enrichment, such as breath samples (especially those from low ^{13}C CHO conditions).

2.6 Magnetic Resonance

2.6.1 Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) is a non-invasive method for imaging tissues, allowing qualitative and quantitative analysis of these tissues. MR methods were used in Chapter 5 in the measurement of liver glycogen content.

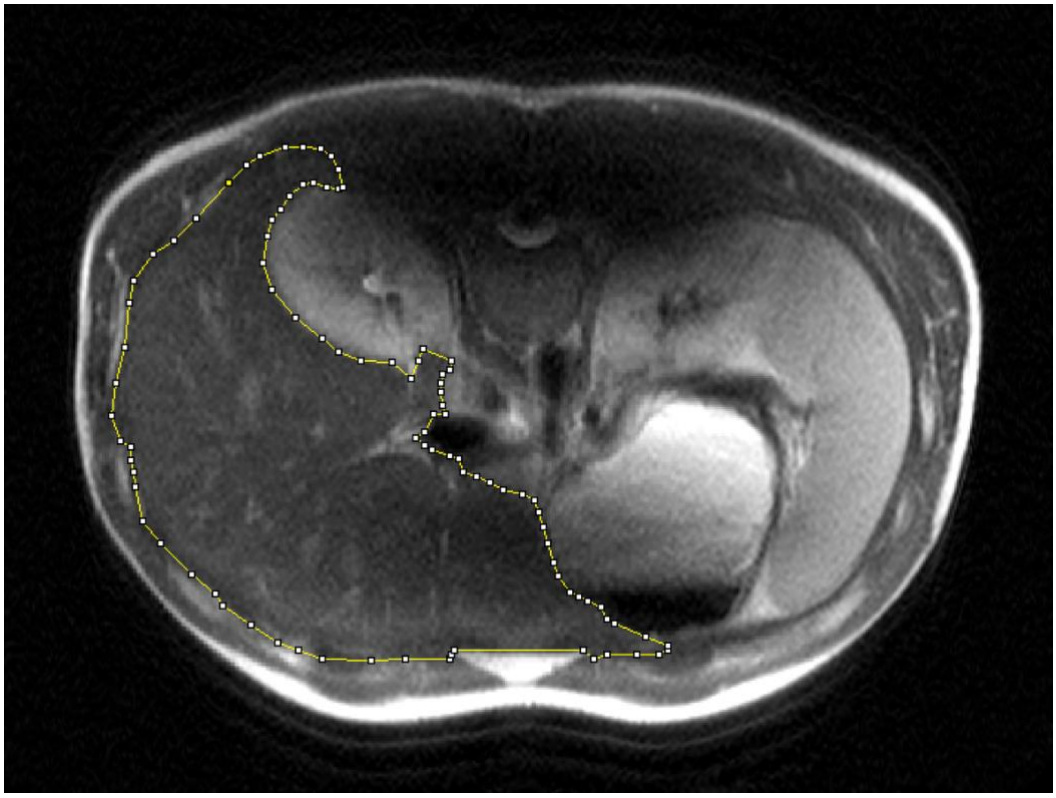
MRI relies on the magnetic properties of the hydrogen atom, a single proton (Berger, 2002). Hydrogen is abundant in both fat and water, making imaging of most tissues feasible. Under normal circumstances, hydrogen atoms spin randomly, with randomly aligned axes. In MRI the body is exposed to a strong magnetic field which aligns the axes of the protons. The interaction of the nuclear magnetic field (i.e. that of the proton) and the scanners magnetic field results in energy exchange termed ‘resonance’ (Alger, 2009). The strength of the magnetic field generated by the scanner is measured in Tesla, with the scanner used in Chapter 5 being a 3 T scanner. The magnetic field results in a magnetic vector, which is aligned with the axis of the MRI scanner. A radio wave is then introduced to the magnetic field, broadcasted by radiofrequency coils in the scanner. The stimulation of the hydrogen atoms results in reversal of their orientation to the magnetic field, and deflection of the magnetic vector (Carr and Grey, 2002). The frequency of the radio waves can be altered depending on the strength of

the magnetic field whilst the strength of the magnetic field is altered by small increments locally to image specific parts of the body. When radio waves emission ends, the magnetic vector returns to its original state, causing the re-emittance of a radio wave to the receiver coils, which determine the time taken and the energy emitted (Berger, 2002). These receiver coils are placed in various positions around the body to improve the detection of these signals. The intensity of these signals is then plotted to form images. Different tissues relax at different rates when radio wave emission ends, and the time taken for the protons to return to their resting state is measured in two ways, T1 and T2 (Serai, 2021), resulting in two types of images:

1. T1 – also known as the longitudinal relaxation time, is a measure of the time taken for protons to realign with the external magnetic field.
2. T2 – also known as the transverse relaxation time, is the time taken for the protons axial spin to return to the resting state.

In T1 images, fat is bright and highlighted, whilst in T2 images both fat and water are bright. T1 images were used in Chapter 5 for the determination of liver volume, and the method for determining liver volume can be found in the methods section of Chapter 5.

Figure 2.1 Representative magnetic resonance image of the abdomen after 4 hours of CHO beverage ingestion, with the liver area outlined.



2.6.2 Magnetic Resonance Spectroscopy

Whilst MRI detects signals produced by water from hydrogen, MRS detects signals from specific compounds to determine their concentration. Other than this, MRS and MRI rely on the same principles of magnetic resonance (Zhou et al., 2020). MRS is analogous to nuclear magnetic resonance (NMR) spectroscopy, which is used widely in other disciplines, but MRS is the term used when it is applied in a biological context (Tognarelli et al., 2015). In addition to the magnetic field generated by the main MRI scanner, MRS requires a second external magnetic field. This external field must have temporal periodicity, i.e., regularly and consistently repeating, as this allows detection of the specific resonance interaction (Alger, 2009). This second magnetic field is known as the radio frequency (rf) field and is produced by the rf coil – a specific coil that is placed directly on or over the part of the body under examination.

The rf coil is turned on and off quickly (pulsed), generating an rf field which resonates with a broad range of atomic nuclei in the tissue of interest. The rf coil emits many pulses, generating a pulse sequence. Because MRS signals for such purposes are weak compared to those used in chemistry or biology, signal detection must be repeated many times, as the signal is weak compared to the background noise (Alger, 2009). The results from many signals are averaged because the desired signal is consistent with every repetition, whilst background noise is random, and thus different on every repeat. When the pulse ends, the interaction between the magnetic fields of the atoms (atomic magnetic field) and the static magnetic field produces distinct electromagnetic signals at the rf coil, at specific frequencies. These frequencies are then processed to produce a magnetic resonance spectrum, from which concentrations of specific metabolites can be calculated (see Chapter 5.3.7).

Only nuclei with an intrinsic nuclear magnetic moment and angular momentum can be examined using MRS, and these include ^{1}H, ^{31}P and ^{13}C, and less commonly ^{7}Li, and ^{19}F. These properties are achieved by the nucleus having an odd number of protons and/or neutrons, making even numbered nuclei inactive for the purposes of MR methods. The MRS signals produced by these isotopes are drastically different, making them easily distinguishable in the magnetic resonance spectrum. The strength of the signal is also affected by the abundance of the nuclei in question in the target tissue. Whilst ^{1}H is ubiquitous, ^{13}C only makes up ~1% of all C atoms. This is one reason that ^{1}H is commonly used in NMR, because it requires the same equipment as MRI (Alger, 2009).

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Chapter 3: Comparable exogenous carbohydrate oxidation from lactose or sucrose during exercise.

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3.1 Abstract:

Ingesting readily oxidized carbohydrates (CHO) such as sucrose during exercise can improve endurance performance. Whether lactose can be utilized as a fuel source during exercise is unknown. The purpose of this study was to investigate the metabolic response to lactose ingestion during exercise, compared to sucrose or water. 11 participants (age, 22 ± 4 years; $\dot{V}O_{2peak}$, 50.9 ± 4.7 $ml \cdot min^{-1} \cdot kg^{-1}$) cycled at 50% W_{max} for 150 min on 5 occasions. Participants ingested CHO beverages (lactose or sucrose; 48 $g \cdot h^{-1}$, 0.8 $g \cdot min^{-1}$) or water throughout exercise. Total substrate and exogenous CHO oxidation were estimated using indirect calorimetry and stable isotope techniques (naturally high ^{13}C-abundance CHO ingestion). Naturally low ^{13}C-abundance CHO trials were conducted to correct background shifts in breath $^{13}CO_2$ production. Venous blood samples were taken to determine plasma glucose, lactate and non-esterified fatty acids (NEFA) concentrations. Mean exogenous CHO oxidation rates were comparable with lactose (0.56 ± 0.19 $g \cdot min^{-1}$) and sucrose (0.61 ± 0.10 $g \cdot min^{-1}$; P=0.49) ingestion. Endogenous CHO oxidation contributed less to energy expenditure

in Lactose (38 ± 14 %) versus Water (50 ± 11 %, P=0.01) and Sucrose (50 ± 7 %, P≤0.05). Fat oxidation was higher in Lactose (42 ± 8 %) than Sucrose (28 ± 6 %, P≤0.01); CHO conditions were lower than Water (50 ± 11 %; P≤0.05). Plasma glucose was higher in Lactose and Sucrose than Water (P≤0.01); plasma lactate was higher in Sucrose than Water (P≤0.01); plasma NEFA were higher in Water than Sucrose (P≤0.01). Lactose and sucrose exhibited similar exogenous CHO oxidation rates during exercise at moderate ingestion rates. Compared to sucrose ingestion, lactose resulted in higher fat and lower endogenous CHO oxidation.

3.2 Introduction

Carbohydrate (CHO) is the predominant respiratory substrate at moderate to vigorous exercise intensities (i.e., ≥ 60 % $\dot{V}O_{2max}$) (Bergman and Brooks, 1999). Endogenous CHO (i.e., liver and muscle glycogen) has a finite storage capacity and depletion of these substrate stores can limit performance in prolonged strenuous exercise (Burke et al., 2011). Ingestion of CHO during exercise is a well-established nutritional strategy for improving endurance exercise performance and capacity, and operates through sparing endogenous CHO utilization, maintenance of blood glucose concentrations and total CHO oxidation, and positively impacting the central nervous system (Cermak and van Loon, 2013). CHOs that are known to be readily oxidised, such as glucose, glucose polymers, combined glucose and fructose or sucrose, form the basis of expert guidelines for CHO feeding during endurance exercise to enhance performance (Burke et al., 2011, Thomas et al., 2016). Lactose, a CHO found in dairy foods such as milk

and yoghurt does not feature in expert guidelines and indeed very little is known about its metabolism in the context of exercise.

Lactose is a disaccharide that consists of a glucose and galactose monomer bound with a β 1-4 glycosidic bond. Galactose has been shown to be oxidised during exercise at ~50-60 % the rate of glucose (Leijssen et al., 1995, Burelle et al., 2006), likely due to the requirement for hepatic metabolism before oxidation. Due to its slow oxidation rate galactose is not generally considered for ingestion during exercise and can result in poorer exercise performance when compared to combined glucose/fructose ingestion (Stannard et al., 2009). Lactose could therefore be less readily available as a fuel source than other CHO's due to the galactose component. However, combined free glucose and galactose ingestion (1:1 ratio) has been shown to improve performance in a ~30 min time trial, completed after 120 min cycling at ~65 $\dot{V}O_{2max}$ to a similar extent to combined glucose and fructose ingestion (80:20 ratio) (Stannard et al., 2009). Furthermore, ingestion of low-fat milk during exercise has been shown to improve time to exhaustion at ~70 % $\dot{V}O_{2peak}$ when compared to water ingestion, and to a similar extent to a glucose beverage (Lee et al., 2008). Milk ingestion does not isolate the effects of the lactose component, but such observations lend support to the idea that lactose may represent a viable energy source during exercise. Indeed, it has been demonstrated that recovery of breath $^{13}CO_2$ after ingestion of high ^{13}C lactose during very light physical activity (cycling at 50W), was comparable to glucose (Stellaard et al., 2000), perhaps implying a similar oxidation rate. Collectively, the evidence suggests that there could be beneficial metabolic effects of lactose ingestion,

but these have not yet been comprehensively studied under exercise conditions representative of moderate to vigorous intensities.

The paucity of research on lactose in an exercise context may in part, be due to concerns surrounding lactose maldigestion and the potential resulting gastrointestinal symptoms (GIS). GIS during endurance exercise are common, occurring due to mechanical, ischemic and nutritional factors, and can have severe impacts upon the performance of athletes (Costa et al., 2017). GIS can be triggered or worsened by the ingestion of CHO during exercise, especially where ingestion rates exceed absorption rates, and unabsorbed CHO passes into the colon (de Oliveira and Burini, 2014). Improvements in exercise performance with CHO ingestion can be dependent upon an absence of, or minimal GIS (O'Brien and Rowlands, 2011). Lactose maldigestion, the inefficient or incomplete digestion of lactose, is a concern for some individuals even under resting conditions. Unhydrolyzed lactose reaching the colon may be fermented by colonic bacteria, producing H_2, CO_2 and CH_4 and short chain fatty acids (SCFAs), resulting in intolerance symptoms, such as diarrhoea, cramping, bloating and flatulence (Misselwitz et al., 2013). This lactose intolerance is present in 5-14 % of Caucasians, and presence is higher in other ethnicities (Misselwitz et al., 2013). However, large boluses of lactose (40-80 g) do not generally trigger symptoms in lactose-tolerant individuals (Stellaard et al., 2000), and even in lactose intolerant individuals, quantities of up to 12-15 g are well tolerated by most (Misselwitz et al., 2013). Nonetheless, knowledge of GIS would be important in understanding the practical utility of lactose as a CHO source for ingestion during exercise.

The aim of the present study was to compare the exogenous CHO oxidation rates of ingested lactose with sucrose, as a representative readily oxidizable CHO, during endurance exercise and more broadly to characterise the metabolic effects of lactose feeding on exercise metabolism and GIS. The primary hypothesis was that lactose and sucrose would exhibit similar rates of exogenous CHO oxidation during exercise.

3.3 Methods:

3.3.1 Participants

8 males (Mean ± SD: age 23 ± 4 years, weight 74.6 ± 10.6 kg, height 179.7 ± 8.3 cm, $\dot{V}O_{2peak}$ 50.8 ± 5.2 ml·min^{-1}·kg^{-1}, W_{max} 302 ± 48 W) and 3 females (21 ± 3 years, weight 55.5 ± 5.5 kg, height 164.1 ± 3.7 cm, $\dot{V}O_{2peak}$ 51.1 ± 4.0 ml·min^{-1}·kg^{-1}, W_{max} 235 ± 30 W) completed the study. One additional male participant commenced the study but withdrew after experiencing severe GIS in his first experimental trial (ingesting lactose). The sample size was selected to be comparable with previous research that has investigated metabolic responses to CHO ingestion during exercise (O'Brien et al., 2013, Wallis et al., 2007, Wallis et al., 2005, O'Brien and Rowlands, 2011). Participants were defined as recreationally active and participated in endurance-type exercise at least three times per week and were included in the study if they attained a $\dot{V}O_{2peak}$ of ≥ 40 ml·min^{-1}·kg^{-1} or ≥ 45 ml·min^{-1}·kg^{-1} for females and males, respectively. Participants were classified as healthy by successful completion of a general health questionnaire and were excluded if they had a known diagnosis of galactosemia or a history of lactose intolerance. Participants gave written informed consent, after having the purpose, risks and practical details explained to them in accordance with the

Declaration of Helsinki. The study was approved by the Science, Technology, Engineering and Mathematics Ethics Committee, University of Birmingham, Birmingham, UK (ERN_17-1283a).

3.3.2 Experimental design

Participants completed 6 visits to the laboratory, including a screening visit and five experimental trials, separated by at least 5 days. Two female participants were using monophasic contraception and completed visits in the active pill consumption phase. One female participant who did not use hormonal contraception self-reported to be regularly menstruating and performed trials in the mid-follicular phase of the menstrual cycle (estimated as days 7-10 from menstruation onset) of successive menstrual cycles. All experimental trials consisted of 150 min of exercise on a cycle ergometer at 50 % W_{max}, whilst ingesting one of five beverages in a randomized order, in a single blind fashion. The five beverages included lactose with high (LacHi) or low (LacLo) natural abundance of ^{13}C stable-isotope, sucrose with high (SucHi) or low (SucHi) natural abundance of ^{13}C, and water. Venous blood and expired breath samples were collected to characterize substrate oxidation and metabolic responses to CHO ingestion. Breath H_2 was measured before and after exercise as a proxy for CHO maldigestion (Misselwitz et al., 2013).

3.3.3 Preliminary testing

Participants' height (Stadiometer Model 220, Seca, Germany) and body mass (Champ II, OHAUSE, Switzerland) were recorded. They then completed a step-incremental exercise test on a cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). The test began at 100 W and increased by 30 W every 2 min until volitional exhaustion, or until a cadence of ≤ 60 revolutions·min^{-1} could not be maintained. Heart rate (HR) was monitored continuously via radio telemetry (Polar H7, Kempele, Finland). Respiratory gas exchange measurements were performed throughout exercise using an online automated gas analyser (Vyntus, Vyaire Medical, Mettawa, IL, US) to determine $\dot{V}O_2$ and $\dot{V}CO_2$. The highest average 30s of $\dot{V}O_2$ was considered to be $\dot{V}O_{2peak}$. W_{max} was calculated as the power output from the final stage completed, combined with the fraction of the time spent in the following stage, multiplied by 30 W, as previously described (Podlogar et al., 2020).

Upon completion of the exercise test, participants completed a questionnaire to assess their habitual lactose intake. The questionnaire was based on the 2015 Nurse's Health Study II questionnaire, Question 28 (Nurses' Health Study II, 2017). Alterations were made, including changing units to metric equivalents, removing some listed foods that contain no lactose (sorbet, margarine) or contain negligible amounts (butter) and condensing items with equivalent lactose content into one item (yoghurts). Daily lactose intake was calculated using the weekly or daily frequency of ingestion of each food or drink item, and reference values for the amount of lactose per serving (Pennington and Spungen, 2009, Nutrition, 1988). Where a range of lactose content was provided, the mid-point of the range was used. Participants habitual lactose intake was 11 ± 10 g·day^{-1}, which is congruent with normal lactose intake of 10-12 g·d^{-1} in

the Western diet (Larsson et al., 2004, Brisbois et al., 2014), with 2 participants reporting no regular lactose intake.

3.3.4 Pre-experimental control

Participants were asked to avoid foods with a high natural abundance of ^{13}C, to minimise the background shift from glycogen stores for 5 days preceding experimental trials. They were also asked to refrain from alcohol and caffeine, as well as foods which may affect H_2 breath concentrations (onions, leeks, garlic, cabbage, beans, pickled vegetables, fibre supplements) for 24 hours preceding experimental trials. Participants also recorded their diet the day preceding their first experimental trial and replicated it the day before subsequent experimental trials.

3.3.5 Experimental trials

Participants attended the laboratory in an overnight fasted state, between 06:00 and 08:30 am. Breath H_2 concentration was measured in duplicate before and after exercise using a handheld monitor (Hydrogenius, Bedfont Scientific Ltd, Maidstone, England) according to manufacturer's instructions, with participants in a seated position. A cannula (Venflon, Becton-Dickinson, Helsingborg, Sweden) was inserted into an antecubital vein and attached to a three-way stopcock (Connecta, Becton-Dickinson, Helsingborg, Sweden) to allow repeated venous blood sampling (LacHi,

SucHi and Water trials only). A basal 10 ml blood sample was collected before exercise and every 30 min during exercise with blood dispensed into an EDTA-containing vacutainer and stored on ice until centrifugation. Resting expired breath samples were collected in duplicate into evacuated 10ml Exetainer tubes (Labco, High Wycombe, United Kingdom) which were filled from a mixing chamber to determine ^{13}C:^{12}C ratio at rest and every 30 min during exercise. The exercise consisted of 150 min of cycling at 50 % W_{max} (151 ± 24 W for male participants, 118 ± 15 W for female participants) on a cycle ergometer. Respiratory gas exchange measurements ($\dot{V}O_2$, $\dot{V}CO_2$ and respiratory exchange ratio (RER)) and HR were measured every 30 min during exercise as described above; ratings of perceived exertion (RPE) (Borg, 1982) were also taken at these time points. Participants completed a GIS questionnaire at rest, and every 30 min during exercise by completing a 100 mm visual analogue scale (VAS) scale for each symptom. GIS were divided into upper GIS (stomach problems, vomiting, belching, stomach burn, bloating and stomach cramps), lower GIS (flatulence, urge to defecate, intestinal cramps, diarrhoea and side aches on the left and right), and other symptoms (nausea, dizziness, headache and urge to urinate) (Jeukendrup et al., 2000). For analysis, an integrated GIS score was derived as the sum of symptoms (in mm from VAS) combined between 30-150 min, for total, upper, lower and other GIS.

3.3.6 Test beverages

Participants ingested a total of 2.4 L of a test beverage in each trial. CHO beverages contained 120 g (w/v) to deliver CHO at a rate of 0.8 g·min^{-1} (48 g·h^{-1}) in line with

recommendations to provide CHO at 30-60 g·h^{-1} for exercise lasting 2-2.5 h (Burke et al., 2011). A 600 ml bolus was provided at exercise onset followed by 200 ml drinks every 15 min. To allow quantification of exogenous CHO oxidation CHOs were selected to have either a high or low natural abundance of ^{13}C (LacHi: –16.29 δ‰ versus Pee Dee Bellemnitella [PDB] [Milk Specialities Global, Minnesota, United States]; LacLo: –27.73 δ‰ [Volac International, Royston, United Kingdom]; SucHi: –11.87 δ‰ [Tate and Lyle, London, United Kingdom]; SucLo: –26.13 δ‰ [Aldi, Coventry, United Kingdom]). Low ^{13}C trials were used exclusively to quantify the background shift in breath $^{13}CO_2$ in order to allow correction and more accurate quantitation of exogenous CHO oxidation.

3.3.7 Plasma and Breath Analyses

Venous blood samples were centrifuged at 1865 g for 15 min at 4 °C. Aliquots of plasma were immediately frozen and stored at –70 °C for later analysis. Plasma was analysed using commercially available kits for glucose, lactate and NEFA concentrations (Glucose kit, Lactate kit, NEFA kit; Randox, London, UK) using an automated photometric clinical chemistry analyser RX Daytona+ (Randox, London, UK). Plasma galactose concentration was quantified using a colorimetric assay (Sigma Aldrich, St Louis. MO, USA) and insulin using an enzyme-linked immunosorbent assay (Ultrasensitive Insulin ELISA, Mercodia, Uppsala, Sweden).

Isotopic enrichment of breath samples was determined using gas chromatography isotope ratio mass spectrometry (IR-MS) (Europa Scientific Hydra 20-20, Crewe,

United Kingdom). ^{13}C enrichment of ingested CHOs was determined by elemental analyser IRMS (Europa Scientific Hydra 20-20, Crewe, United Kingdom).

3.3.8 Calculations

Fat and total CHO oxidation rates were calculated using the below equations (Frayn, 1983), with protein oxidation assumed to be negligible.

$$\text{Total CHO oxidation} = 4.55\ \dot{V}CO_2 - 3.21\ \dot{V}O_2$$

$$\text{Fat oxidation} = 1.67\ \dot{V}O_2 - 1.67\ \dot{V}CO_2$$

In which $\dot{V}CO_2$ and $\dot{V}O_2$ are measured in $L \cdot min^{-1}$ and oxidation rates in $g \cdot min^{-1}$. Energy expenditure (EE) was calculated using the modified Weir equation (Weir, 1949).

The isotopic enrichment of expired breath samples was expressed as δ per milliliter difference between the ^{13}C:^{12}C of the sample and a known laboratory reference standard using an established formula (Mosora et al., 1976). $\delta^{13}C$ was then related to an international standard (PDB).

The rate of exogenous CHO oxidation was calculated using the following formula:

$$\text{Exogenous CHO oxidation} = VCO_2 \times \left(\frac{\delta\text{Exp} - \delta\text{Exp}_{\text{bkg}}}{\delta\text{Ing} - \delta\text{Exp}_{\text{bkg}}}\right) \times \left(\frac{1}{k}\right)$$

In which δExp is the ^{13}C enrichment of the expired air at various time-points in the high ^{13}C (LacHi, SucHi) conditions, δIng is the enrichment of the ingested beverage, δExp_{bkg}

is the ^{13}C enrichment of the expired air at the corresponding time-points in the low ^{13}C (LacLo, SucLo) conditions, and k is the amount of CO_2 (in litres) produced by the complete oxidation of 1 g of glucose (k = 0.7467 L). Comparisons of mean and peak exogenous CHO oxidation rates were made only using data from 60-150 min during which time recovery of breath $^{13}CO_2$ from oxidation approaches 100 %, when dilution in the bicarbonate pool becomes negligible (Robert et al., 1987, Pallikarakis et al., 1991). Endogenous CHO oxidation was calculated by subtracting exogenous CHO oxidation from total CHO oxidation. Subsidiary analyses were also made between different methods of background correction for the calculation of exogenous CHO oxidation as the study design permitted these comparisons. Calculation of exogenous CHO oxidation is often corrected by using the corresponding time-points during a water trial (Wallis et al., 2007). In the present study, a low ^{13}C CHO condition was used for background correction, and the two methods were compared.

Area under the curve (AUC) was calculated for glucose, lactate, NEFA and insulin using the trapezoidal method: $AUC = (C_1 + C_2 / 2) (t_2 – t_1)$, where C_1 and C_2 represent the concentrations of the analyte being calculated, and t_2 and t_1 are the corresponding time points. AUC data are presented as the time-averaged summary value by dividing AUC by the duration of the observation period (i.e., 150 min).

3.3.9 Statistical Analysis

Experimental data are presented as mean ± standard deviation (SD). Low ^{13}C trials were used exclusively to quantify the background shift in breath $^{13}CO_2$ and therefore

are not included in the statistical analysis. Statistical analysis was performed using SPSS Statistics for Macintosh, Version 25.0 (IBM Corp. Armonk, NY: IBM Corp). Differences in mean and peak exogenous CHO oxidation rates between CHO conditions and mean exogenous CHO oxidation using the different background correction methods were compared using paired t-tests. Differences between conditions for GIS, HR, RPE, AUC for all plasma analytes, energy and substrate utilization as a proportion of energy expenditure (EE) were all analysed using one-way repeated measures analysis of variance (ANOVA). Mauchly's test for sphericity was employed, and in cases where this assumption was violated, the Greenhouse-Geisser correction was used. When a main effect was observed pairwise comparisons were made to locate the source of difference using paired t-tests with Bonferroni corrections applied to account for multiple comparisons. Two-way repeated measures ANOVA was used to analyse differences over time (30-150 min) and between conditions for breath hydrogen, substrate utilization and plasma metabolites. Where significant interaction (time × condition) effects were detected by ANOVA, post-hoc pairwise comparisons were made between time-points and conditions using paired t-tests and the Bonferroni correction to account for multiple comparisons. Statistical significance was set at $P<0.05$. Cohen's D was calculated as previously described (Cohen, 1988) for comparisons between two conditions, with the pooled variance used for within-subject comparisons.

3.4 Results:

3.4.1 Physiological and perceptual characteristics of exercise bouts

As shown in Table 3.1 there were no significant differences between conditions for either absolute ($L \cdot min^{-1}$) or relative ($\%\dot{V}O_{2peak}$) oxygen consumption or EE. RER was significantly higher in Sucrose than Water ($P \leq 0.01$) and showed a trend to be lower in Lactose than Sucrose ($P=0.09$) but higher than Water ($P=0.09$). RPE was significantly lower in Sucrose than Water ($P \leq 0.01$) and showed a trend to be lower in Lactose than Water ($P=0.09$). HR was not significantly different between conditions ($P=0.17$).

Table 3.1 Metabolic, physiological and perceptual responses to 150 min exercise at 50 % W_{max} with ingestion of lactose, sucrose or water.

	Lactose	Sucrose	Water
$\dot{V}O_2$ ($L \cdot min^{-1}$)	2.29 ± 0.44	2.20 ± 0.38	2.27 ± 0.40
$\%\dot{V}O_2$ peak	66 ± 4	63 ± 4	65 ± 4
EE (kcal)	1677 ± 330	1607 ± 275	1643 ± 294
RER	0.87 ± 0.03	0.90 ± 0.03†	0.85 ± 0.03
RPE	12.5 ± 1.8	12.1 ± 1.8†	13.4 ± 1.5
HR ($beats \cdot min^{-1}$)	142 ± 4	135 ± 5	140 ± 4

† - Significant ($P<0.05$) difference between Sucrose and Water.

3.4.2 Exogenous and endogenous substrate utilization

There was a significant interaction effect ($P \leq 0.01$; time × condition; η^2_p: 0.38) in total CHO oxidation (Fig. 3.1A) and total CHO oxidation was significantly higher in Sucrose

than Water at all time-points (all P≤0.05). Total CHO oxidation was higher in Lactose than Water from 60 min onwards (all P≤0.05) and higher in Sucrose than Lactose at 120 min (P≤0.05). Mean exogenous CHO oxidation (between 60-150 min; Fig. 3.1B) was not significantly different between Lactose (0.56 ± 0.19 g·min^{-1}) and Sucrose (0.61 ± 0.10 g·min^{-1}; P=0.49; *d*=0.33). No differences in peak exogenous CHO oxidation between Lactose (0.65 ± 0.19 g·min^{-1}) and Sucrose (0.71 ± 0.13 g·min^{-1}; P=0.45; *d*=0.33) were observed. There was a significant interaction effect (P≤0.05; time × condition; η^2_p: 0.32) in exogenous CHO oxidation, and a trend towards higher exogenous CHO oxidation in Sucrose (0.31 ± 0.15 g·min^{-1}) than in Lactose (0.18 ± 0.19 g·min^{-1}) at the 30 (P=0.06) and 60 min (P=0.09) time-point. There was a significant interaction effect (P≤0.05; time × condition; η^2_p: 0.26) in endogenous CHO oxidation (Fig. 3.1C) which was significantly lower in Lactose than Sucrose at 90 min (P≤0.05), and lower in Lactose than Water from 90-150 min (all P<0.05). There was a significant interaction effect (P≤0.05; time × condition; η^2_p: 0.411) in fat oxidation (Fig. 3.1D) which was significantly higher in Water than Sucrose from 30 min onwards (all P≤0.05) and higher in Lactose than Sucrose from 60 to 120 min (all P≤0.05). One subject could not fully complete the Water trial due to fatigue, and ceased exercise shortly after 120 min, despite completing other trials. Therefore, data from measures of substrate utilization except exogenous CHO oxidation, were from 0-120 min (n=11) and 120-150 min (n=10).

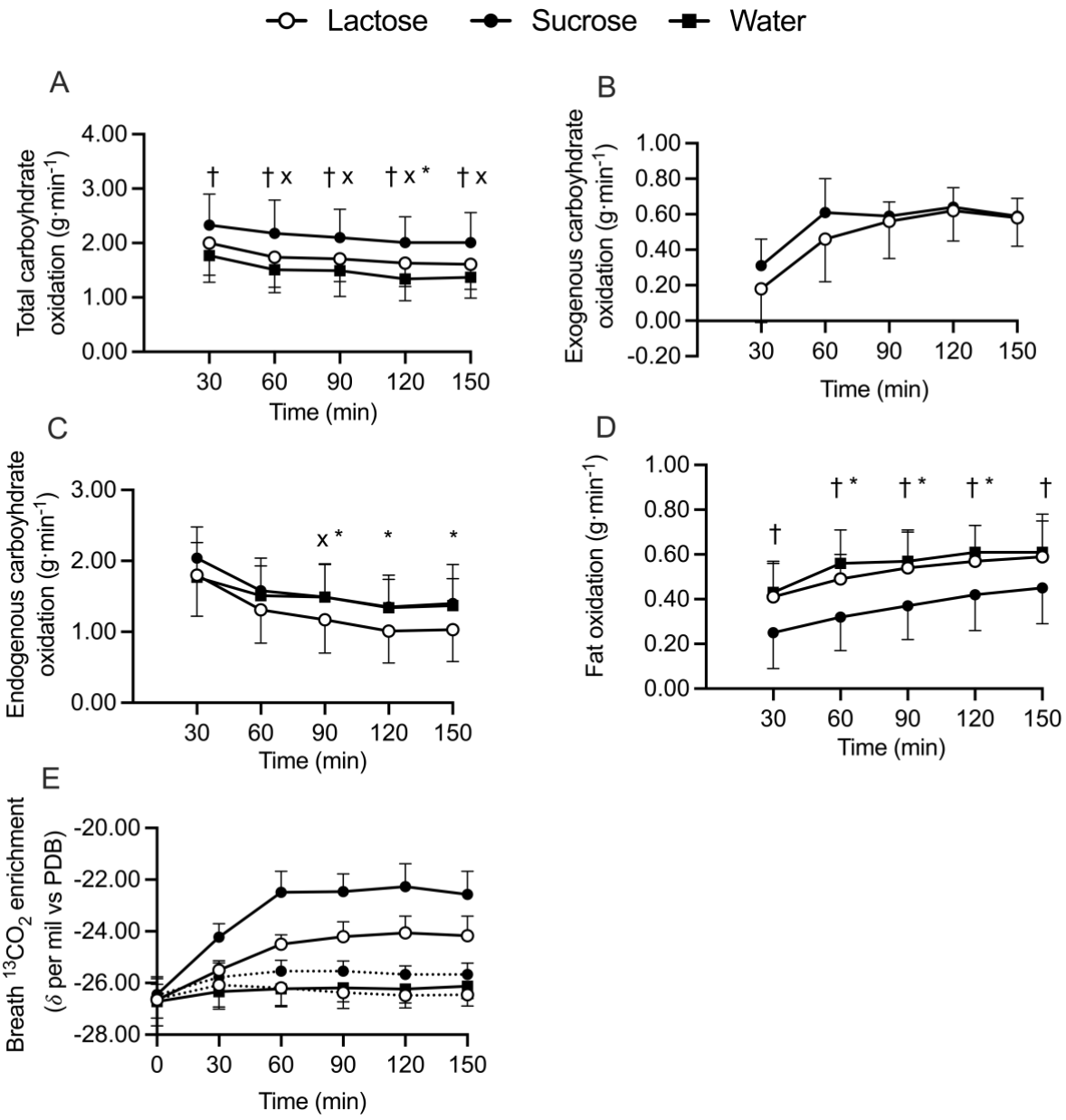

Figure 3.1 Total CHO (A), exogenous CHO (B), endogenous CHO (C), fat oxidation (D) and change in breath $^{13}CO_2$ enrichment (E) between 30-150 min of cycling at 50 % Wmax, ingesting either Lactose, Sucrose or Water, in high ^{13}C substrate (—) or low ^{13}C substrate (•••)

*, a significant difference (P<0.05) between Lactose and Sucrose at this time-point. x, a significant difference (P<0.05) between Lactose and Water at this time-point. †, a significant difference (P<0.05) between Sucrose and Water at this time-point.

Substrate utilization from 60-150 min expressed as a proportion of total EE is shown in Figure 3.2. Exogenous CHO oxidation contributed comparably to EE in both Lactose (20 ± 8 %) and Sucrose (22 ± 5 %; P=0.35, *d*= 0.32). Endogenous CHO oxidation contributed least to EE in Lactose (38 ± 14 %), compared to Sucrose (50 ± 7 %; P≤0.05) and Water (50 ± 11 %; P≤0.01; η^2_p: 0.44). Fat oxidation contributed most to EE in Water (50 ± 11 %), greater than both Lactose (42 ± 8 %, P≤0.05) and Sucrose (28 ± 8 %; P≤0.01; η^2_p: 0.73), which were also significantly different from one another (P≤0.01).

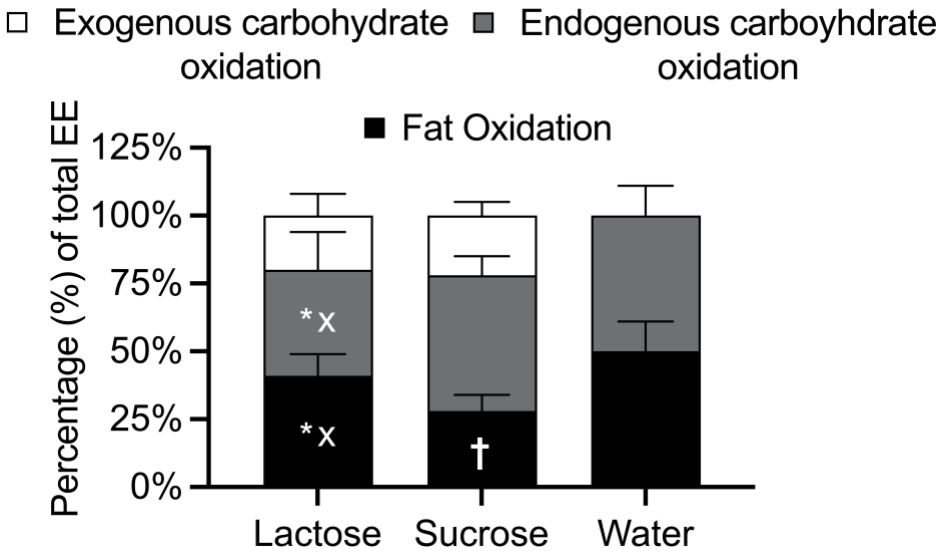

Figure 3.2 Substrate contributions to total energy expenditure from 60-150 min

*, a significant difference (P<0.05) between Lactose and Sucrose. x, a significant difference (P<0.05) between Lactose and Water. †, a significant difference (P<0.05) between Sucrose and Water.

Comparison of background correction methods in the calculation of exogenous CHO oxidation rates revealed significantly higher estimation of sucrose oxidation rates using the water correction (0.69 ± 0.15 g·min^{-1}) compared to the low ^{13}C CHO correction (0.61 ± 0.10 g·min^{-1}, P≤0.01). Lactose oxidation rates were not significantly different between water-corrected (0.51 ± 0.16 g·min^{-1}) and low ^{13}C CHO-corrected (0.56 ± 0.19 g·min^{-1}; P=0.22). The 95% confidence intervals (i.e., lower, upper) for the mean difference between water-corrected and low ^{13}C CHO-corrected exogenous CHO oxidation rates for Sucrose and Lactose were 0.03 to 0.13 g.min^{-1} and -0.12 to 0.02 g.min^{-1}, respectively.

3.4.3 Plasma Metabolites

As shown in Figure 3.3A, there was a significant interaction effect (P≤0.01; time × condition; η^2_p: 0.45) in plasma glucose which was higher in Sucrose than Water from 30-60 min and 120-150 min, and higher in Lactose than Water at 30 min and from 120-150 min. Glucose AUC was significantly higher in Lactose (5.2 ± 0.4 mmol·L^{-1}, P≤0.01) and Sucrose (5.4 ± 0.4 mmol·L^{-1}, P≤0.01) than Water (4.6 ± 0.4 mmol·L^{-1}·150 min^{-1}) with no difference between CHOs (P=0.45). There was a significant interaction effect (P≤0.05; time × condition; η^2_p: 0.21) in plasma lactate concentration, which was higher in Sucrose than Water from 30-60 min (Fig. 3.3B). There was no main effect of

condition (P=0.14) in lactate AUC. There was a significant interaction effect (P≤0.001; time × condition; η^2_p: 0.391) in plasma NEFA concentration which was higher in Water than Sucrose than from 60 min onwards (all P≤0.05) and higher in Water than Lactose at 90 min. (Fig. 3.3C). NEFA AUC was significantly higher in Water (1.1 ± 0.3 mmol·L^{-1}) than Sucrose (0.5 ± 0.2 mmol·L^{-1}, P≤0.01), and tended to be higher than Lactose (0.7 ± 0.3 mmol·L^{-1}, P=0.08) with no difference between CHOs (P=0.62). There was a significant interaction effect (P≤0.01; time × condition; η^2_p: 0.51) in plasma insulin concentration (Fig. 3.3D), which was significantly higher in Sucrose than Water from 30-60 min, tended to be higher in Lactose than Water (P=0.08) at 30 min, and was significantly higher at 60 min and 150 min. Insulin AUC was significantly higher in Sucrose (6.8 ± 3.4 μU·L^{-1}) than Water (1.9 ± 1.2 μU·L^{-1}, P≤0.05), which tended to be lower than Lactose (4.5 ± 2.6 μU·L^{-1}, P≤0.05) with no significant difference between CHOs (P=0.14). Analysis for plasma galactose was conducted in full in the Lactose condition (n=10), as the only condition where galactose was expected to be present, unlike Sucrose and Water (n=1). Plasma galactose concentrations rose from baseline with Lactose ingestion but did not deviate substantially from baseline with Sucrose or Water ingestion (Fig. 3.3E). All comparisons of plasma metabolites were n=10, because a cannula could not be placed in the Lactose condition in one subject, except insulin which was n=8 due to limited plasma aliquots.

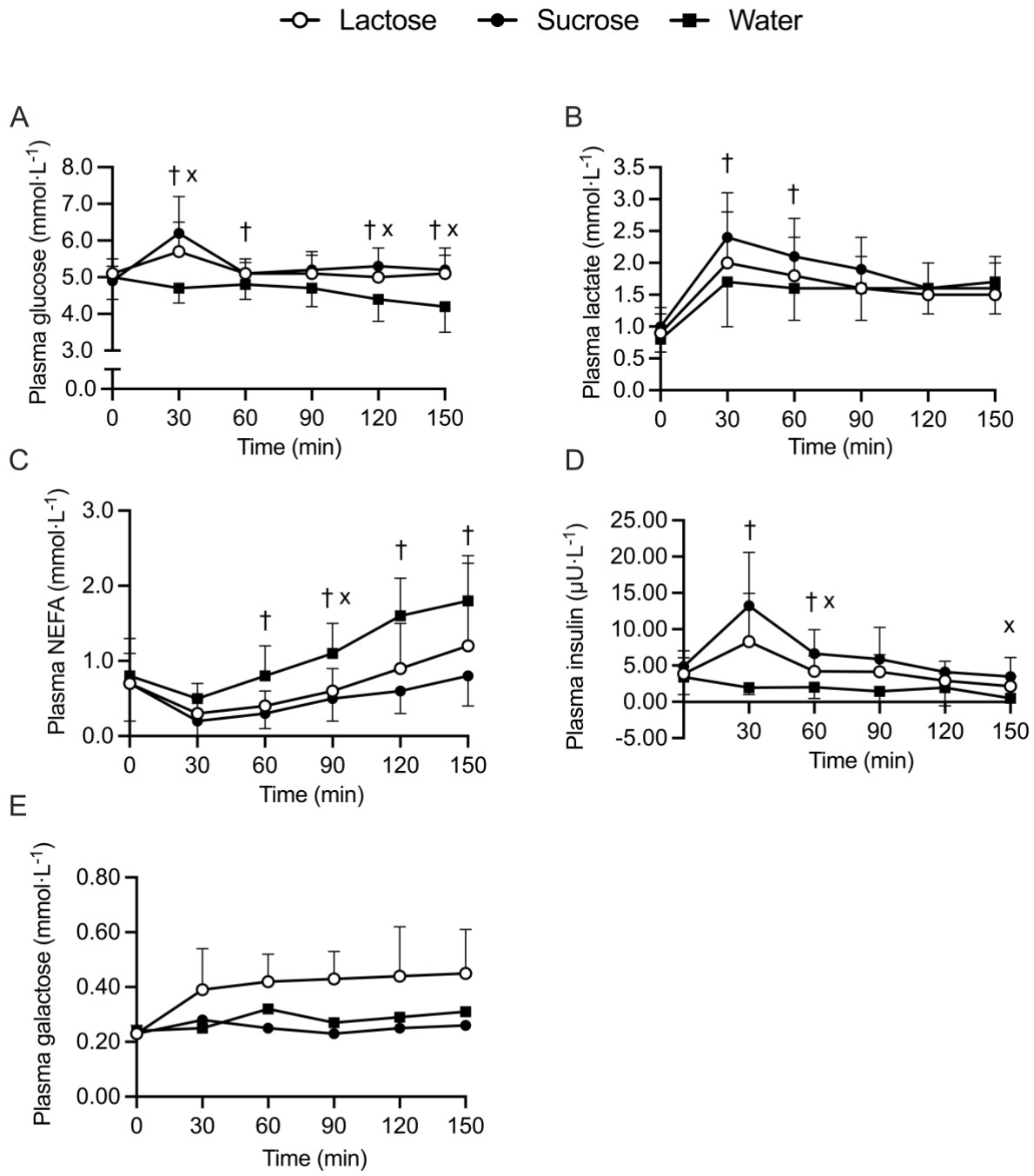

Figure 3.3 Plasma glucose (A), lactate (B), NEFA (C), Insulin (n=8) (D) and galactose (E) concentrations between 30-150 min of cycling at 50 % Wmax, ingesting either Lactose, Sucrose or Water

*, a significant difference (P<0.05) between Lactose and Sucrose at this time-point. x, a significant difference (P<0.05) between Lactose and Water at this time-point. †, a significant difference (P<0.05) between Sucrose and Water at this time-point.

3.4.4 Gastrointestinal discomfort and maldigestion

The maximum scores for upper, lower and other GIS were 3000 mm, 3000 mm and 2000 mm respectively. There were no differences in upper GIS between any conditions (Lactose, 12 ± 20 mm, Sucrose 10 ± 22 mm or Water 10 ± 22 mm, P=0.90; η^2_p: 0.01), nor were there any differences in lower GIS (Lactose, 20 ± 32 mm, Sucrose 29 ± 53 mm or Water 11 ± 23 mm, P=0.42; η^2_p: 0.08) or other GIS (Lactose, 91 ± 88 mm, Sucrose 104 ± 64 mm or Water 74 ± 58 mm, P=0.10; η^2_p: 0.21). Thus, GIS were overall minimal, although one participant experienced severe symptoms, including diarrhea and stomach pain during their first trial (LacHi) and was therefore excluded from the study. One subject experienced milder GIS after the lactose trials, but remained in the study.

There was a significant interaction (time × condition) for breath H_2 concentration (P≤0.05; η^2_p: 0.37; Figure 3.4). Breath H_2 concentration significantly reduced from pre-exercise (26 ± 21 ppm) to post-exercise in Sucrose (11 ± 11 ppm; P≤0.01) and Water (23 ± 16 ppm to 6 ± 6 ppm; P≤0.01). The apparent increase from pre-exercise (25 ± 20 ppm) to post-exercise (60 ± 67 ppm) in the Lactose condition was non-significant (P=0.14).

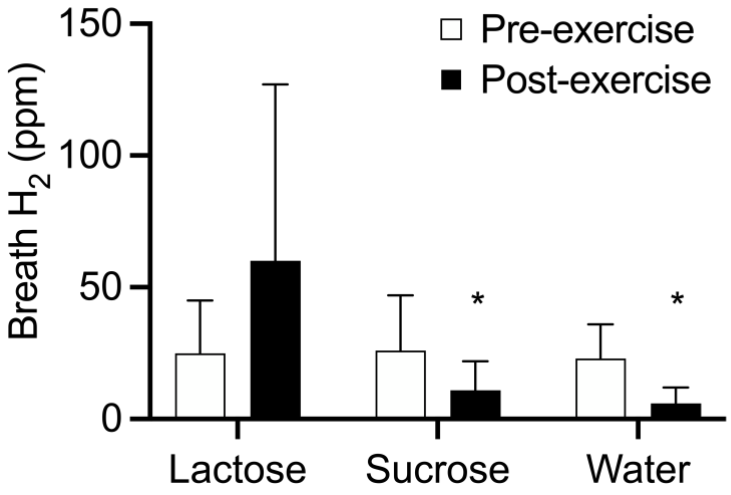

Figure 3.4 Breath H_2 concentration (ppm) before and after 150 min of cycling at 50 % Wmax and ingestion of either Lactose, Sucrose or Water (*n*=11).

* represents a significant (P<0.05) reduction from pre- to post-exercise.

3.5 Discussion

The aim of this study was to characterise the metabolic effects of lactose ingestion during endurance exercise as compared to sucrose or water. The primary finding was that mean exogenous CHO oxidation rates from ingested lactose (0.56 ± 0.19 g·min^{-1}) were comparable to those of sucrose (0.61 ± 0.10 g·min^{-1}) when ingested at moderate rates (<60 g·h^{-1}). Furthermore, as compared to sucrose, lactose ingestion resulted in increased fat oxidation and reduced endogenous CHO oxidation, while GIS were largely comparable.

The finding that the oxidation rates of ingested lactose and sucrose are similar was in line with the hypothesis. A previous investigation of combined galactose and glucose ingestion during exercise showing similar performance benefits to combined glucose and fructose ingestion would suggest that oxidation of the CHOs could be comparable (Stannard et al., 2009). However, Stannard et al. (2009) did not measure exogenous CHO oxidation rates and used free galactose and glucose as opposed to lactose. Stellaard et al. (2000) investigated the ingestion of 80 g lactose before light physical activity and showed comparable recovery of breath $^{13}CO_2$ after ingestion of high ^{13}C glucose or lactose suggesting similar oxidation of the two substrates. It was also shown that the digestion of lactose was not limiting upon its oxidation at this ingestion rate. However, as previously mentioned the intensity of the protocol (50 W) was very low and along with the fact that oxidation rates were not directly determined, understanding of the viability of ingested lactose as a fuel source for exercise remained limited. Thus, the present study is the first to show that lactose is readily oxidized during moderate-vigorous intensity endurance exercise and is comparable to other typical CHO types when consumed at doses (i.e., 30-60 $g \cdot h^{-1}$) in line with expert guidelines for CHO ingestion during exercise lasting 2-2.5 h (Burke et al., 2011, Hulston et al., 2009).

The hypothesis in the present study was that lactose and sucrose would be similarly and readily oxidized during exercise. Such a hypothesis may have been questioned given the slow oxidation rate of galactose (~50-60 % the rate of glucose) alone (Leijssen et al., 1995, Burelle et al., 2006). O'Hara et al. (2012) demonstrated that galactose oxidation was lower than glucose in the first 60 min of a 120 min steady state cycling protocol, but predominated in the second hour, suggesting a delay in galactose

oxidation. However, participants in that study ingested a bolus of CHO pre-exercise, as opposed to regular ingestion during exercise, and therefore the metabolic impacts of the CHO ingestion are not comparable. Nevertheless, the metabolism of galactose is known to be altered when co-ingested with glucose at rest, such that the presence of glucose more than doubles the first pass splanchnic clearance of galactose, and almost completely ablates the rise in plasma galactose that normally characterises galactose ingestion (Williams et al., 1983, Segal and Blair, 1961). A blunted rise in plasma galactose has also been demonstrated to occur also with lactose ingestion as compared to galactose only ingestion, implying even with intact lactose, glucose may facilitate galactose metabolism (Williams et al., 1983). The oxidation rates of lactose during exercise suggest that galactose metabolism has been augmented such that lactose and sucrose were oxidized to a comparable extent. However, whether glucose facilitates galactose metabolism remains to be tested in an exercise context. Regardless, the oxidation rates of both lactose and sucrose observed herein are entirely consistent with those observed previously with glucose or glucose-fructose ingestion at similar doses (0.8 $g \cdot min^{-1}$), exercise intensity (55 % W_{max}) and duration (150 min) (Hulston et al., 2009) further supporting the potential of lactose as a readily oxidizable fuel substrate.

Digestion of lactose is limited in many individuals at rest, due to low activity or absence of lactase (Misselwitz et al., 2013), and was therefore a potential concern in the present study. Indeed, there was a trend towards higher sucrose oxidation in the first 60 min, which could suggest a delay in lactose digestion. It is possible that this may be attributable to the higher K_m of sucrase (~142 mM) than lactase (~14 mM) (Naim and

Lentze, 1992, Gray and Ingelfinger, 1966), which at the very least implies the scope for increasing above moderate ingestion rates is greater in sucrose versus lactose. Nonetheless, there is methodological uncertainty surrounding saturation of the bicarbonate pool in the early stages of exercise (Pallikarakis et al., 1991, Robert et al., 1987), and oxidation rates of lactose and sucrose were not ultimately different as exercise duration progressed, with no significant difference in mean exogenous CHO oxidation rates, and a small (*d*=0.33) effect size. This suggests any potential digestion limitation with lactose at the ingestion rates used herein was transient in respect of the overall effect on exogenous carbohydrate oxidation.

There were markedly different responses of breath H_2 to lactose ingestion, with 5 subjects experiencing a reduction from pre- to post-exercise, whilst the remaining 6 increased breath H_2. By comparison, breath H_2 reduced significantly from pre to post-exercise in both Sucrose and Water conditions. Although the increase in breath H_2 with lactose ingestion was not significant, it suggests that digestion was incomplete and fermentation of lactose in the colon occurred in some individuals. Indeed, both subjects who experienced GIS markedly increased breath H_2, though some non-symptomatic subjects increased breath H_2 to a similar or greater extent. This variable response of breath H_2 suggests that its measurement may not be an accurate proxy for symptomatic CHO maldigestion during exercise. It is possible that some of the recovered breath $^{13}CO_2$ following lactose ingestion may be of colonic origin, from fermentation of lactose by bacteria such as Bifidobacteria (Szilagyi, 2015). Fermentation may produce CO_2, which is absorbed into the circulation and expired, and would be detected as breath $^{13}CO_2$. It is possible that this could lead to slight

overestimation of rates of exogenous CHO oxidation of lactose, though the extent to which this occurred in the present study cannot be determined, and the quantities of CO_2 arising from fermentation are not known. In addition, fermentation may have resulted in the production of SCFAs, which could be oxidised during exercise and increase recovery of breath $^{13}CO_2$. Regardless, lactose ingestion resulted in physiological bioavailability, evidenced by increase in plasma glucose, galactose and insulin. Importantly, the beneficial changes in plasma glucose are comparable to sucrose, which is an important and expected outcome of effective CHO supplementation in exercise and may suggest an at least similar potential for ergogenic effects of lactose and sucrose.

Given the importance of gut comfort for exercise performance (de Oliveira and Burini, 2014) and the presence of lactose intolerance in some individuals, investigation of GIS was particularly important. Upper, lower and other GIS were predominantly mild, with no differences between conditions. However, two subjects experienced noteworthy symptoms with lactose ingestion. One experienced severe lactose intolerance symptoms during exercise (flatulence, urge to vomit and diarrhoea) and was excluded from the study. The other participant experienced similar but milder symptoms in the hours post-exercise. This participant demonstrated a discrepancy in mean exogenous CHO oxidation rates between lactose (0.34 $g \cdot min^{-1}$) and sucrose (0.61 $g \cdot min^{-1}$). Both subjects reported regular dairy and lactose ingestion. In another participant a marked disparity in mean exogenous CHO oxidation between lactose (0.15 $g \cdot min^{-1}$) and sucrose (0.73 $g \cdot min^{-1}$) was observed alongside large increases in breath H_2 (17 to 208 ppm) with lactose, yet GIS were mild and comparable in both conditions. Collectively,

these preliminary observations are interpreted to mean that GIS with the moderate dose lactose feeding protocol used herein are unlikely to be problematic for most individuals, although caution should be taken as some apparently lactose tolerant individuals may experience symptoms.

Higher fat oxidation and lower estimated endogenous CHO oxidation were observed with lactose ingestion than sucrose ingestion. Fat oxidation is known to be suppressed with CHO ingestion during exercise, which in part occurs due to suppression of lipolysis by insulin, which reduces plasma free fatty acids (Horowitz et al., 1997). However, there is also a direct effect of CHO ingestion and resulting insulinemia upon muscle, which suppresses mitochondrial long chain fatty acid oxidation (Coyle et al., 1997). Although plasma insulin levels were not significantly different between the CHO conditions, insulinemia was greater at 30 min in Sucrose than Water, but not Lactose. There is also an evident visual disparity in insulin concentrations and AUC between Sucrose and Lactose. The observed effect size (η^2_p: 0.51) was large, which may suggest that despite the lack of significant difference (perhaps due to the limited sample size, *n*=8) between CHOs, there may be a genuine difference. Lactose is known to be less insulinogenic than CHOs such as glucose at rest (Ercan et al., 1993) and this may extend into exercise. It is possible that the higher insulin levels in the Sucrose condition suppressed fat oxidation, therefore increasing reliance on endogenous CHO oxidation, whereas an attenuated insulin response with Lactose ingestion did not trigger such extensive suppression of fat oxidation. The presence of plasma lactate has also been demonstrated to reduce lipolysis during exercise (Boyd et al., 1974). Higher concentrations of lactate were present in the Sucrose condition

than Water, which may contribute to the lower fat oxidation observed in the Sucrose condition. The maintenance of high fat oxidation rates with lactose ingestion may have permitted the lower endogenous CHO oxidation with lactose ingestion versus sucrose, which represents glycogen sparing. Because CHO (both muscle and liver glycogen) is the most important substrate in moderate to vigorous exercise intensities, glycogen depletion can contribute to poorer endurance exercise performance, and therefore reducing reliance on endogenous CHO oxidation may benefit exercise performance (Romijn et al., 1993, Jeukendrup, 2004, Cermak and van Loon, 2013). However, the observed sparing of endogenous CHO with lactose ingestion versus water ingestion is of unclear origin and necessitates further investigation.

Finally, due to the design of the present study it was possible to perform a subsidiary analysis to compare different methods of background correction in the calculation of exogenous CHO oxidation. Using a water trial for correction is commonplace to account for the background shift in breath $^{13}CO_2$:$^{12}CO_2$ that occurs during exercise, which is attributable to the oxidation of residual ^{13}C in endogenous CHO stores. The present study demonstrated that a water-correction significantly overestimated exogenous CHO oxidation of sucrose by approximately 15 %, and underestimated lactose oxidation rates by 10 %, though non-significantly. Ingesting the corresponding CHO with low natural abundance of ^{13}C for each CHO accounts for the sugar-specific effects of CHO ingestion on metabolism and the subsequent shift in breath $^{13}CO_2$, which a water-correction fails to do. Furthermore, using a water-correction increases the possibility of participants fatiguing in the water trial due to a lack of carbohydrate, which occurred to one participant in the present study. Using a low ^{13}C CHO correction

reduces the possibility of fatigue due to a lack of carbohydrate in background correction trials. If a water-correction had been utilized in the present study a difference in exogenous CHO oxidation rates between the sugars would have been concluded due to the significant over-estimation of sucrose oxidation. The absence of a clear influence of background correction method on calculated lactose oxidation rates may be attributable to the effects of lactose ingestion on the overall metabolic response being less pronounced than those of sucrose. These findings suggest that past studies using a water-correction and naturally high abundance ^{13}C CHOs may have, depending on the nature of the CHOs investigated, overestimated exogenous CHO oxidation rates. Future investigations utilising naturally high abundance ^{13}C CHOs should consider using a CHO specific low ^{13}C background trial to more accurately quantify exogenous CHO oxidation.

In summary, the present study demonstrated that lactose ingested during exercise is oxidised at a comparable rate to sucrose and maintains plasma glucose similarly at moderate ingestion rates. Furthermore, lactose ingestion reduced endogenous CHO oxidation and increased fat oxidation relative to sucrose ingestion. Future investigations could seek to determine the source of endogenous CHO sparing (i.e., liver and/or muscle glycogen), the potential for lactose ingestion at higher rates, and whether lactose ingestion during exercise can beneficially influence exercise performance.

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Chapter 4: Using an improved ^{13}C oral tracer methodology to assess muscle and liver glycogen oxidation with lactose or sucrose ingestion during exercise: a pilot study.

4.1 Abstract:

Lactose ingestion has previously been shown to result in similar exogenous carbohydrate oxidation rates to sucrose during exercise. However, lactose has also been shown to result in increased fat oxidation and reduced endogenous carbohydrate oxidation, compared to sucrose. The aim of this study was to investigate the source of the previously observed glycogen sparing and determine whether the reduction in endogenous carbohydrate oxidation was of hepatic or muscular origin. Due to COVID-19 restrictions, it was not possible to complete the study with the planned number of participants, leading to incomplete comparisons and limited ability to answer the physiological question. Data from 6 subjects (Mean ± SD: age 21 ± 2 years, 68.6 ± 12.9 kg, 176.2 ± 12.1 cm, $\dot{V}O_{2peak}$ 50.2 ± 7 ml·min^{-1}·kg^{-1}, W_{max} 293 ± 81 W) were included. Participants cycled for 150 min at 50% W_{max}, while ingesting in a randomized order, one of 6 lactose or sucrose beverages, with high or low natural abundance ^{13}C, and a trial with added U-^{13}C glucose tracer, all at a total rate of 0.8 g·min^{-1} in an improved methodology to assess muscle and liver glycogen oxidation. Exogenous CHO oxidation rates were similar with lactose (n=5) and sucrose ingestion (n=3), 0.22 ± 0.11 g·min^{-1} and 0.22 ± 0.05 g·min^{-1}, respectively, though lower than previously observed. Plasma glucose oxidation appeared higher with sucrose oxidation compared to lactose, perhaps suggesting that the observed glycogen sparing with lactose

ingestion is of hepatic origin, though it was not possible to confirm this. The improved ^{13}C oral tracer methodology offered a number of benefits compared to existing methods, particularly for investigating exercise metabolism in carbohydrates that are not exclusively glucose based.

4.2 Introduction

Carbohydrate (CHO) ingestion during exercise is a well-established method for the improvement of exercise performance and capacity (Jeukendrup, 2008). Ingestion of exogenous carbohydrate leads to improved performance through a number of mechanisms, including maintaining stable blood [glucose], positively impacting the central nervous system, maintaining high total CHO oxidation rates and the sparing of endogenous CHO, as glycogen (Cermak and van Loon, 2013). Glycogen sparing can originate in the active muscle or the liver. Whilst there are reports of muscle glycogen sparing with CHO ingestion, (Tsintzas et al., 1996, Hargreaves et al., 1987, Hargreaves et al., 1984, Tsintzas et al., 2001), the preponderance of evidence suggests that muscle glycogen is largely unaffected by carbohydrate ingestion (Coyle et al., 1986, Flynn et al., 1987, Mitchell et al., 1989, Hargreaves et al., 1987, Coggan and Coyle, 1987, Karelis et al., 2010). However, hepatic glucose production is reduced with CHO ingestion during exercise (Jeukendrup et al., 1999, Smith et al., 2010, McConell et al., 1994, Wallis et al., 2007) which implies liver glycogen sparing, and one report provides direct evidence of liver glycogen sparing (Gonzalez et al., 2015).

In Chapter 3 it was demonstrated that ingested lactose can be oxidised at a comparable rate to ingested sucrose when ingested at an average rate of 0.80 g·min^{-1} (0.56 ± 0.19 g·min^{-1}) and sucrose (0.61 ± 0.10 g·min^{-1}). Lactose ingestion also resulted in significantly lower rates of endogenous CHO oxidation compared to sucrose ingestion, contributing to total energy expenditure 38 ± 14% and 50 ± 11% respectively. This glycogen sparing with lactose ingestion was accompanied by a concomitant increase in fat oxidation which was postulated to be caused by two possible factors. Plasma [lactate] was ~15% higher (though non-significantly) with sucrose ingestion compared to lactose, and lactate is known to suppress lipolysis during exercise (Boyd et al., 1974). Similarly, plasma [insulin] area under the curve (AUC) was ~50% higher, though non-significantly, with sucrose ingestion than lactose. Insulin is known to suppress lipolysis and long-chain fatty acid oxidation during exercise, resulting in lower fat oxidation rates when CHO is ingested during exercise (Horowitz et al., 1997, Coyle et al., 1997). Although non-significant, the sample size may have been insufficient to detect statistically significant differences between conditions in these measures (*n*=10 and *n*=8 respectively).

It is not clear whether the observed reduction in endogenous CHO oxidation with lactose ingestion resulted from reduced muscle or liver glycogen oxidation (LGO). As previously discussed, most instances of reduced endogenous CHO oxidation with exogenous CHO ingestion during exercise are caused by reduced liver glycogenolysis. However, Horowitz et al. (1997) demonstrated that although glucose ingestion before exercise resulted in suppressed lipolysis, this could be partially ‘rescued’ by the infusion of heparin and triglycerides, which also resulted in a reduction in muscle

glycogen oxidation (MGO). Therefore, it is possible that lactose ingestion, which may also result in higher lipolysis and certainly elicits higher fat oxidation than sucrose ingestion, could spare muscle glycogen rather than liver glycogen.

The original aim of this chapter was to build on the observations in Chapter 3 by investigating whether lactose ingestion during exercise results in the sparing of muscle and/or liver glycogen by calculation of the oxidation of these endogenous CHO sources. The hypothesis is that lactose ingestion results in reduced liver glycogen oxidation, which may be accompanied by a reduction in muscle glycogen. To answer the physiological question, ^{13}C tracer methods were to be employed to calculate plasma glucose (PG) oxidation and exogenous CHO oxidation, as have been applied in previous studies (O'Hara et al., 2017, Wallis et al., 2007). An improvement upon the approach used in previous studies, and based on observations from Chapter 3, was also to be performed using 3 trials per CHO condition. Specifically, by including a low ^{13}C CHO condition for each CHO as a background correction the aim was to attain more valid estimates of PG and exogenous carbohydrate oxidation which would ultimately enable more accurate assessment of LGO and MGO (see Figure 4.1). However, due to constraints imposed by COVID-19 restrictions, it was not possible to answer the physiological research question, and therefore the remainder of this chapter is related to providing more detail about the approach and discussing its feasibility for improving the accuracy of the estimation of PG oxidation.

4.3 Methods

4.3.1 Statement of disruption

Due to the national lockdown from March of 2020, the data collection phase of the study ended abruptly. Although plans were made to recommence data collection, the repeated lockdowns prevented research on human participants from restarting in a timely manner, in line with the deadline of the PhD project. The decision was taken to proceed with analysis of samples that had been collected. Therefore, the sample size of this study is limited, with inconsistent numbers of comparisons between conditions due to the long duration of the study (~7 weeks minimum). However, these data do show the feasibility of an improved methodology and also demonstrates the intellectual and analytical skills advancement needed to develop and test the approach. Although the data do not provide reliable insight into the physiological questions posed by the original aims of the research, limited qualitative interpretation is attempted.

Due to the incomplete nature of the data, there are three primary outcomes for this chapter. Other metabolic, physiological, and perceptual responses to lactose and sucrose ingestion during exercise have already been characterised in Chapter 3, with a larger sample size and complete comparisons between conditions. Therefore, the outcomes for this Chapter are:

1. The rates of exogenous carbohydrate oxidation of lactose and sucrose
2. The rate of PG oxidation with ingestion of lactose and sucrose.
3. Oxidation rates of muscle and liver glycogen with ingestion of lactose and sucrose.

4.3.2 Participants

Although 8 participants were recruited, only the data from 6 could be used to contribute to primary outcomes. Exogenous CHO oxidation could be calculated for lactose (n=5) and sucrose (n=3). PG oxidation could be calculated for lactose (n=4 and sucrose (n=3). MGO and LGO could only be calculated in 1 participant in both conditions.

6 subjects (Mean ± SD: age 21 ± 2 years, 68.6 ± 12.9 kg, 176.2 ± 12.1 cm, $\dot{V}O_{2peak}$ 50.2 ± 7 $ml \cdot min^{-1} \cdot kg^{-1}$, W_{max} 293 ± 81 W) comprising 3 males and 3 females participated in the study. Participants were excluded if they suffered from lactose intolerance or galactosemia. Participants were recreationally active and included in the study if they attained a $\dot{V}O_{2peak}$ of ≥ 40 $ml \cdot min^{-1} \cdot kg^{-1}$ or ≥ 45 $ml \cdot min^{-1} \cdot kg^{-1}$ for females and males, respectively. Participants gave informed consent in accordance with the Declaration of Helsinki, and the study was approved by the Science, Technology, Engineering and Mathematics Ethics Committee, University of Birmingham, Birmingham, UK (ERN_17-1283b).

4.3.3 Experimental design

Participants visited the laboratory on 7 occasions, including a screening visit and 6 experimental trials, separated by at least 5 days. Female subjects using monophasic contraception (n=3) completed experimental trials in the active pill consumption phase. Experimental trials involved 150 min of cycling at 50% W_{max} on a cycle ergometer, while ingesting in a randomized order, one of 6 beverages (Figure 4.1). Beverages

contained lactose in three trials, and sucrose in three trials. Beverages contained high natural abundance of ^{13}C (LacHi, SucHi), low natural abundance of ^{13}C (LacLo, SucLo) or low natural abundance of ^{13}C with an added U-^{13}C glucose tracer (LacLo+G, SucLo+G)

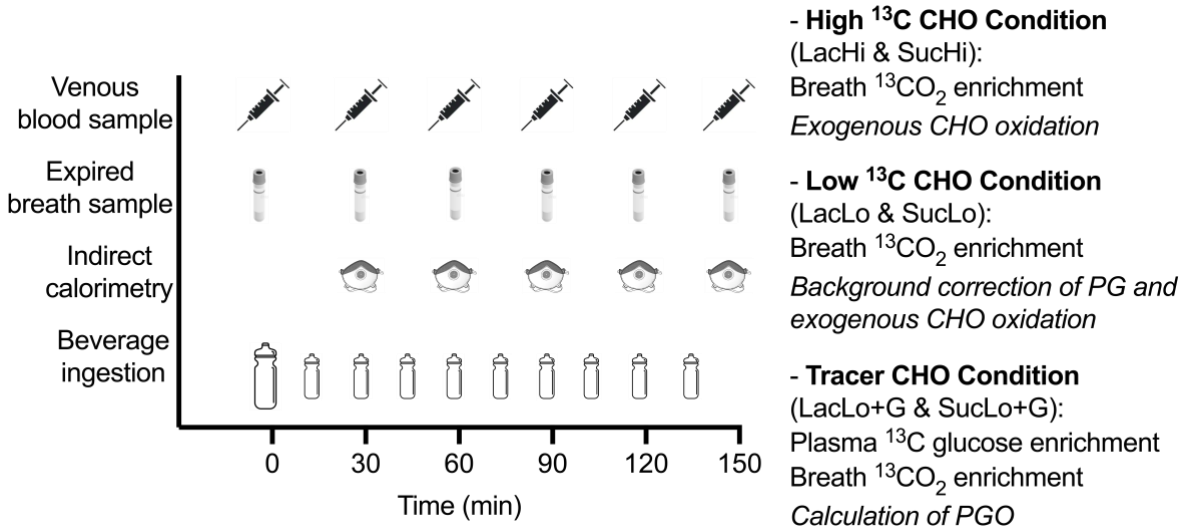

Figure 4.1 Schematic of protocol, detailing the measurements taken in experimental trials, and the outcomes associated with each measurement. PG; plasma glucose, CHO; carbohydrate, PGO; plasma glucose oxidation

4.3.4 Preliminary testing

Participants' height (Stadiometer Model 220, Seca, Germany) and body mass (Champ II, OHAUSE, Switzerland) were measured. Participants then performed an incremental test to task failure on a cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands), as described in Chapter 3. After completing the exercise test, if the criterion $\dot{V}O_{2peak}$ was achieved, participants completed a questionnaire to assess habitual lactose intake, as described in Chapter 3, which was 19 ± 17 g·day^{-1}.

4.3.5 Pre-experimental control

For the 5 days preceding experimental trials, participants were asked to avoid foods with a high natural abundance of ^{13}C to minimise the background shift in breath $^{13}CO_2$ from the oxidation of glycogen. Participants were also asked to refrain from alcohol and caffeine for 24 hours preceding experimental trials. Participants recorded their diet and exercise activity 24 h before the first experimental trial, which they replicated the day before subsequent experimental trials.

4.3.6 Experimental trials

Participants attended the laboratory between 06:00 and 08:00 in an overnight fasted state. A cannula (Venflon, Becton-Dickinson, Helsingborg, Sweden) was placed in an antecubital vein and attached to a three-way stopcock (Connecta, Becton-Dickinson, Helsingborg, Sweden) to permit repeated venous blood sampling in the two trials which included a U-^{13}C glucose tracer (LacLo+G, SucLo+G). A 10 ml blood sample was taken before exercise, and every 30 minutes during exercise. Blood was dispensed into an ethylenediaminetetraacetic acid (EDTA) containing vacutainer (6 ml) or a fluoride oxalate vacutainer (4 ml) and stored on ice until centrifugation. Blood samples in EDTA vacutainers were centrifuged at 1865 g for 10 minutes, whilst those in fluoride oxalate vacutainers were centrifuged at 1300 g. Expired breath samples were collected in

duplicate into evacuated 10ml Exetainer tubes (Labco, High Wycombe, United Kingdom) filled from a mixing chamber to determine ^{13}C: ^{12}C ratio, at rest and every 30 minutes during exercise. Exercise consisted of 150 min of cycling at 50% W_{max} on a cycle ergometer. Gas exchange measurements ($\dot{V}O_2$ and $\dot{V}CO_2$) and ratings of perceived exertion (RPE) (Borg, 1982) were measured every 30 min.

4.3.7 Test beverages

Test beverages totaled 1.7 L in each trial, and delivered 120 g (w/v) of lactose or sucrose, at an average rate of 0.80 g·min^{-1} (48 g·h^{-1}), in line with recommendations to ingest 30-60 g·h^{-1} of carbohydrate during exercise lasting 2-2.5 h (Thomas et al., 2016, Burke et al., 2011). Participants ingested a 440 ml bolus at the onset of exercise and ingested 140 ml beverages every 15 min thereafter.

Carbohydrates had either a high or low natural abundance of ^{13}C. LacHi: -16.29 δ‰ *versus* Pee Dee Bellemnitella [PDB] [Milk Specialities Global, Minnesota, United States], LacLo: -26.72 δ‰ [Volac International, Royston, United Kingdom], SucHi: -12.15 δ‰ [Tate and Lyle, London, United Kingdom], SucLo: -26.29 δ‰ [Silver Spoon, Peterborough, United Kingdom]). All were measured by elemental analyser isotope ratio mass spectrometry (Europa Scientific, 20-20, Iso-Analytical Ltd, Crewe, UK). In both LacLo+G and SucLo+G, 2.4 g (2 %) of U-^{13}C glucose tracer (Cambridge Isotope Laboratories, MA, USA) was added to the low ^{13}C CHO of each condition to permit calculation of PGO. All outcome measures, such as $\dot{V}O_2$ and $\dot{V}CO_2$ were obtained from

the LacLo+G and SucLo+G trials. High ^{13}C CHO trials were used to quantify the shift in breath $^{13}CO_2$ associated with exogenous CHO oxidation. Low ^{13}C CHO trials were used exclusively to quantify the background shift in breath $^{13}CO_2$ (See Figure 4.1).

4.3.8 Plasma analyses

Gas chromatography-mass spectrometry (GC-MS) was used to determine plasma ^{13}C glucose enrichment (measured as tracer/tracee ratio (TTR)) using a butaneboronic acid (BBA) derivatisation method. Samples were deproteinized, by adding 150 µL of distilled water (with added hydrochloric acid to achieve a pH of 2) to a 50 µL plasma aliquot in a glass vial and mixed on a plate shaker. 3 ml of methanol:chloroform (2.3:1) was added to each sample and placed on a plate shaker for 5 minutes. Samples were then centrifuged (Heraeus Multifuge X1R, Thermo Scientific, Loughborough, UK) at 4 °C and 1780 g for 15 minutes. The supernatant was separated and placed in a new glass tube. 2 ml of chloroform and 1 ml of pH2 distilled water were then added, followed by 15 minutes on a plate shaker. Samples were then centrifuged again at 4 °C and 1780 g for 15 minutes and the resulting supernatant separated into a new glass tube. The samples were then dried in a heated nitrogen evaporator (MULTIVAP 118, Organomation Associates Inc, Berlin, MA, USA) at 40 °C until dry (~3 h). To derivatise the samples, 150 µL of BBA (10 mg in 1 ml of pyridine) was added to each sample, which were placed on a plate shaker for 15 minutes before incubation at 95 °C for 30 minutes. 150 µL of acetic anhydride was then added to each sample, before being placed on a plate shaker for 90 minutes. The samples were then dried under nitrogen at 40 °C (~45 minutes). Samples were prepared for the GC-MS by adding 150 µL of ethylacetate and mixing for 10 minutes. These were further diluted with ethylacetate

(10:1). 2 µL of glucose derivative was injected into the GC with a split ratio of 1:15 and vaporised at 250 °C. The peak areas for the ions *m/z* 297 (natural glucose) and 303 (^{13}C glucose) were measured using an Agilent 6890N GC with 5973N MS (Agilent Technologies, Stockport, UK). A standard curve of 8 samples was generated using $^{12}C_6$ glucose (Sigma-Aldrich, Gillingham, Dorset, UK) and U-^{13}C glucose tracer, with TTRs from 0.000 to 0.052. Each sample was analysed in biological duplicate and repeated if the coefficient of variation between the two samples exceeded 10%. Enrichment was calculated and expressed as TTR.

4.3.9 Breath sample analysis

$^{13}CO_2/^{12}CO_2$ ratio of expired breath samples was determined using gas chromatography isotope ratio mass spectrometry (Europa Scientific Hydra 20-20, Iso-Analytical Ltd, Crewe, UK).

4.3.10 Calculations

Total carbohydrate and fat oxidation were calculated using the following equations (Frayn, 1983), assuming protein oxidation to be negligible. $\dot{V}CO_2$ and $\dot{V}O_2$ were measured in L·min^{-1} and substrate oxidation rates in g·min^{-1}.

$$\text{total CHO oxidation} = 4.55 \cdot \dot{V}CO_2 - 3.21 \cdot \dot{V}O_2$$

$$\text{total fat oxidation} = 1.67 \cdot \dot{V}O_2 - 1.67 \cdot \dot{V}CO_2$$

Exogenous carbohydrate oxidation rates in g·min^{-1} were calculated using the equation below, where δExp is the ^{13}C enrichment of expired air at specific time-points, δExp$_{bkg}$ is the ^{13}C enrichment of expired air at the corresponding time-point from the low ^{13}C condition, δIng is the ^{13}C enrichment of the ingested CHO and k is the volume of CO_2 liberated from the complete oxidation of 1 g of glucose (0.7467 L). Where low ^{13}C conditions were not completed by participants, the mean ^{13}C enrichment of expired air at the corresponding time-point from other participants was used in the calculations.

$$\text{exogenous CHO oxidation} = \dot{V}\text{CO}_2 \times \left(\frac{\delta Exp - \delta Exp_{bkg}}{\delta Ing - \delta Exp_{bkg}}\right) \times \left(\frac{1}{k}\right)$$

Endogenous CHO oxidation rates were calculated by subtracting exogenous CHO oxidation rates from total CHO oxidation rates. PG oxidation (in g·min^{-1}) was calculated using the equation below, in which δPG is the PG ^{13}C enrichment and δPG$_{bkg}$ is the PG ^{13}C enrichment before exercise. Calculation of PG oxidation used TTR, and δ^{13}C ‰ versus PDB was converted to TTR to calculate exogenous CHO oxidation.

$$\text{plasma glucose oxidation} = \dot{V}\text{CO}_2 \times \left(\frac{\delta Exp - \delta Exp_{bkg}}{\delta PG - \delta PG_{bkg}}\right) \times \left(\frac{1}{k}\right)$$

TTR was calculated from δ13C ‰ versus PDB using the below equation (Slater et al., 2001).

$$\text{TTR} = [(\delta^{13}\text{C‰}/1000) + 1] \times 0.112372$$

Because PG oxidation is comprised of glucose derived from the liver (from glycogenolysis and gluconeogenesis) and exogenous carbohydrate from the gut, muscle glycogen and LGO can be derived from the following formulae:

$$\text{liver glycogen oxidation} = \text{plasma glucose oxidation} - \text{exogenous CHO oxidation}$$

$$\text{muscle glycogen oxidation} = \text{total CHO oxidation} - \text{plasma glucose oxidation}$$

4.3.11 Statistics

Data are presented as means ± standard deviation (SD), except for mean exogenous CHO oxidation is presented as the median ± the interquartile range. Mean exogenous CHO oxidation was analysed using the non-parametric Mann Whitney U test (Microsoft Excel, version 16.53) with an alpha level of 0.05. Other outcomes could not be analysed statistically due to the small sample sizes, and lack of paired comparisons within participants. Therefore, qualitative comparisons between conditions should be understood in the context of non-paired data.

4.4 Results

4.4.1 Breath $^{13}CO_2$ enrichment and exogenous CHO oxidation

Mean resting breath $^{13}CO_2$ enrichment ranged from -26.30 ‰ to -25.19 ‰. Breath $^{13}CO_2$ enrichment increased substantially in the conditions with the U-^{13}C glucose

tracer (LacLo+G and SucLo+G), with a smaller increase in the conditions with natural high abundance CHOs (LacHi and SucHi), and a small shift in the background conditions (LacLo and SucLo).

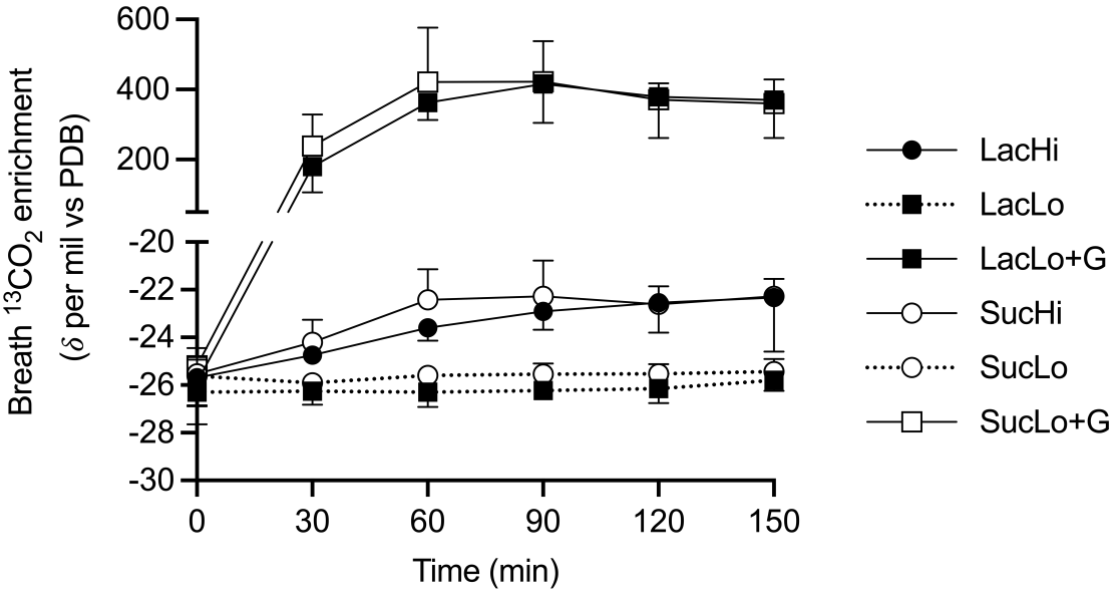

Figure 4.2 Change in breath $^{13}CO_2$ enrichment during 150 min of cycling at 50% W_{max}, ingesting either lactose (●) or sucrose (○), with high natural abundance ^{13}C substrate (—), low natural abundance ^{13}C substrate (⋯), or with a U-^{13}C glucose tracer (■, □). PDB; Pee Dee Bellemnitella.

Mean exogenous CHO oxidation (60-150 min) was similar with lactose (n=5) and sucrose ingestion (n=3), 0.22 ± 0.11 g·min^{-1} and 0.22 ± 0.05 g·min^{-1}, respectively (see Figure 4.3).

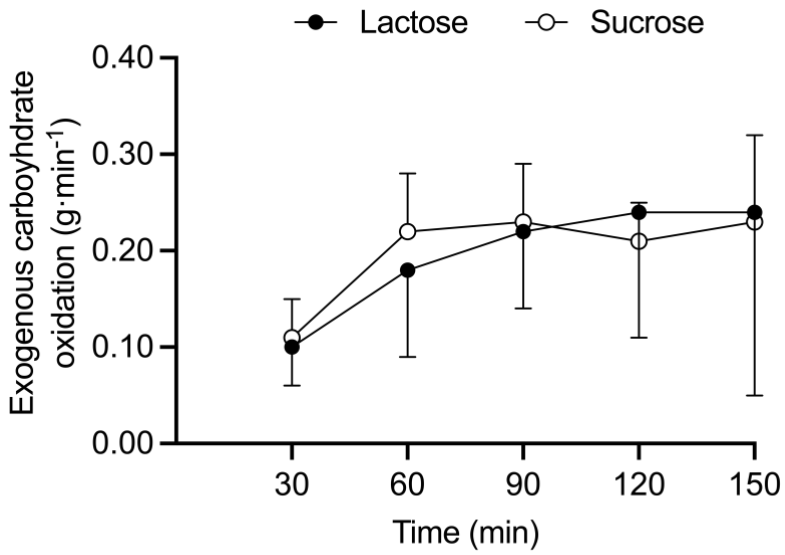

Figure 4.3 Exogenous carbohydrate oxidation rates between 30 and 150 min of cycling at 50% W_{max}, ingesting either lactose (●; n=5) or sucrose (○; n=3). Values are means ± SD.

Mean (60-150 min) exogenous CHO oxidation was not significantly different with lactose (0.15 ± 0.14 g·min^{-1}; *n*=5) or sucrose (0.24 ± 0.04 g·min^{-1}; *n*=3) ingestion (medians ± interquartile range; *P*=0.82), with three paired comparisons.

4.4.2 Plasma glucose oxidation

Plasma ^{13}C glucose enrichment is shown in Figure 4.4. Mean PG oxidation appeared higher with sucrose ingestion (1.30 ± 0.07 g·min^{-1}; n=3) than lactose ingestion (0.93 ± 0.31 g·min^{-1}; n=4).

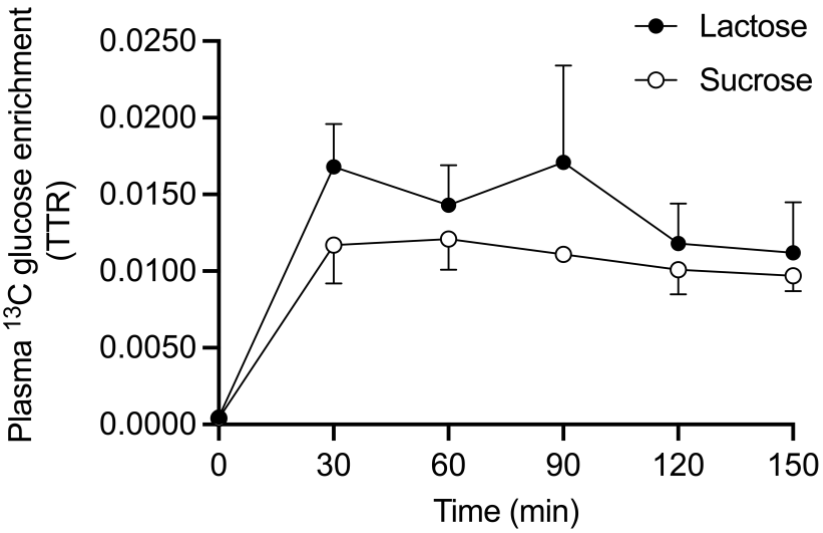

Figure 4.4 Plasma glucose tracer:tracee ratio (TTR) over 150 min of cycling at 50% Wmax, ingesting either lactose (●; n=4) or sucrose (○; n=3). Values are means ± SD.

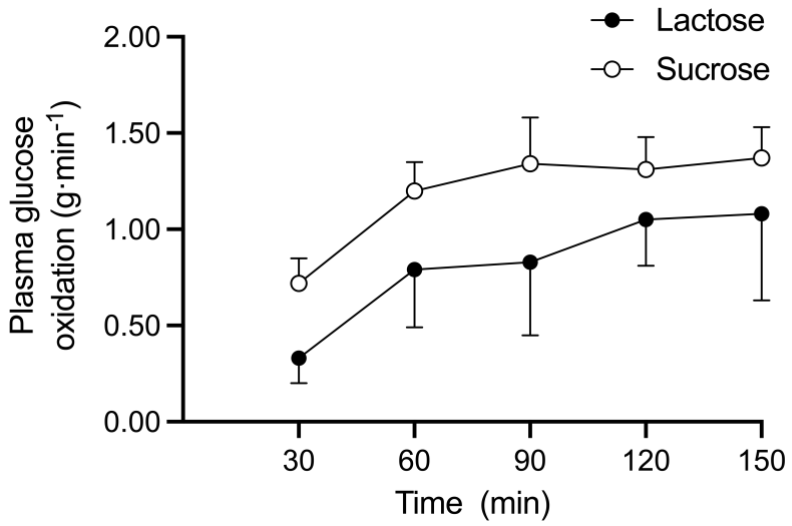

Figure 4.5 Plasma glucose oxidation rates between 30 and 150 min of cycling at 50% Wmax, ingesting either lactose (●; n=4) or sucrose (○; n=3).

4.4.3 Muscle and liver glycogen oxidation

MGO appeared slightly higher with lactose ingestion (0.98 ± 0.30 g·min^{-1}; n=4) than sucrose ingestion (0.75 ± 0.27 g·min^{-1}; n=3).

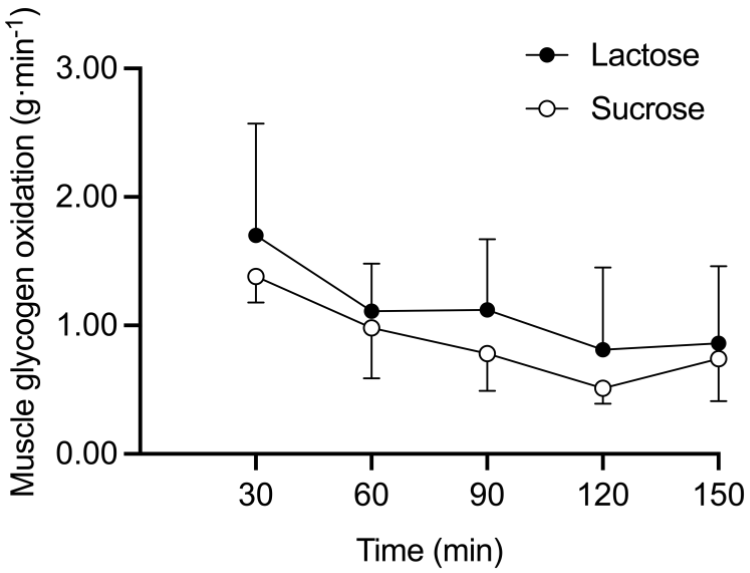

Figure 4.6 Muscle glycogen oxidation over 150 min of cycling at 50% W_{max}, ingesting either lactose (●; n=4) or sucrose (○; n=3).

In the single participant for whom all conditions were completed, with all time-points, it was possible to parse out the sources of oxidised substrates, which are displayed as % of total energy expenditure (EE; Figure 4.7).

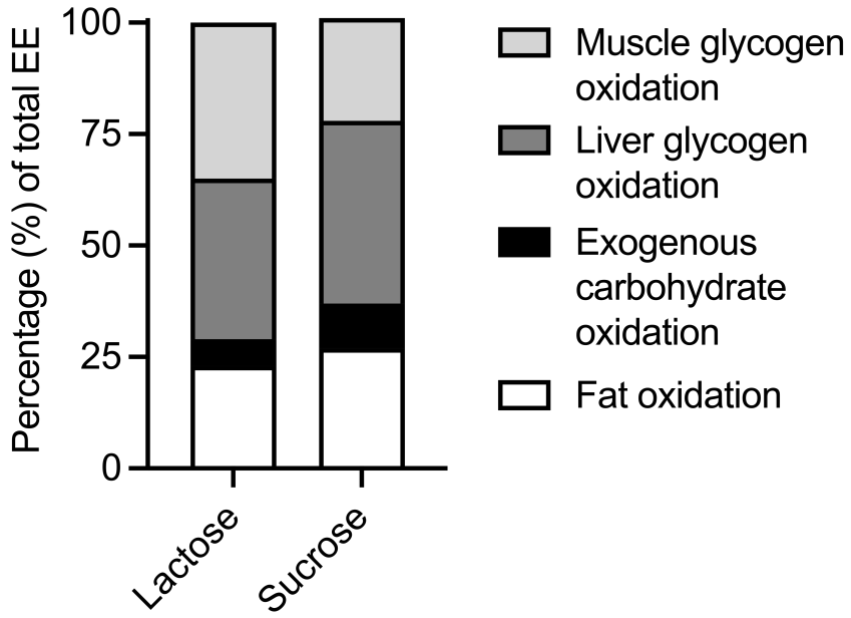

Figure 4.7 Substrate contributions to total energy expenditure (EE) from 60-150 min (n=1).

4.5 Discussion

In this study it was demonstrated that detailed substrate oxidation rates can be calculated using an ingested ^{13}C glucose tracer, with exogenous lactose or sucrose ingestion. This represents a development in the methods used for assessing CHO metabolism during exercise. This section will discuss the methods used, the strengths and weaknesses of the approach, as well as comparisons to other methods. There is limited scope for interpretation and discussion of the metabolic impacts of lactose or sucrose ingestion due to the limited sample size, but tentative qualitative discussion can be attempted.

There are a number of strengths to this modified method, compared both to previous approaches using tracers, but also other techniques for assessing muscle and liver glycogen. This approach allows detailed parsing out of substrate oxidation by increasing the number of experimental trials. Exogenous CHO oxidation is calculated using naturally high abundance ^{13}C CHOs, which are cheaper than synthesised ^{13}C tracers, and for lactose and sucrose, these tracers are prohibitively expensive. A separate trial is required for calculating exogenous CHO oxidation because the inclusion of a ^{13}C glucose tracer would prevent calculation of exogenous CHO oxidation given that the CHO is not exclusively glucose based. PG oxidation is calculated using a condition using a ^{13}C glucose tracer, combined with a low natural abundance CHO, which is required to prevent the CHO contributing to breath and plasma ^{13}C enrichment.

The addition of an authentic low ^{13}C CHO background correction condition instead of using a resting, pre-exercise background sample allows correction in the calculation of both PG oxidation and exogenous CHO oxidation. However, the impact of this correction is likely small as the background shift in breath $^{13}CO_2$ enrichment was small (~0.2-0.5 δ‰ *versus* PDB) compared to the change in breath $^{13}CO_2$ enrichment in the conditions with ^{13}C glucose tracer (~420 δ‰ *versus* PDB). However, the impact of an authentic background correction on exogenous CHO oxidation using naturally high abundance ^{13}C CHOs is much greater. This results in a more accurate estimation of LGO, which is calculated as exogenous CHO oxidation subtracted from PG oxidation. Therefore, if an authentic background correction is made for exogenous CHO oxidation, then the data can easily also be used in the PG oxidation calculation.

Alternative methodological approaches could have been used to assess the physiological outcomes of this study, that is, to determine whether lactose ingestion during exercise results in reduced LGO or MGO. Muscle biopsies are commonly used to assess muscle glycogen concentration, and have been used in similar studies to determine whether muscle glycogen is spared during exercise (Coyle et al., 1986, Hargreaves et al., 1984). Although they are more invasive than procedures such as phlebotomy, they are feasible in such research. However, biopsies of the liver to assess glycogen concentration are not feasible due to their highly invasive nature, with risk of serious complications including serious internal bleeding, organ perforation or sepsis. Both liver and muscle glycogen can be measured non-invasively using ^{13}C magnetic resonance (MR) spectroscopy, combined with MR imaging, and have been used to compare post-exercise muscle and liver glycogen content after ingestion of different CHOs during exercise (Gonzalez et al., 2015). However, cost is often prohibitively expensive for MR techniques. Use of infused tracers rather than oral tracers would also allow estimation of MGO and LGO. However, intravenous infusion carries a higher risk of complications, and may require medical supervision, which represents a barrier to its use in some institutions. Therefore, this method is less expensive and less invasive than alternative approaches to answer the same research question.

However, this method does have a number of limitations. The number of experimental trials required is higher than previous studies assessing MGO and PG oxidation using oral tracers (Wallis et al., 2007), due to both the requirement for an authentic low ^{13}C background correction condition, and an additional trial to assess exogenous CHO oxidation separately to PG oxidation.. Measurement of exogenous CHO oxidation and

PGO in two CHO types (or doses) could be performed with just three trials (one per CHO condition with a ‘water-correction’ condition), provided both CHOs conditions are exclusively glucose-based. Both biopsy and MR methods would have required as few as two total trials to compare muscle and liver glycogen with ingestion of two CHO types or doses. However, the current study required six. This is a substantial increase in time commitment and burden for both participant and researcher and could affect participant recruitment.

This approach also assumes that the metabolic response to the conditions is consistent. Although the experimental trials were identical with regard to time of day, exercise duration and intensity, and involved replication of food and drink intake, as well as exercise patterns in the 48 hours preceding trials, there may be some variation. Subtle changes in cardiorespiratory fitness over the 7-week study period may manifest as marginally different metabolic responses to the exercise bouts. Similarly, there may be an effect of differences in sleep between visits which could impact metabolism (Konishi et al., 2013). Other minor differences between conditions, including blood sampling being performed and the presence of the U-^{13}C glucose tracer in the beverage in LacLo+G and SucLo+G could also contribute to variation between conditions. Cycling cadence was self-determined by participants and was not measured or replicated between trials, though it has been shown to influence substrate utilization (Beneke and Alkhatib, 2015). Such inconsistencies between conditions could contribute to inaccurate estimations of the metabolic processes.

This method has a further limitation in that it does not assess the contribution of lactate and other 3C compounds from exogenous CHO, in this case the fructose from sucrose.

Exogenous fructose results in both glucose and lactate production (Dietze et al., 1976), both of which can be oxidised by the working muscle. The oxidation of exogenous lactate is captured in the estimation of exogenous CHO oxidation because both the fructose and glucose molecules are naturally abundant in ^{13}C and result in $^{13}CO_2$ production when oxidised. However, the use of a ^{13}C glucose tracer without a ^{13}C lactate tracer means that it is not possible to trace lactate oxidation, and there is no contribution to breath $^{13}CO_2$ production in the U-^{13}C glucose tracer conditions. Therefore, the measure of PG oxidation likely underestimates the contribution of exogenous CHO to energy demands. This may lead to a slight underestimation of LGO and overestimation of MGO in the sucrose condition, though the tracing of plasma glucose likely accounts for the majority of exogenous substrate from sucrose ingestion. This limitation may be even less impactful in the lactose condition, as galactose predominantly results in glucose production in the liver, rather than 3C compounds such as lactate (Coss-Bu et al., 2009).

Although the exogenous CHO oxidation equation has an authentic low ^{13}C background correction, the PG oxidation equation could also use an authentic correction for plasma ^{13}C glucose enrichment, if additional blood samples were collected in the low ^{13}C CHO conditions, instead of using a pre-exercise resting sample. However, any shift in plasma glucose ^{13}C enrichment with the ingestion of a low ^{13}C CHO would be very small, possibly requiring a more sensitive method of measurement such as IR-MS. The additional participant burden of collecting blood samples and analysis costs in these conditions would likely not be justifiable.

This chapter has demonstrated the feasibility of the method, but in this form, cannot answer the physiological question. Due to the limited sample size, there is limited ability to assess metabolic or physiological differences between conditions. However, tentative qualitative discussion can be attempted. Mean exogenous CHO oxidation rates were identical with lactose and sucrose ingestion (0.22 $g \cdot min^{-1}$), which is in accordance with the results of Chapter 3. However, the rates observed in this chapter were less than half those observed in Chapter 3 for lactose and sucrose (0.56 ± 0.19 $g \cdot min^{-1}$ and 0.61 ± 0.10 $g \cdot min^{-1}$, respectively), despite identical mean ingestion rates (0.80 $g \cdot min^{-1}$), relative exercise intensity (50% W_{max}) and similar absolute workloads (147 W compared to 142 W in Chapter 3). The reason for this disparity is unclear. Breath $^{13}CO_2$ enrichment changed to a similar extent in this Chapter and Chapter 3, with ingestion of similarly enriched CHOs, both in low and high ^{13}C CHO conditions (see Figure 4.2). There is marked variability between individuals in exogenous CHO oxidation rates, with mean exogenous CHO oxidation rates with lactose ingestion in Chapter 3 ranging from 0.15-0.85 $g \cdot min^{-1}$. Therefore, it is possible that the small number of participants recruited in this study were dominated by individuals on the lower end of this range.

PG oxidation appeared higher with sucrose ingestion than lactose which suggests that lactose ingestion reduced hepatic glucose output compared to sucrose. This may contribute to liver glycogen sparing, though LGO could only be calculated in one participant in both conditions (see Figure 4.7). MGO appeared slightly higher with lactose ingestion compared to sucrose, despite the higher total CHO oxidation observed with sucrose ingestion in Chapter 3 and this chapter. Therefore, these data

suggest that the glycogen sparing observed with lactose ingestion is likely hepatic in origin.

In summary, this study has demonstrated that PG oxidation rates can be calculated using an oral ^{13}C glucose tracer, with the ingestion of lactose and sucrose. Furthermore, detailed parsing out of muscle and LGO is possible using this approach. Limited qualitative assessment of the physiological objectives was possible, which suggest that PG oxidation was lower with lactose ingestion, which may result in reduced LGO. Exogenous CHO oxidation rates with lactose and sucrose were lower than observed in Chapter 3, but the CHOs were similar in both studies.

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Chapter 5: The effect of combined lactose and fructose ingestion on post-exercise liver glycogen synthesis

5.1 Abstract:

Liver glycogen is important for blood glucose control during exercise and increasing liver glycogen storage post-exercise can improve subsequent exercise capacity. Combining glucose with fructose or galactose enhances post-exercise liver glycogen synthesis above glucose alone. The aim of this study was to investigate the effect of lactose and fructose (LAC+FRU) ingestion, providing glucose, fructose and galactose, on liver glycogen synthesis, compared to sucrose (SUC), providing glucose and fructose. 10 cyclists and triathletes completed two experimental trials, each involving a 93-minute intermittent cycling exercise bout, followed by combined magnetic resonance imaging and spectroscopy (MRI/S) to assess liver glycogen content. Participants ingested 1.5 $g \cdot kg^{-1} \cdot h^{-1}$ of LAC+FRU or SUC over 4-hours of recovery, alongside two boluses of 0.4 $g \cdot kg^{-1}$ of whey protein. Blood samples were taken hourly, and a second MRI/S measurement made after 4-hours. Liver glycogen content increased from baseline to post-exercise in LAC+FRU (36 ± 11 g to 79 ± 17 g) and SUC (36 ± 16 g to 70 ± 23 g), but this did not differ between conditions (both P=0.39). There was no significant difference in the change in liver glycogen content from baseline in LAC+FRU (43 ± 14 g) and SUC (34 ± 17 g; P=0.098). SUC resulted in higher plasma [glucose] and [insulin] (6.3 ± 0.8 $mmol \cdot L^{-1}$, 105 ± 58 $\mu U \cdot L^{-1}$ respectively) than LAC+FRU (5.5 ± 0.6 $mmol \cdot L^{-1}$, 61 ± 34 $\mu U \cdot L^{-1}$, both P<0.05) from 60-240 min. LAC+FRU resulted in higher plasma [lactate] (2.5 ± 0.4 $mmol \cdot L^{-1}$) than SUC (2.0 ± 0.4

mmol·L^{-1}, *P*=0.001) from *t*=120 min. Gastrointestinal symptoms were similar in both conditions. In conclusion, a combination of ingested glucose, fructose and galactose was not clearly more effective at restoring liver glycogen after exercise than glucose and fructose.

5.2 Introduction:

Carbohydrate (CHO) is the main respiratory substrate for extended and high intensity exercise (Romijn et al., 1993, Bergman and Brooks, 1999). Endogenous CHO is stored as glycogen, in muscle tissue and in the liver (Taylor et al., 1996, Taylor et al., 1993). Muscle glycogen is found in three sites in the muscle, intermyofibrillar, intramyofibrillar and subsarcolemmal, all of which are utilised by the working muscle during exercise (Burke et al., 2017). Liver glycogen is responsible for blood glucose homeostasis through glycogenolysis which can be stimulated by glucagon and epinephrine (Taylor et al., 1996, Gonzalez et al., 2016). Postabsorptive liver and muscle glycogen contents are ~100 g and ~400 g respectively (Wasserman, 2009). However, glycogen content varies with nutrition and exercise, such that liver glycogen may range from close to 0 to 160 g and muscle glycogen from 300-700 g (Murray and Rosenbloom, 2018). Whilst athletes display higher resting muscle glycogen concentrations compared to the non-athletic population, this is not the case for liver glycogen (Gonzalez et al., 2016). Liver glycogen concentrations are much higher than muscle glycogen concentrations, with liver glycogen molecules being up to 100 times larger than muscle units, with up to ~50,000 glycosyl units (Adeva-Andany et al., 2016).

Glycogen stores are generally sufficient to provide glucose for exercise at moderate intensities for up to ~90 min, though at higher exercise intensities and/or longer durations glycogen availability can limit exercise capacity and performance (Burke et al., 2011). Because glycogen stores are so impactful on exercise performance, rapid restoration of glycogen stores is a priority, especially if subsequent exercise will be performed in a short time frame, for example in < 24 hours (Gonzalez et al., 2016). Rapid restoration of glycogen stores is particularly important for athletes training multiple times per day, tournament settings and competitions such as stage races. Muscle glycogen can be synthesised rapidly in a short timeframe (4-6 h) with ingestion of ≥1.2 $g \cdot kg^{-1} \cdot h^{-1}$ of CHO, whilst the addition of protein can increase muscle glycogen resynthesis when ingestion rates are suboptimal (Alghannam et al., 2018). However, remarkably less is known about optimal nutritional strategies for liver glycogen restoration, despite its link with subsequent exercise capacity (Casey et al., 2000). Accentuated restoration of post-exercise liver glycogen has been shown to increase exercise capacity in cycling and running (Maunder et al., 2018, Gray et al., 2020). Furthermore, in mice overexpressing protein targeting to glycogen with substantially increased liver glycogen content, exercise capacity was increased, alongside prevention of hypoglycaemia and higher end-exercise liver glycogen (Lopez-Soldado et al., 2021).

Whilst glucose alone is sufficient to maximise muscle glycogen resynthesis post-exercise, liver glycogen resynthesis is slow if glucose alone is ingested (Décombaz et al., 2011, Fuchs et al., 2016). However, the addition of a CHO that is metabolised by the liver – fructose or galactose – can roughly double rates of liver glycogen synthesis

post-exercise. Décombaz et al. (2011) demonstrated that with ingestion of ~0.93 $g \cdot kg^{-1} \cdot h^{-1}$ of glucose, glucose and fructose or glucose and galactose, over 6.5 h post-exercise, combined glucose and galactose, and combined glucose and fructose result in higher liver glycogen synthesis rates (8.6 ± 0.9 $g \cdot h^{-1}$ and 8.1 ± 0·6 $g.h^{-1}$ respectively) than glucose alone (3.7 ± 0.5 $g \cdot h^{-1}$). Similarly, Fuchs et al. (2016) found that ingestion of 1.5 $g \cdot kg^{-1} \cdot h^{-1}$ of sucrose (glucose and fructose) resulted in faster liver glycogen resynthesis (6.6 ± 3.3 $g \cdot h^{-1}$) than glucose (3.3 ± 3.0 $g \cdot h^{-1}$). Importantly, Fuchs et al. (2016) confirmed that ingestion of glucose and fructose (as sucrose) did not compromise muscle glycogen repletion rates at these ingestion rates, confirming previous work (Wallis et al., 2008, Casey et al., 2000). Although a handful of studies failed to observe an increase in liver glycogen synthesis with sucrose ingestion compared to glucose, these negative findings may be attributable to suboptimal ingestion rates, small sample sizes and methodological problems (Moriarty et al., 1994, Casey et al., 2000).

The additive effects of galactose and fructose over glucose on liver glycogen resynthesis are attributable to their requirement for hepatic metabolism. Glucose is a poor substrate for liver glycogen synthesis, both when infused (Nilsson and Hultman, 1973,) and ingested (Décombaz et al., 2011), with the majority entering the systemic circulation and synthesised to form muscle glycogen. In contrast, fructose is taken up by the liver where it is phosphorylated and converted to lactate and pyruvate, after which glucose-1-phosphate can be synthesised to undergo glycogenesis (Mayes, 1993). Although a potentially physiologically relevant amount of fructose enters the systemic circulation and escapes hepatic fructolysis, the absolute quantities are orders

of magnitude lower than glucose (Francey et al., 2019). Galactose is also taken up by the liver, where it enters the Leloir pathway for conversion into uridine diphosphate (UDP) glucose and glucose-1-phosphate (Holden et al., 2003). Approximately 67% of ingested galactose (when ingested in isolation) is converted to glucose via a direct (6C) pathway, whilst the remaining 33% is converted via indirect (3C) pathways (Coss-Bu et al., 2009). Although ingestion of galactose in isolation results in substantial increases in plasma [galactose], co-ingestion with glucose (either as lactose or the free monosaccharides) blunts this rise (Williams, 1986). This has been suggested to be attributable to enhanced splanchnic galactose extraction, possibly resulting in liver glycogen synthesis (Sunehag and Haymond, 2002). Because both galactose and fructose undergo hepatic metabolism resulting in substantial liver glycogen synthesis, it is possible that ingestion of both galactose and fructose, alongside glucose would result in greater liver glycogen synthesis than one such CHO alone, though this has not been investigated.

Therefore, aim of this study was to investigate the impact of ingesting two CHOs that are hepatically metabolised (fructose and galactose; alongside glucose), compared to one such CHO (fructose, alongside glucose) on liver glycogen synthesis after glycogen-depleting exercise. The hypothesis was that the provision of lactose and fructose (LAC+FRU) would accelerate liver glycogen synthesis, above the provision of sucrose alone (SUC).

5.3 Methods:

5.3.1 Participants

10 healthy, recreationally active male (mean ± SD: age, 28 ± 5 years; body mass, 75.6 ± 5.4 kg; height, 181.2 ± 1.7 cm; $\dot{V}O_2$peak, 52.4 ± 4.9 mL·min^{-1}·kg^{-1}; W_{max}, 327 ± 36 W, n=7) and female (mean ± SD: age, 33 ± 12 yr; body mass, 61.1 ± 1.5 kg; height, 173.7 ± 6.6 cm; $\dot{V}O_2$peak, 48.8 ± 0.7 min^{-1}·kg^{-1}; W_{max}, 259 ± 24 W, n=3) volunteers completed the study. Eligibility criteria were a $\dot{V}O_{2peak}$ of ≥ 40 ml·min^{-1}·kg^{-1}or ≥ 45 ml·min^{-1}·kg^{-1} for females and males, respectively, regularly performing aerobic exercise ≥3 times per week, and an absence of cardiometabolic disease, galactosemia and lactose intolerance. Participants provided their informed consent after having the purpose, risks and practical details explained to them in accordance with the Declaration of Helsinki. The study was approved by the Faculty of Medical Sciences Research Ethics Committee of Newcastle University (project number 2134/12045). Three participants withdrew from the study due to scheduling constraints; one was excluded in their first visit (SUC) due to vomiting during the recovery period and one was excluded after experiencing claustrophobia in the scanner, and these participants data were not included in the study.

5.3.2 Experimental Design

Participants completed 3 trials, including a screening visit, and 2 experimental trials, which were separated by at least 6 days. Female participants using monophasic contraception completed visits in the active pill consumption phase (n=2), and female participants not using hormonal contraception performed trials in the self-reported mid-

follicular phase of sequential menstrual cycles (n=1). Experimental trials involved participants completing 93-min glycogen-reducing exercise bout, followed by magnetic resonance spectroscopy (MRS) and magnetic resonance imaging (MRI) to determine liver glycogen concentration and volume. Participants then ingested isocaloric beverages over a 4-hour recovery period, containing either sucrose (SUC) or a combination of lactose and fructose (LAC+FRU), with two boluses of whey protein at *t*=0 and 120 min, in a randomised, single-blind manner. After the 4-hour recovery period liver glycogen concentration and volume was measured again. A baseline MRI/MRS measurement was not required because this exercise bout has been demonstrated to result in substantial reductions in liver and muscle glycogen (Detko et al., 2013, Bowtell et al., 2000) and the cost of an additional MRI/MRS measurement is substantial. Changes in breath H_2 were measured, and venous blood samples were taken every 60 minutes.

5.3.3 Screening visit

Height (Seca 213, Seca, Hamburg, Germany) and body mass (Seca 704, Seca, Hamburg, Germany) were measured, before beginning a step-incremental exercise test to exhaustion on a cycle ergometer (Velotron, RacerMate, Seattle, WA) to determine $\dot{V}O_{2peak}$ and W_{max}. The test began at 100 W and every 2 min increased by 30 W until volitional exhaustion. Heart rate (HR) was monitored throughout using radio telemetry (Polar FT7, Kempele, Finland). Gas exchange measurements were made continuously during exercise using an automated online gas analyser (Vyntus, Vyaire Medical, Mettawa, IL, USA) to measure $\dot{V}O_2$ and $\dot{V}CO_2$. The highest average $\dot{V}O_2$ over

30 s was taken to be $\dot{V}O_{2peak}$. W_{max} was calculated as the power output during the final stage completed, combined with the fraction of the time spent in the subsequent stage, multiplied by 30 W, as described in Chapter 3. After successful completion of the test, participants rested for 5-10 min, before undertaking a familiarisation exercise bout. This involved cycling at 55 % W_{max} for 20 min, followed by three 1 min bouts at 110 % W_{max} with 2 min of rest between each bout, and a further 16 min at 55 % W_{max} (45 min total). This protocol confirmed participants' ability to perform extended aerobic exercise and their suitability for performing subsequent glycogen-reducing exercise bouts. Upon completion of this second exercise bout, participants were included in the study.

5.3.4 Experimental trials

Participants attended the Newcastle Magnetic Resonance Centre at 07:00-07:30 in an overnight fasted state (10 h), having been asked to refrain from alcohol and caffeine ingestion, as well as vigorous intensity exercise for 24 h. They were asked to record and replicate their diet and exercise patterns for the 48h before experimental trials, which were analysed for energy and macronutrient intake (Myfitnesspal, San Francisco, USA). Participants began a standardised glycogen-reducing exercise bout on a cycle ergometer (Velotron, RacerMate, Seattle, WA; see Figure 5.1). Participants cycled for 30 min at 55% W_{max} , followed by six 1-min 'bursts' at double the intensity (110% W_{max}) separated by 2-min rest periods, before completing a further 45 min at 55% W_{max} (Bowtell et al., 2000). This protocol has a specific duration (93 min), unlike many glycogen depleting protocols, which have significant inter- and intra-individual variation (Fuchs et al., 2016). This exercise protocol has been shown to reduce both

liver (Detko et al., 2013) and muscle (Bowtell et al., 2000) glycogen. Water intake was provided ad libitum and the volume replicated in the subsequent trial (1.02 ± 0.20 L).

Post-exercise, participants were permitted a brief (<5 min) shower, before undergoing a post-exercise MRI and MRS measurement for ~45 min to determine liver volume and glycogen concentration. Participants then completed a gastrointestinal symptom (GIS) questionnaire and underwent a breath H_2 measurement (Hydrogenius, Bedfont Scientific Ltd, Maidstone, England). A catheter (Vasofix Safety 20G, B. Braun, Melsungen, Germany) was inserted into an antecubital forearm vein to allow repeated blood sampling using a 3-way stopcock (Discofix C, B. Braun, Melsungen, Germany) and a basal (10 ml) blood sample was taken. Participants then ingested the first CHO beverage and the first protein beverage (t = 0 min) and began the 4-hour recovery period, during which they remained seated. Venous blood samples were collected every 60 min. The GIS questionnaire was completed every 60 minutes, and a breath H_2 assessment was made at t= 120- and 240-min. CHO beverages were provided every 30 min, each delivering 0.75 $g \cdot kg^{-1}$ of CHO. Protein beverages (each delivering 0.4 $g \cdot kg^{-1}$ of protein) were provided at t= 0 and 120 min. After the 4-hour recovery period, participants underwent a second MRI and MRS measurement to determine changes in liver volume and glycogen concentration.

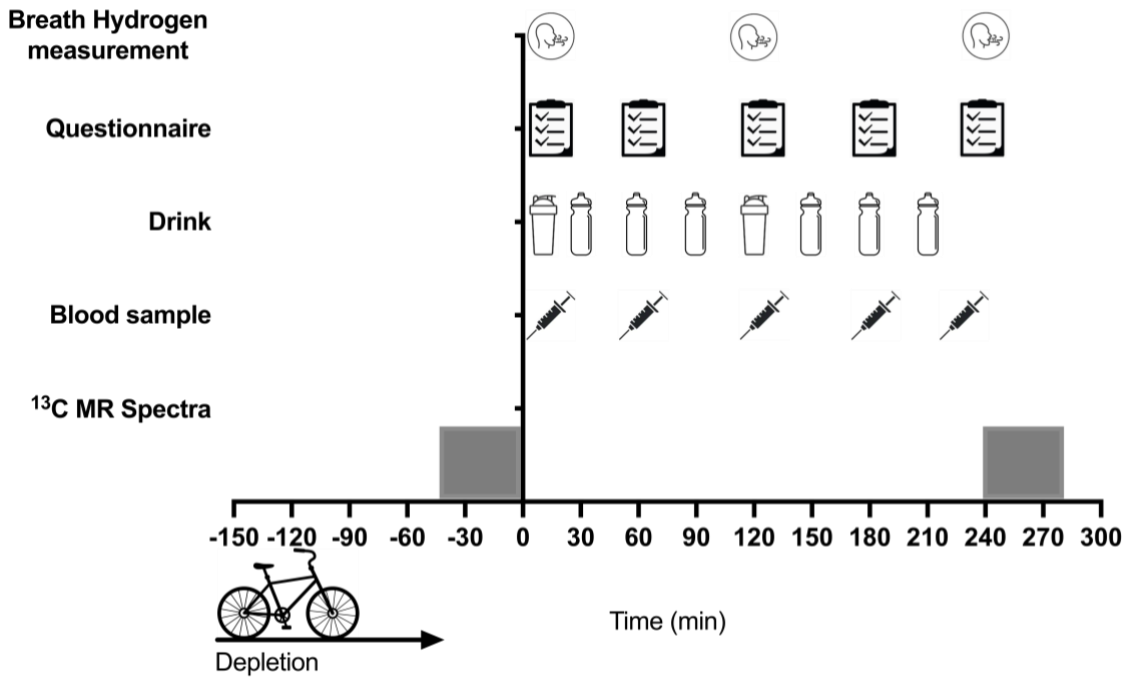

Figure 5.1 Schematic of the protocol. Glycogen depleting exercise was followed by measurement of liver volume and glycogen concentration using MRI and MRS post-exercise, and after a 240 min recovery period. Blood samples were taken, and GIS questionnaires complete

5.3.5 Beverages

CHO beverages delivered 1.5 $g \cdot kg^{-1} \cdot h^{-1}$ of total CHO. The SUC condition contained 1.5 $g \cdot kg^{-1} \cdot h^{-1}$ of sucrose, whilst the LAC+FRU delivered 0.75 $g \cdot kg^{-1} \cdot h^{-1}$ of fructose and 0.75 $g \cdot kg^{-1} \cdot h^{-1}$ of lactose (see Table 5.1). Ingestion of fructose (from sucrose or as free fructose) at a rate of 0.75 $g \cdot kg^{-1} \cdot h^{-1}$ is likely sufficient to maximise liver glycogen resynthesis from this monosaccharide (Gonzalez et al., 2017). Therefore, the additional ingestion of 0.375 $g \cdot kg^{-1} \cdot h^{-1}$ of galactose directly examines the effect of the addition of a second hepatically metabolised CHO. Although this replaced an equivalent amount of glucose in the LAC+FRU condition this was deemed unlikely to

compromise liver glycogen synthesis, though may have affected muscle glycogen synthesis. CHOs were of low natural abundance ^{13}C to minimise differences in the carbon isotope ratios, with sucrose (–26.65 δ‰ vs Pee Dee Bellemnitella; Silver Spoon, Peterborough, UK) and fructose (–26.25 δ‰; Peak Supps, Bridgend, UK) derived from sugar beet, and lactose (–26.46 δ‰; Volac International, Royston, UK) from predominantly grass-fed cows.

A separate protein beverage containing 0.4 $g \cdot kg^{-1}$ of sugar-free whey protein concentrate (Volac International, Royston, UK) bolus was provided at *t*=0 and *t*=120 min. Whey protein is known to increase the insulin response to CHO ingestion (Jentjens et al., 2001), and this dose is sufficient to maximise muscle protein synthesis (Moore, 2019). CHO beverages were prepared as an 18% solution and flavoured to taste (Flavdrops™, Myprotein, The Hut Group, Cheshire, UK) and protein beverages as a 15% solution (w/v).

Table 5.1 Macronutrient and energy compositions of beverages

Condition	Fructose ($g \cdot kg^{-1} \cdot h^{-1}$)	Glucose ($g \cdot kg^{-1} \cdot h^{-1}$)	Galactose ($g \cdot kg^{-1} \cdot h^{-1}$)	Protein ($g \cdot kg^{-1}$)	Energy content ($kcal \cdot kg^{-1} \cdot h^{-1}$)
SUC	0.750*	0.750*	0.000	0.40	7.6
LAC+FRU	0.750	0.375†	0.375†	0.40	7.6

* - represents monosaccharides from sucrose

† - represents monosaccharides from lactose

5.3.6 Gastrointestinal discomfort and breath H_2

The GIS questionnaire was comprised of a 16 item list of GIS divided into upper GI symptoms (stomach problems, vomiting, belching, stomach burn, bloating and stomach cramps), lower GI symptoms (flatulence, urge to defecate, intestinal cramps, diarrhoea and side aches on the left and right), and other symptoms (nausea, dizziness, headache and urge to urinate), scored on a 1-10 scale, as described previously (Fuchs et al., 2016). Breath H_2 concentration was measured in duplicate at *t*=0, 120 and 240. Two participants were unable to ingest all of the CHO beverages in their first trials (both SUC) due to a high urge to vomit (5-8 out of 10). 298 ml and 546 ml of the final beverage (s) were not ingested resulting in 53.6 g (0.70 $g \cdot kg^{-1}$) and 98.3 g (1.38 $g \cdot kg^{-1}$) of CHO not being consumed, respectively. This reduction was replicated in the subsequent trial, with participants experiencing negligible urge to vomit (both 1 out of 10) with LAC+FRU. The CHO ingestion rates for these participants, although lower than 1.5 $g \cdot kg^{-1} \cdot h^{-1}$, were still in line with previous investigations into liver glycogen synthesis (≥ 1.2 $g \cdot kg^{-1} \cdot h^{-1}$), which resulted in high rates of liver glycogen synthesis, and so the inclusion of these participants should not affect the primary outcomes.

5.3.7 Measurement of liver volume and calculation of liver glycogen

MRI and MRS measurements were made using a Philips Achieva 3T whole body scanner (Philips Medical Systems, Best, The Netherlands) and a custom-built $^{13}C/^{1}H$ surface coil comprising of a 12cm diameter circular ^{13}C surface coil and two oval ^{1}H coils operating in quadrature. Subjects lay in a supine position in the scanner and the $^{13}C/^{1}H$ coil was placed over the liver. ^{1}H scout images were acquired to confirm coil positioning and the position was marked on the participants skin to aid coil repositioning for the subsequent scan. The correct coil location was confirmed by

comparison of scout ^{1}H images. The ^{13}C spectra were acquired over 10 minutes using a non-localised ^{1}H decoupled ^{13}C pulse- pulse acquisition sequence (TR 250 ms, spectral width 8 kHz, 2,400 averages, ^{1}H WALTZ decoupling with a nominal ^{13}C tip angle of 130°). ^{1}H decoupling was applied for 60 % of the ^{13}C signal acquisition to allow a relatively fast TR of 250 ms to be used within the specific absorption rate safety limitations. A ^{13}C flip angle calibration over 4 nominal flip angles (25°, 50°, 100° and 200°) was also acquired by observing the variation in signal from a fiducial marker located in the coil housing, containing a sample exhibiting ^{13}C signal with short T_1 (213 mM [2-^{13}C]-acetone and 25 mM $GdCl_3$ in water).

Tissue glycogen concentration was calculated from the area of the C1-glycogen ^{13}C signal using the java-based magnetic resonance user interface (jMRUI version 4.0, Universitat Autònoma de Barcelona, Spain) with the advanced method for accurate, robust and efficient spectral fitting non-linear least square fitting algorithm and in-house software in Matlab (Mathworks, Natick, MA, USA). For each subject the separation between radiofrequency coil and liver tissue was measured from the ^{1}H scout images, and ^{13}C coil loading assessed from the ^{13}C flip angle calibration data for each subject. Tissue glycogen concentration was determined by comparison of glycogen signal amplitude to spectra acquired from a liver phantom filled with aqueous solutions of glycogen (100 mM) and potassium chloride (70 mM). Phantom data were acquired at a range of flip angles and separation distances between the coil and phantom. Quantification of each human ^{13}C spectrum employed a phantom dataset matched to body geometry and achieved flip angle so that differences in coil sensitivity profile and loading were accounted for each subject. Slice thickness was 10 mm with a 0 mm gap,

and the area of each slice was measured in ImageJ (National Institutes of Health, Bethesda, Maryland, USA). The areas of all slices was added, and multiplied by the slice thickness. The total number of liver slices used for liver volume measurement was 18, which varied between participants due to anatomical differences. Liver glycogen content was calculated by multiplying liver glycogen concentration and liver volume, with millimolar units converted to grams using the molar mass of a glycosyl unit (162 $g.M^{-1}$). Both liver glycogen volume and concentration were analysed in a double-blinded manner.

5.3.8 Plasma analyses

Blood samples (10 ml) were collected into EDTA-containing vacutainers (Becton Dickinson, Helsingborg, Sweden) were stored on ice and then centrifuged at 3000 rpm for 10 min at 4°C, and frozen at –70°C for later analysis. Plasma [insulin] was measured using an enzyme-linked immunosorbent assay (Insulin Human ELISA Kit #KAQ1251, Invitrogen, Paisley, UK). Plasma [glucose] and [lactate] were measured using an automated photometric clinical chemistry analyser (RX Daytona+, Randox, London, UK) and commercially available kits (Glucose kit, Lactate kit, Randox). Due to issues with blood sampling in one participant, data are *n*=9 for all plasma analyses.

5.3.9 Statistics

Sample size was calculated using G*Power (Franz Faul, Universitat Kiel, Germany), based on a previous study investigating the effect of ingesting an additional

carbohydrate source that is preferentially metabolised by the liver (Fuchs et al., 2016). The expected effect size was calculated from the difference in liver glycogen synthesis rates after sucrose ingestion (6.6 $g.h^{-1}$ ± 3.3 $g \cdot h^{-1}$) compared to glucose (3.3 ± 3.0 $g \cdot h^{-1}$). A sample size of 10 in a crossover design would provide statistical power of >80% statistical power with an α-level of 0.05.

Two-way repeated measures analysis of variance (ANOVA) was used to assess the changes in liver glycogen concentration, liver volume and liver glycogen content, breath H_2, plasma [glucose], [lactate] and [insulin] between conditions and over time, with Bonferroni post-hoc tests used to locate differences. If normality assumptions were violated, the data were log transformed and analysed, with the raw data expressed in text and in figures, and statistical reporting referring to the log transformed data. If the assumption of sphericity was violated, the Greenhouse-Geisser correction was employed. Liver glycogen concentration repletion rates, liver glycogen content, liver glycogen synthesis, total GIS and other GIS were analysed using paired T-tests, whilst upper and lower GIS data were analysed using Wilcoxon signed rank tests. Data are expressed as means ± SD unless otherwise stated. Data were analysed using SPSS Statistics for Macintosh, Version 27.0 (IBM Corp., Armonk, NY) or Excel Version 16.53 (Microsoft, Redmond, WA). The alpha level was set as $P<0.05$. Cohen's D was calculated as previously described (Cohen, 1988) for comparisons between two conditions, with the pooled variance used for within-subject comparisons.

5.4 Results:

5.4.1 Liver glycogen concentration

There was a significant main effect of time (P<0.001; η^2_p: .91), with both LAC+FRU and SUC increasing liver glycogen concentration from t=0 to 240 (both P<0.001; see Table 5.2). There was no main effect of condition (P=0.62; η^2_p: 0.03) or interaction condition*time (P=0.21; η^2_p: 0.17). Liver glycogen concentration repletion rates were not significantly different between LAC+FRU (35 ± 12 mmol·L^{-1}·h^{-1}) and SUC (29 ± 14 mmol·L^{-1}·h^{-1}; P=0.21; d=0.44).

5.4.2 Liver glycogen volume

There was a significant main effect of time (P<0.001; η^2_p: 0.71), with both LAC+FRU and SUC increasing liver volume from t=0 to 240 (both P=0.001; see Table 5.2). There was no main effect of condition (P=0.32; η^2_p: 0.11) or interaction condition*time (P=0.12; η^2_p: 0.25).

Table 5.2 Liver glycogen concentration and volume

	Time (min)	
	0	240
Liver glycogen concentration (mmol·L^{-1})		
LAC+FRU	143 ± 39	283 ± 60†
SUC	143 ± 69	261 ± 85†
Liver glycogen volume (ml)		
LAC+FRU	1 553 ± 158	1 723 ± 198†
SUC	1 558 ± 148	1 671 ± 211†

Values are means ± SD· Liver glycogen concentration (mmol·L^{-1}) and liver volume (ml) at *t*=0 post-exercise and *t*=240 with ingestion of 1.5 g·kg^{-1}·h^{-1} of either lactose and fructose (LAC+FRU) or sucrose (SUC), both *n*=10. †; significantly different from baseline (*P*≤0.001)

5.4.3 Liver glycogen content

There was a significant main effect of time (*P*<0.001), with both LAC+FRU and SUC increasing liver glycogen content from *t*=0 to 240 (both *P*=0.001; see Figure 5.2). There was no main effect of condition (*P*=0.39) or interaction condition*time (*P*=0.43). Liver glycogen synthesis rates were not significantly different between LAC+FRU (10.7 ± 3.4 g·h^{-1}) and SUC (8.6 ± 4.3 g·h^{-1}; *P*=0.10; *d*= 0.53). There was no significant difference in the change in liver glycogen content from 0 min to 240 min in LAC+FRU (43 ± 14 g, 95% CIs 34.3-51.7) and SUC (34 ± 17 g, 95% CIs 23.5-44.5; *P*=0.098; η^2_p: 0.53; See Figure 5.3).

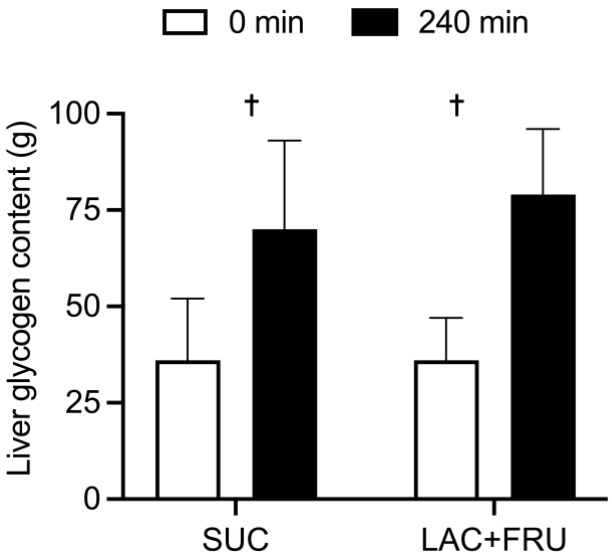

Figure 5.2 Liver glycogen content before (0 min) and after (240 min) a 4-hour post-exercise recovery period, with ingestion of either 1.5 g·kg^{-1}·h^{-1} of either sucrose (SUC) or lactose and fructose (LAC+FRU).

† - represents a significant difference from baseline values (P<0.05).

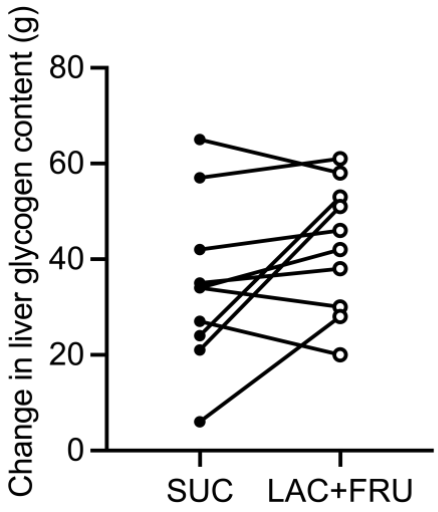

Figure 5.3 Individual changes from baseline in liver glycogen content after a 4-hour post-exercise recovery period, with ingestion of either 1.5 g·kg^{-1}·h^{-1} of either sucrose (SUC) or lactose and fructose (LAC+FRU).

5.4.4 Plasma analyses

Plasma [glucose] from 60-240 min was significantly higher in SUC (6.3 ± 0.8 mmol·L^{-1}) than LAC+FRU (5.5 ± 0.6 mmol·L^{-1}) with a main effect of condition (P=0.04; η^2_p: 0.53), time (P=0.017; η^2_p: 70) and interaction (P=0.046; η^2_p: 0.34; see Figure 5.4A). Plasma [lactate] was significantly higher in LAC+FRU (2.5 ± 0.4 mmol·L^{-1}) than SUC (2.0 ± 0.4 mmol·L^{-1}), from 120 min onwards with a main effect of condition (P=0.001; η^2_p: 0.76), time (P<0.001; η^2_p: 0.69) and interaction (P=0.027; η^2_p: 0.38; see Figure 5.4B). Plasma [insulin] was significantly higher in SUC (105 ± 58 µU·L^{-1}) than LAC+FRU (61 ± 34 µU·L^{-1}), from 60 min onwards with a main effect of condition (P=0.002; η^2_p:0.73), time (P=0.002; η^2_p: 0.62) and interaction (P=0.033; η^2_p:0.37; see Figure 5.4C).

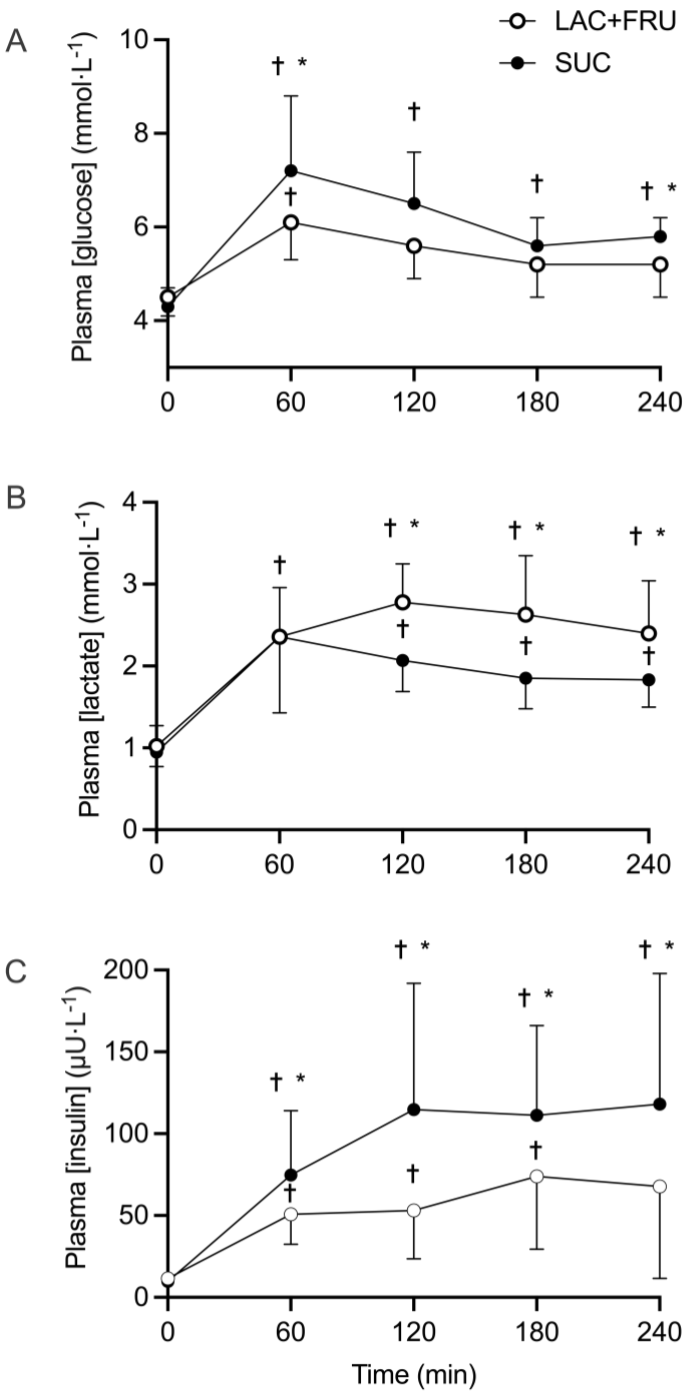

Figure 5.4 Plasma glucose (A), lactate (B) and insulin (C) concentrations over 4 hours of post-exercise recovery, with ingestion of either 1.5 $g \cdot kg^{-1} \cdot h^{-1}$ of either sucrose (SUC) or lactose and fructose (LAC+FRU).

† - represents a significant difference from baseline values (P<0.05). * - represents a significant difference between conditions at this time-point (P<0.05).

5.4.5 GIS and breath H_2

There were no significant differences between LAC+FRU and SUC in total GIS (96 ± 26 and 90 ± 19, respectively *P*=0.48, *d*=0.27), other GIS (29 ± 10 and 29 ± 10 *P*=1.00, *d*=0.00), upper GIS (32 ± 11 and 30 ± 8, *P*=0.33, *d*=0.42, median ± IQR) or lower GIS (24 ± 15 and 27 ± 6, *P*=0.42, *d*=0.28, median ± IQR), out of maximum scores of 800, 200, 300 and 300 respectively There was no significant difference in breath H_2 between conditions, with no main effect of condition (*P*=0.286; η^2_p: 0.13) and no interaction effect (*P*=0.69; η^2_p: 0.04; see Figure 5.5). There was a significant main effect of time (*P*=0.017; η^2_p: 0.42), but no significant differences between any time point.

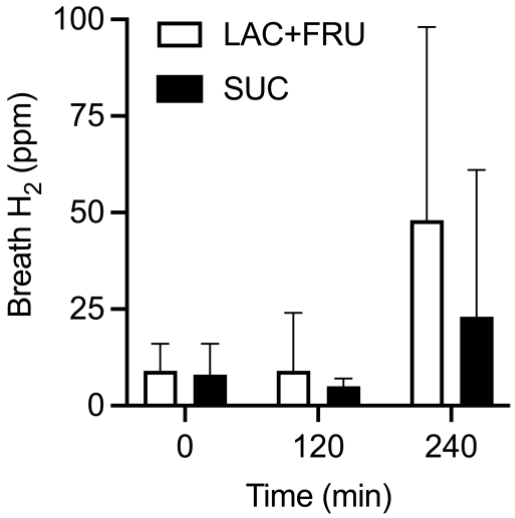

Figure 5.5 Breath H2 concentrations during 4 hours of post-exercise recovery, with ingestion of either 1.5 $g \cdot kg^{-1} \cdot h^{-1}$ of either sucrose (SUC) or lactose and fructose (LAC+FRU).

5.4.6 Dietary analysis

Energy intake was comparable in the 48 hours and 24 hours before LAC+FRU (2074 ± 493 and 2386 ± 725 kcal respectively) and SUC (2055 ± 430 and 2251 ± 728 kcal respectively). Average macronutrient intakes over the entire 48-hour period were also similar between LAC+FRU (protein; 1.3 ± 0.3 $g \cdot kg^{-1} \cdot d^{-1}$, CHO; 4.0 ± 0.9 $\cdot kg^{-1} \cdot d^{-1}$ and fat 1.2 ± 0.5 $\cdot kg^{-1} \cdot d^{-1}$) and SUC (protein; 1.3 ± 0.4 $g \cdot kg^{-1} \cdot d^{-1}$, CHO; 3.8 ± 1.0 $\cdot kg^{-1} \cdot d^{-1}$ and fat 1.1 ± 0.5 $\cdot kg^{-1} \cdot d^{-1}$).

5.5 Discussion:

The aim of this study was to investigate the effect of combined lactose and fructose ingestion compared to sucrose on post-exercise liver glycogen resynthesis. The primary finding was that LAC+FRU did not elicit superior liver glycogen resynthesis to SUC, which is in contrast to the hypothesis. LAC+FRU resulted in lower plasma [insulin] and [glucose], but higher [lactate] than SUC, with comparable GIS in both conditions.

Post-exercise liver glycogen concentrations and content reported in this study for LAC+FRU (143 ± 39 $mmol \cdot L^{-1}$; 36 ± 11 g) and SUC (143 ± 69$mmol \cdot L^{-1}$; 36 ± 16 g) are comparable to previous values measured using MRI/S (Décombaz et al., 2011, Casey et al., 2000). Despite the exercise protocol being of a set duration and not exhaustive, post-exercise liver glycogen concentration and content were not only similar to studies using the same protocol (Detko et al., 2013), but also those using protocols to exhaustion (Fuchs et al., 2016). Liver volume was comparable to values for the general population (Moraes de Oliveira et al., 2020), though lower than previous studies

involving post-exercise resynthesis which may reflect the inclusion of female participants.

Ingestion of both LAC+FRU and SUC resulted in substantial increases in liver glycogen concentration, content and volume from *t*=0 to 240 min. However, there was no significant difference in these measures between conditions. The addition of fructose or galactose to glucose ingestion post-exercise has been shown to increase liver glycogen synthesis beyond glucose ingestion alone (Décombaz et al., 2011, Fuchs et al., 2016). Both CHOs are preferentially metabolised in the liver, with fructose undergoing fructolysis to produce glucose-1-phosphate, lactate and pyruvate (Mayes, 1993, Gonzalez and Betts, 2018), which can be locally converted into liver glycogen. Similarly, galactose is predominantly converted into glucose via the Leloir pathway, which is readily converted into liver glycogen. It was hypothesised that the provision of two CHOs (fructose and galactose) that are hepatically metabolised would accelerate liver glycogen synthesis, though this was not apparent. The study was powered based on effect sizes from Fuchs et al. (2016) with the ingestion of sucrose compared to glucose, which resulted in doubling of liver glycogen synthesis rates. However, a smaller magnitude of difference between conditions in the present study could be expected, given that the increase in hepatically metabolised CHO was just 0.375 $g \cdot kg^{-1} \cdot h^{-1}$, whilst in Fuchs et al. (2016) the difference was double this. The effect size in the present study for change in liver glycogen content (*d*=0.53) was moderate in size, suggesting that the superior effect of LAC+FRU over SUC was potentially meaningful, though non-significant. Therefore, although there was no significant difference between conditions, a larger sample size may be required to conclude this with

certainty. Because liver glycogen is a key regulator of exercise capacity, which is likely regulated through maintenance of blood [glucose], both in mice (Lopez-Soldado et al., 2021) and humans (Gray et al., 2020), it is not clear whether there would be a benefit to exercise capacity with LAC+FRU ingestion, and the impact of LAC+FRU ingestion on exercise performance is discussed in Chapter 6.2.

In the LAC+FRU condition, total fructose and galactose ingestion was 1.125 $g \cdot kg^{-1} \cdot h^{-1}$. Comparable liver glycogen synthesis rates are observed with combined glucose and fructose (free or as sucrose) ingestion with fructose ingestion rates from 0.125 $g \cdot kg^{-1} \cdot h^{-1}$ to 0.750 $g \cdot kg^{-1} \cdot h^{-1}$ (Gonzalez et al., 2017). Therefore, if galactose and fructose both result in production of the same substrates for liver glycogen synthesis (pyruvate, lactate and ultimately UDP-glucose), a rate-limiting step may exist that prevents further liver glycogen synthesis (Tang et al., 2016, Gonzalez and Betts, 2018). Given that higher rates of fructose ingestion do not appear to accelerate liver glycogen synthesis, there is likely a rate limiting step in the process of glycogenesis from fructose-derived substrates. Considering the lack of additional liver glycogen synthesis with galactose, this suggests that this rate limiting step may be located in the shared pathway of hepatic fructose and galactose metabolism. Muscle glycogen synthesis (i.e. the transfer of glucose from UDP-glucose to an amylose chain) is thought to be the rate-limiting step in muscle glycogen synthesis (Jentjens and Jeukendrup, 2003). This may be the case in liver glycogen synthesis, which might suggest that both conditions in the present study maximised production of UDP-glucose for liver glycogen synthesis, either from fructose and glucose, or fructose, galactose and glucose.

Although the rates of liver glycogen synthesis, were not statistically different between conditions, they were somewhat higher than previously observed. Liver glycogen synthesis rates were 10.7 ± 3.4 and 8.6 ± 4.3 $g \cdot h^{-1}$ in LAC+FRU and SUC, respectively. Fuchs et al. (2016) observed rates of 6.6 ± 3.3 $g \cdot h^{-1}$ and 3.3 ± 3.0 $g \cdot h^{-1}$ with sucrose and glucose ingestion respectively, with identical CHO ingestion rates in the same laboratory. However, Décombaz et al. (2011) observed rates of 8.1 ± 0.6 $g \cdot h^{-1}$, 8.6 ± 0.9 $g \cdot h^{-1}$ and 3.7 ± 0.5 $g \cdot h^{-1}$ with fructose and glucose, galactose and glucose and glucose ingestion, respectively, despite lower ingestion rates (1.2 $g \cdot kg^{-1} \cdot h^{-1}$). Detko et al. (2013) compared the effect of adding protein and amino acids to a glucose and galactose beverage, on post-exercise liver glycogen restoration. The protein co-ingestion condition matched liver glycogen restoration despite the protein and amino acids replacing an isocaloric amount of glucose (0.4 $g \cdot kg^{-1} \cdot h^{-1}$). This is similar to the effect on muscle glycogen synthesis which is attributed to the accentuated insulinemia it causes (Burke et al., 2017). Insulin has a powerful effect on liver glycogen synthesis, with half-maximal rates at a portal vein [insulin] of 160 $pmol \cdot L^{-1}$ and a plateau at 200 $pmol \cdot L^{-1}$ (Roden et al., 1996). Portal vein [insulin] is approximately 3-fold higher than systemic concentrations (Horowitz et al., 1975), meaning that both LAC+FRU and SUC exceeded this 200 $pmol \cdot L^{-1}$, threshold, (~1300 and ~2200 $pmol \cdot L^{-1}$ respectively) as did both conditions in Fuchs et al. (2016). Therefore, the effect of protein co-ingestion with CHO on liver glycogen synthesis is unlikely to be due to augmented insulin secretion. Post-exercise combined CHO and protein ingestion does not appear to suppress glucagon to a greater extent than CHO alone, which also could have contributed (Rustad et al., 2016, van Hall et al., 2000). Therefore, protein co-ingestion with CHO can accelerate liver glycogen synthesis when CHO intake is limited (0.8 $g \cdot kg^{-1} \cdot h^{-1}$).

However, it remains unclear whether it retains this effect when CHO ingestion rates are optimal (1.2-1.5 $g \cdot kg^{-1} \cdot h^{-1}$), though this could explain the high liver glycogen synthesis rates observed herein. Comparisons between studies are difficult due to differences in exercise protocols, lengths of recovery periods and CHO ingestion rates. The impact of protein co-ingestion with optimal CHO on liver glycogen requires further investigation.

Lower plasma [glucose] were observed with LAC+FRU ingestion compared to SUC, which is explicable by the lower glucose ingestion in this condition (0.375 $g \cdot kg^{-1} \cdot h^{-1}$) compared to SUC (0.75 $g \cdot kg^{-1} \cdot h^{-1}$). This difference extended into plasma [insulin], which was higher with SUC and was likely caused by the higher plasma [glucose]. Plasma [insulin] were higher than previous investigations, which may be explained by the very high CHO intake (1.5 $g \cdot kg^{-1} \cdot h^{-1}$) combined with whey protein ingestion (Detko et al., 2013, Fuchs et al., 2016). It is possible that the lower glucose content in the LAC+FRU condition, which extended to reduced circulating [glucose] and [insulin] might limit liver glycogen synthesis. However, previous work has shown that replacing glucose ingestion with fructose or galactose is effective at accelerating liver glycogen synthesis, even with reduced plasma [glucose] and [insulin] (Fuchs et al., 2016, Décombaz et al., 2011).

Plasma [lactate] was elevated post-exercise in both conditions, though from *t*=120 min [lactate] was higher in LAC+FRU. Fructose ingestion results in lactate production, and subsequent elevated plasma [lactate] which was observed in both conditions (Mayes, 1993). Galactose ingestion does result in 3C compound production in the liver,

resulting in increased plasma [lactate] when ingested in isolation (Gannon et al., 2001) and when co-ingested with fat, in this latter case to a similar extent fructose (Watkins et al., 2020, Gonzalez and Betts, 2018). Therefore, the increased plasma [lactate] may result from galactose, via other 3C compounds such as pyruvate. If this occurs, this may result in increased fatty acid and triglyceride production, which is observed with galactose and fat ingestion (Watkins et al., 2020). It is also possible that lactate clearance was compromised with LAC+FRU compared to SUC, though the observations that plasma [lactate] can be increased with galactose ingestion suggest that this may be a more likely cause.

GIS were similar and generally mild in both conditions despite the large amounts of CHO ingested. An average of 214 g (range 180-249 g) of lactose was ingested by participants over the 4h recovery period, a comparable ingestion rate to that used in Chapter 3 of 120 g over 2.5h during exercise. This supports previous suggestions that, in lactose tolerant individuals, even very large amounts of lactose can be ingested without GIS worse than another CHO in the same quantity (Stellaard et al., 2000), which was also observed in Chapters 3 and 4. Although breath H_2 did not change significantly in either condition, there is a clear rise from baseline in both conditions, which is driven by a few individuals with substantial breath H_2 responses to the CHO ingestion. This suggests that in some participants in SUC, fructose malabsorption may have occurred (though sucrose maldigestion cannot be ruled out). Fructose malabsorption is characterised by limited intestinal transport capacity of fructose, resulting in reduced absorption and GIS (Ebert and Witt, 2016). There may have been a larger breath H_2 response in LAC+FRU, which may represent some lactose

maldigestion and subsequent fermentation, though no statistical difference was apparent. In the absence of a significant difference between conditions, this supports previous suggestions that positive H_2 breath tests for lactose maldigestion can occur in 10-20% of participants without the presence of GIS (De Geyter et al., 2021).

In conclusion, a combination of lactose and fructose providing a full spectrum of dietary monosaccharides, did not result in statistically greater replenishment of liver glycogen after a glycogen-depleting exercise session, than sucrose. This occurred despite higher plasma [insulin] and [glucose] with SUC ingestion. Furthermore, LAC+FRU ingestion did not elicit worse GIS than SUC ingestion.

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Chapter 6: General discussion

The overarching theme of this thesis was to identify novel applications for lactose in human nutrition, with three specific aims:

1. To investigate whether lactose represents a viable energy source for exercise.
2. To further understand the metabolism of lactose, and its specific effects on exercise metabolism.
3. To investigate whether lactose can play a role in post-exercise liver glycogen resynthesis, specifically as a delivery vehicle for galactose and glucose, alongside fructose.

The General Discussion will involve a brief summary of the key findings of each chapter. Further sections will discuss each chapter in greater detail, with reference to how they meet the specific aims outlined above. General themes and concepts that are apparent across multiple chapters, though are not stated aims of the thesis will then be discussed individually. Limitations of the thesis will also be discussed, followed by suggestions for future work in this field.

6.1 Summary of key findings

Chapter 3 was the first comprehensive investigation into lactose as an energy source in an exercise context, addressing the first aim of the thesis. Exogenous carbohydrate

(CHO) oxidation rates for lactose were compared to sucrose at moderate ingestion rates (0.80 $g \cdot min^{-1}$). The novel finding was that exogenous CHO oxidation rates were comparable with lactose and sucrose ingestion. Furthermore, lactose resulted in increased fat oxidation, and subsequently a reduction in endogenous CHO oxidation. This chapter also showed that gastrointestinal discomfort is unlikely to be an issue for the majority of lactose tolerant individuals, even with lactose ingestion at the high doses required for sport. It was also possible to use a superior authentic background correction in the calculation of exogenous CHO oxidation. Therefore, this chapter addressed the first aim of the thesis, showing that lactose can be a viable energy source for exercise, and highlighted its potential for a positive impact on exercise performance.

The aim of Chapter 4 was to further elucidate the unique effect of lactose ingestion on exercise metabolism, by calculating plasma glucose oxidation (PGO), liver glycogen oxidation (LGO) and muscle glycogen oxidation (MGO), and contrasting lactose to the same comparator CHO, sucrose. However, due to the impact of COVID-19 restrictions, it was not possible to address this physiological research question adequately. The limited data that were generated suggest that the glycogen sparing observed in Chapter 3 was hepatic in origin, though further investigation is warranted. It was also possible to use a modified method to calculate PGO with ingestion of lactose and sucrose by adding an experimental trial for each CHO. This also allowed an authentic background correction to be used, improving the accuracy of exogenous CHO oxidation calculation, and thus LGO as well.

Chapter 5 investigated lactose in a post-exercise recovery nutrition setting, testing the hypothesis that two hepatically metabolised CHOs (fructose and galactose) are superior to one (fructose) for liver glycogen restoration. Because lactose is essentially the only major source of galactose in the human diet, lactose acted as the only feasible 'delivery vehicle' for galactose and glucose (furthermore, even galactose powder is not readily available as a food grade raw material). This might be relevant for athletes, as increased restoration of liver glycogen post-exercise is important for subsequent exercise capacity. The primary finding was that lactose and fructose ingestion did not significantly accelerate liver glycogen synthesis above sucrose. This demonstrates that although lactose did not appear to offer a specific benefit in this scenario, it can play a role in post-exercise recovery nutrition. The study also provided limited supporting evidence for the idea that protein can accelerate liver glycogen synthesis at high CHO ingestion rates, though this requires further substantiation.

6.2 Comparable Exogenous Carbohydrate Oxidation from Lactose or Sucrose during Exercise

The first aim of this thesis was to determine whether lactose represents a viable energy source for exercise. This is a relevant question for sports nutrition for two main reasons. Firstly, athletes may ingest more lactose than the general population, due to their higher absolute energy requirements, recommendations for dairy product ingestion for their protein and calcium content (e.g. yoghurt) and the common use of whey and casein protein powders among athletes (Bianco et al., 2011). Therefore, understanding whether lactose from such foods can contribute to CHO requirements during exercise

could be valuable. Secondly, if lactose can contribute to CHO requirements during exercise, it may be possible to utilise lactose for sports nutrition products, in the manner that other CHOs are used (e.g. for gels or CHO drinks). Because lactose is a low-value side stream product of the whey protein industry, this would represent a new commercial application for lactose.

Although there were limited suggestions that lactose might be oxidised in exercise (Stellaard et al., 2000, Lee et al., 2008), direct investigations to estimate the oxidation of exogenous lactose during exercise had not been performed. Digestion of lactose is certainly limited in lactose intolerant (LI) individuals at doses of >15 g, though not at doses of ~25 g in lactose tolerant individuals (Shaukat et al., 2010). Slow or limited digestion of lactose might impede its utility in sports nutrition through reduced exogenous CHO oxidation rates and thus poorer exercise performance, which has been observed with the poorly digested CHO isomaltulose, compared to free glucose and fructose (Oosthuyse et al., 2015). The Km of lactase (~14 mM) is substantially lower than that of sucrase (~142 mM), which at least suggests that there is greater scope for higher sucrose ingestion rates than lactose (Gray and Ingelfinger, 1966, Naim and Lentze, 1992).

Chapter 3 demonstrated that ingested lactose is oxidised at comparable rates to ingested sucrose ((0.56 ± 0.19 $g \cdot min^{-1}$ and 0.61 ± 0.10 $g \cdot min^{-1}$ respectively) when ingested at a moderate rate of 0.8 $g \cdot min^{-1}$ or 48 $g \cdot h^{-1}$. Sucrose is an appropriate CHO to compare lactose to, as both are disaccharides of a glucose, combined with a hepatically metabolised CHO (fructose and galactose, respectively). Combinations of

glucose and fructose are also recommended for both high rates of CHO ingestion to maximise exogenous CHO oxidation rates, and at moderate rates to reduce GIS (Rowlands et al., 2015). Although lactose may have been oxidised marginally slower in the first 60 min of exercise, from 90 min onwards, oxidation rates were essentially identical. That there is some discrepancy between the two CHOs is perhaps unsurprising given the order of magnitude difference in the respective enzymes Km. CHO ingestion during exercise can also prevent hypoglycaemia, and both lactose and sucrose resulted in higher plasma [glucose] than water, both in the early and late stages of the exercise bout. Lactose ingestion resulted in higher fat oxidation and reduced endogenous CHO oxidation compared to sucrose, though discussion of this unique impact of lactose on exercise metabolism is discussed in a later section of the general discussion. Together, these data show that lactose can act as a viable exogenous energy source and substrate for oxidation during exercise.

Lactose can therefore be included in sports nutrition recommendations as a possible CHO source for ingestion during exercise at moderate ingestion rates. It is possible that ingestion rates of up to 1 $g \cdot min^{-1}$ (60 $g \cdot h^{-1}$) would be feasible. However, above this ingestion rate transport from the intestinal lumen into the hepatic portal vein via SGLT1 might be limited, as both glucose and galactose use this transporter, which is only capable of absorbing ~60 $g \cdot h^{-1}$ of glucose (Jeukendrup, 2010), even if lactose digestion was not limiting. Lactose may also be feasibly ingested as part of a pre-exercise meal or snack for its CHO content, for example as milk with cereal or oats, or as a specific CHO beverage.

6.3 Using an improved ^{13}C oral tracer methodology to assess muscle and liver glycogen oxidation with lactose or sucrose ingestion during exercise: a pilot study.

Chapter 4 was intended to be a direct follow up to Chapter 3, with aim of investigating further one of the novel findings of this chapter. The observation that fat oxidation was higher, and endogenous CHO oxidation lower with lactose ingestion compared to sucrose ingestion was unexpected. This difference was attributed to elevated insulin and lactate with sucrose ingestion, compared to water ingestion. Though there were no significant differences in these outcomes compared to lactose, it is possible that the small sample size limited detection of a difference. Both lactate and insulin can suppress lipolysis (Horowitz et al., 1997, Boyd et al., 1974), which could limit fat oxidation and result in increased reliance on endogenous CHO. Therefore, Chapter 4 aimed to better characterise the metabolism of lactose during exercise, by attempting to determine whether lactose ingestion results in reduced hepatic or muscle glycogen oxidation, or some combination of both. Due to COVID-19 restrictions, the study was truncated, and it was not possible to fully explore the physiological question, and the focus of Chapter 4 is on the improved ^{13}C oral tracer methodology that was used, which is discussed in a later section of the General Discussion alongside the other methodological advances explored in Chapter 3.

Limited interpretation was possible, which suggest that plasma glucose oxidation (PGO) was higher with sucrose ingestion than lactose, though this was not analysable statistically due to unpaired samples and low sample sizes. This supports the idea that lactose may result in sparing of liver glycogen. Exogenous CHO oxidation rates were

also calculated for lactose and sucrose, resulting in similar rates for both CHOs. However, rates were substantially lower than were observed in Chapter 3, the reason for which is not apparent.

6.4 The effect of combined lactose and fructose ingestion on post-exercise liver glycogen synthesis

Chapter 5 demonstrated that there was no significant additional effect of ingesting two hepatically metabolised CHOs (fructose and galactose; LAC+FRU) with glucose, over just one (fructose; as sucrose; SUC) on post-exercise liver glycogen synthesis. The reason for this is unclear, given that both fructose and galactose accelerate post-exercise liver glycogen synthesis when co-ingested with glucose (Décombaz et al., 2011). However, the findings of this chapter do meet the third aim of the thesis, demonstrating that lactose can play a role in post-exercise liver glycogen resynthesis, specifically as a delivery vehicle for galactose, which represents a novel application for lactose in human nutrition.

In Chapter 5 it was hypothesised that the lack of a significant additive effect on liver glycogen synthesis with LAC+FRU ingestion compared to SUC may be due to galactose and fructose ingestion both resulting in production of the same substrates for liver glycogen synthesis (pyruvate, lactate and ultimately UDP-glucose). Figure 6.1 shows the hepatic metabolism of the three main dietary monosaccharides. All CHOs ultimately result in UDP-glucose production before glycogen synthesis via glycogen

synthase occurs. Because similar rates of liver glycogen synthesis are observed with a variety of rates of fructose ingestion (0.125-0.750 $g \cdot kg^{-1} \cdot h^{-1}$) with glucose (Gonzalez et al., 2017), it is possible that the degree of substrate availability for liver glycogen synthesis is not a limiting factor. That the addition of galactose to fructose and galactose does not further enhance liver glycogen synthesis further supports this hypothesis, and also suggests that the rate limiting step may be in a shared pathway of hepatic metabolism for fructose and galactose. This may be in the action of glycogen synthase (highlighted) in transferring glucose from UDP-glucose to an amylose chain, which is considered the rate-limiting step in muscle glycogen synthesis (Jentjens and Jeukendrup, 2003).

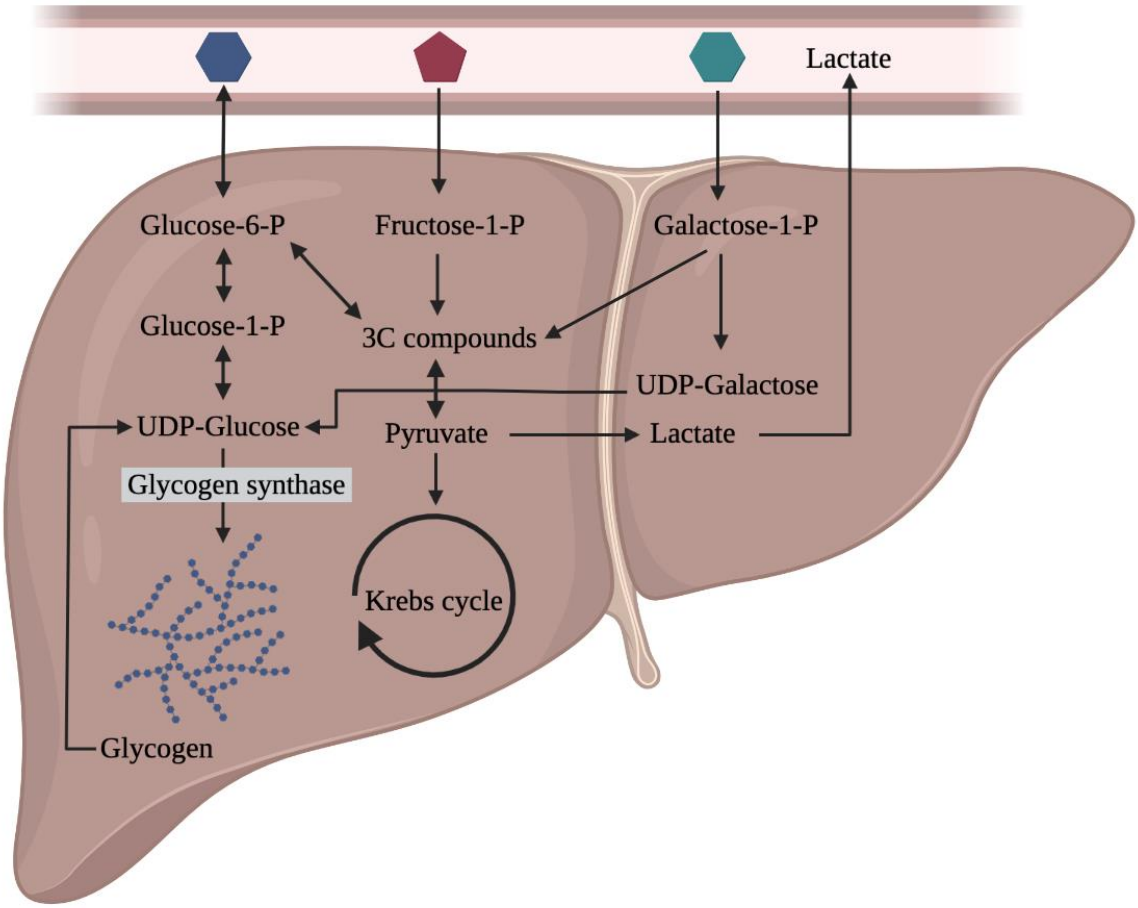

Figure 6.1 The hepatic metabolism of dietary monosaccharides. P – phosphate, 3C – 3-carbon, UDP – uridine diphosphate. Created with BioRender.com.

Enhanced post-exercise restoration of liver glycogen has been shown to improve endurance exercise capacity in some, (Maunder et al., 2018, Gray et al., 2020) but not all investigations (Casey et al., 2000, Podlogar and Wallis, 2020), through the ingestion of combined fructose and glucose, compared to glucose alone. The improvements in exercise capacity is attributable to increased hepatic glycogen storage, and subsequently increased CHO oxidation rates (Maunder et al., 2018). Although in Chapter 5 there were no differences in liver glycogen between conditions, it is not clear whether muscle glycogen restoration may be different. Replacing substantial amounts of glucose with fructose in the post-exercise period does not compromise muscle glycogen resynthesis (Fuchs et al., 2016, Wallis et al., 2008). However, the LAC+FRU beverages contained just 0.375 $g \cdot kg^{-1} \cdot h^{-1}$ of glucose, with the other 0.375 $g \cdot kg^{-1} \cdot h^{-1}$ of the glucose content from the SUC condition consisting of galactose. Galactose does not appear to be an ideal substrate for muscle glycogen synthesis (Podlogar et al., 2020). However, protein co-ingestion with sub-optimal amounts of CHO (<1.2 $g \cdot kg^{-1} \cdot h^{-1}$) can accelerate glycogen synthesis through amplified insulin secretion at a wide variety of CHO ingestion rates (Margolis et al., 2021). Therefore, it is possible that muscle glycogen restoration was similar between conditions, though a superior effect with SUC cannot be ruled out.

The improvement to subsequent exercise capcity after post-exercise glucose and fructose ingestion is attributed to improved liver with similar muscle glycogen synthesis (Maunder et al., 2018, Gray et al., 2020). Given that LAC+FRU resulted in comparable

liver glycogen synthesis to SUC, without knowing the effect of LAC+FRU on muscle glycogen synthesis, it is unclear what effect LAC+FRU would have on subsequent exercise performance. If muscle glycogen stores are comparable after both CHOs, subsequent exercise performance and capacity would likely be similar. Whilst glucose and fructose can be easily obtained in a post-exercise setting, using CHO drinks, or gels, or whole foods, substantial amounts of galactose cannot be added to the diet without ingestion of large quantities of lactose. Chocolate milk is one of the few foods or beverages that contains a mixture of glucose, fructose and galactose in a 2:1:1 ratio, through the addition of sucrose (Amiri et al., 2018). Studies involving post-exercise chocolate milk ingestion have shown positive effects on post-exercise muscle glycogen restoration and subsequent exercise performance, suggesting that blends of all three monosaccharides are capable of restoring muscle glycogen (Karfonta et al., 2010, Ferguson-Stegall et al., 2011).

6.5 Methodological improvements

Although not a stated aim of the thesis, in Chapters 3 and 4 it was possible to make methodological improvements to existing calculations used in exercise metabolism. Chapter 3 investigated the use of an authentic background correction in the calculation of exogenous CHO oxidation, compared to a water correction. A background correction is required in the calculation of exogenous CHO oxidation to account for the background shift in breath $^{13}CO_2$ that occurs when glycogen is oxidised, which occurs even after a low ^{13}C CHO diet. The authentic background correction involved a condition with the ingestion of naturally low abundance ^{13}C CHOs instead of a condition

with water ingestion. The water correction resulted in overestimation of exogenous sucrose oxidation by 15 %, and underestimation of lactose oxidation by 10 %. That the authentic correction resulted in overestimation with sucrose ingestion and underestimation with lactose ingestion might be due to the more marked effect of sucrose ingestion on endogenous CHO oxidation, resulting in greater $^{13}CO_2$ production that did not arise from the exogenous CHO. Previous studies using a water correction with naturally high abundance ^{13}C CHOs may have overestimated exogenous CHO oxidation rates, especially in CHOs which result in greater endogenous CHO oxidation, such as galactose (Leijssen et al., 1995). This makes comparison of exogenous CHO oxidation rates between studies potentially misleading.

Future investigations calculating exogenous CHO oxidation with naturally high abundance CHOs should use an authentic background correction to avoid inaccuracies. However, this does lead to a substantially higher participant and researcher burden, both in terms of time and cost. For example, a study involving two experimental conditions (such as two different CHO types or doses) would need an additional two authentic background trials, whilst a water-correction would only require one additional trial. Research involving ^{13}C CHO tracers instead of naturally high abundance CHOs may not require an authentic background correction, as the background shift that occurs with a water correction and an authentic CHO correction (≤1 δ‰ versus Pee Dee Bellemnitella) is negligible compared to the increase observed with ^{13}C CHO tracer ingestion (~400 δ‰). Although the cost of a synthetic ^{13}C CHO tracer is higher than using naturally high abundance ^{13}C CHOs, this may be more financially viable than the increased analysis costs, as well as reducing time

commitments. However, synthetic tracers are not feasible for all CHO types, as some ^{13}C CHO tracers are not commercially available. Therefore, researchers must balance the additional cost of using a ^{13}C CHO tracer against the time savings its use provides, compared to a naturally high abundance CHO method, with an authentic background correction.

Chapter 4 also used an authentic low ^{13}C CHO correction, because accurate exogenous CHO oxidation rates were required for subsequent calculations (e.g. liver glycogen oxidation; LGO). However, alterations were also made to the method of estimating plasma glucose oxidation (PGO). Firstly, the breath $^{13}CO_2$ data from the authentic background correction were used in calculating PGO instead of a baseline sample. This likely only had a small effect on the calculated figure for PGO, as the background shift was small (~0.2-0.5 δ‰ *versus* PDB) compared to the change in breath $^{13}CO_2$ enrichment in the conditions with ^{13}C glucose tracer (~420 δ‰). Secondly, an additional trial was used to separate the calculation of exogenous CHO oxidation and PGO. If CHOs had been exclusively glucose based, then one trial using a ^{13}C CHO tracer could have been used to calculate exogenous CHO oxidation and PGO. These changes contribute to more accurate estimation of PGO, LGO, muscle glycogen oxidation (MGO), exogenous and endogenous CHO oxidation. Although the calculation of PGO is a less commonly performed technique in similar studies than the calculation of exogenous CHO oxidation, the improvements in this method contribute to current methodological options for assessing exercise metabolism.

6.6 Gastrointestinal considerations for lactose ingestion, in an exercise context

Lactose metabolism varies between individuals, with lactose tolerance status dictating the presence or absence of GIS upon its ingestion. LI individuals have low levels and activity of the digestive enzyme lactase, resulting in limited digestive capability. Undigested lactose enters the colon, increasing the osmotic load, causing diarrhoea and abdominal pain, as well as flatulence and bloating from fermentation by local bacterial flora (Rangel et al., 2016). LI individuals experience GIS with ingestion of more than 12-15 g, though some experience them at smaller amounts (Shaukat et al., 2010). Although there is a clear threshold for LI individuals at which symptoms occur, it is unclear whether such a threshold might exist for lactose tolerant individuals, above which the digestive capabilities of lactase are saturated. Lactose intake in the general population is ~12 g (Brisbois et al., 2014, Larsson et al., 2004), though there is considerable inter-individual variation. However, this amount is far removed from the quantities of CHO required for sports nutrition, for before (1-4 $g \cdot kg^{-1}$), during (30-90 $g \cdot h^{-1}$) or after (1.2 $g \cdot kg^{-1} \cdot h^{-1}$) exercise (Burke et al., 2011).

Research into milk and chocolate milk in sports nutrition suggested that lactose may be able to contribute to CHO requirements for sports nutrition. Lee et al. (2008) fed participants milk during cycling exercise, with ingestion of ~30 $g \cdot h^{-1}$ of lactose, without worse GIS than a CHO-electrolyte beverage. In a post-exercise setting, Costa et al. (2020) fed participants 1.2 $g \cdot kg^{-1}$ of a chocolate milk beverage, and did not observe a difference in GIS compared to water ingestion. However, ingestion of lactose in dairy foods is compounded by the presence of other nutrients as part of the ‘dairy matrix’

(Thorning et al., 2017), such as fat which can slow gastric emptying and reduce symptoms of LI (Jellema et al., 2010). One small (*n*=7) investigation observed no difference in GIS with 80 g lactose ingestion, compared to 80 g glucose ingestion before light (cycling at 50 W) physical activity (Stellaard et al., 2000). However, due to the limited sample size and low exercise intensity, it was not clear how relevant these data are to sports nutrition.

Chapters 3 and 4 both involved ingestion of 120 g of lactose (at a rate of 0.8 g·min^{-1} or 48 g·h^{-1}) during exercise at 50% W_{max}. GIS were similar with ingestion of lactose, sucrose or water, and of the combined sample of Chapters 3 and 4 (*n*=12 and *n*=8, respectively) for participants who completed at least one condition involving lactose ingestion, only two experienced GIS indicative of LI. One participant experienced severe symptoms and dropped out of the study, whilst another experienced milder symptoms in the hours post-exercise. Interestingly, the latter participant demonstrated markedly lower exogenous CHO oxidation rates for lactose (0.37 g·min^{-1}), compared to sucrose (0.70 g·min^{-1}), which further suggests limited digestion of lactose, resulting in reduced subsequent oxidation. These data suggest that even in some lactose tolerant individuals, there can be a threshold at which lactase activity could limit lactose digestion. In Chapter 5, larger absolute amounts of lactose (214 g, range 180-249 g) were ingested without a significant difference between LAC+FRU and SUC in GIS. However, rates of lactose ingestion (~54 g·h^{-1}) were comparable to those of Chapters 3 and 4.

Breath H_2 was measured to provide a quantitative assessment of CHO maldigestion. Lactose ingestion in Chapter 3 resulted in increased breath H_2 (though non-significantly), whilst breath H_2 significantly reduced with sucrose and water ingestion. This suggests that lactose maldigestion does occur in some individuals, as responses were highly variable. However, many participants experienced increased breath H_2 without accompanying GIS. Similarly, with LAC+FRU ingestion in Chapter 5, breath H_2 increased (though non-significantly), with no difference in GIS between conditions and participants experiencing substantial increases in breath H_2 without accompanying GIS. Although measuring breath H_2 has prognostic capabilities for LI in clinical settings, the value of measuring breath H_2 in the studies outlined in this thesis is limited, except perhaps for indicative purposes. It is not clear what quantity of lactose must escape digestion to result in changes in breath H_2 or GIS, and even with clinical testing of lactose maldigestion, 10-20 % of individuals with positive H_2 breath test results may not experience GIS with a 25 g dose (De Geyter et al., 2021). Therefore, GIS do not appear to be an area for concern with lactose ingestion at rates commensurate with sports nutrition guidelines, both during and after exercise. Intolerant individuals or those who experience GIS with large amounts of lactose who desire to ingest lactose, perhaps as a source of galactose, could use lactase enzyme to expedite lactose hydrolysis. Individual responses to lactose ingestion should be monitored even in lactose tolerant individuals, as with any nutritional intervention or practice.

6.7 Unexpected metabolic impacts of lactose ingestion

Chapter 3 demonstrated that lactose ingestion during exercise resulted in higher fat oxidation and concomitantly, lower endogenous CHO oxidation compared to sucrose. This was hypothesised to be due to elevated insulin and lactate with sucrose ingestion compared to water ingestion, both of which can suppress lipolysis (Horowitz et al., 1997, Boyd et al., 1974), potentially limiting fat oxidation and resulting in increased reliance on endogenous CHO. The limited sample size may have made detection of statistically significant differences between the CHOs difficult, however lactose has been shown previously to be a less insulinogenic CHO than glucose (Ercan et al., 1993). Lactose may be unique in this manner, in that it is not a highly insulinogenic CHO, but is readily oxidised. CHOs that elicit a lower plasma insulin response such as galactose or trehalose are generally oxidised more slowly, as the limited insulin response may be caused by limited digestion or limited capacity to synthesise glucose from the CHO (Venables et al., 2008, Stannard et al., 2009). The impact of lactose on exercise performance or capacity has not been investigated, however, the reduction in endogenous CHO oxidation might translate into improved exercise capacity or performance in prolonged exercise. The findings of Chapter 4 suggest that the glycogen sparing might be hepatic in origin, perhaps allowing for better maintained blood [glucose] in the later stages of exercise. However, one study involving free glucose and galactose ingestion in a 1:1 ratio showed no difference in cycling performance between glucose and galactose, and a combination of glucose and fructose (Stannard et al., 2009). However, it is not clear whether hydrolysed lactose results in similar metabolic effects to intact lactose in such scenarios, and the possibility of a performance benefit to lactose ingestion remains.

In Chapter 5, a further unexpected metabolic impact of lactose ingestion was observed. Plasma [lactate] was higher with LAC+FRU ingestion than SUC, from 120 min of the recovery period onwards. Fructose is well known to cause lactate production, as a result of fructolysis (Tappy and Lê, 2010). However, although galactose ingestion has been shown to result in increased plasma [lactate] (Gannon et al., 2001), it has not mechanistically been demonstrated to result in hepatic lactate production. It has only been demonstrated that 3C can be produced with galactose ingestion, though which 3C compounds these are has not been specifically examined (Coss-Bu et al., 2009). The addition of 0.375 $g \cdot kg^{-1} \cdot h^{-1}$ of galactose to 0.75 $g \cdot kg^{-1} \cdot h^{-1}$ of fructose may have contributed to lactate production, suggesting that synthesis of lactate (and 3C precursors of lactate such as pyruvate) is not maximised even at high fructose ingestion rates. Though it is still unclear whether lactate clearance may be involved. The metabolic fate of the additional lactate is not clear, though in this post-exercise setting it could contribute to muscle glycogen synthesis (Fournier et al., 2002). Alternatively, the lactate could contribute to *de novo lipogenesis*, as lactate is the main precursor of fatty acids with fructose intake (Carmona and Freedland, 1989). This could have implications for metabolic health, and further research into the metabolic impacts of lactose and galactose from a health perspective is warranted.

6.8 Milking it; implications and applications of this work

The findings of the thesis culminate in the improvement in the understanding of lactose ingestion for sports nutrition, which is commensurate with the aims of the thesis. Chapter 3 first showed that lactose can be used by athletes during exercise as a readily

oxidisable exogenous CHO source, with a number of positive and unique attributes, with Chapter 4 substantiating this. Chapter 5 demonstrated that lactose ingestion post-exercise can contribute to liver glycogen synthesis and as a delivery vehicle for galactose. Recommendations for sports nutrition can be altered to include consideration of lactose as a CHO that is comparable to others and in some cases, desirable for athletes.

Athletes who eat dairy likely already ingest lactose on a regular basis, and it may form part of their pre- or post-exercise nutrition regimens. However, the quantities of lactose that have been used in the chapters of this thesis are likely too large to be achievable by athletes through the consumption of dairy foods alone. For example, in Chapters 3 and 4, 48 $g \cdot h^{-1}$ of lactose was ingested, whilst in Chapter 5 average lactose ingestion was ~54 $g \cdot h^{-1}$, requiring approximately 1 L of milk to achieve these hourly rates. Dairy foods could contribute lactose to form a portion of a milieu of CHO sources around exercise, for example through the combination of milk to a sucrose-containing cereal. However, to ingest large quantities of lactose it is likely that lactose powder would be required. To the authors knowledge, there are no commercial sports nutrition products containing lactose as a main or active ingredient. However, there is a sports nutrition CHO-electrolyte beverage containing milk permeate (a high lactose dairy ingredient) available (GoodSport Nutrition, Chicago, IL, USA). It combines the naturally occurring electrolytes from milk, alongside lactose which is hydrolysed with added lactase. Manufacturers of dairy ingredients, such as the industrial partner of this PhD studentship, Volac International, could find new revenue streams from lactose sales for sports nutrition uses, as opposed to limited sales in confectionary or for animal feed.

Any existing CHO product could be designed to include lactose, such as ready to drink CHO beverages, drink mixes or gels. However, the presence of lactose may cause GIS for LI consumers, and possibly lactose tolerant consumers if used in substantial quantities. Therefore, manufacturers of sports nutrition products could include lactase enzyme to circumvent possible issues around intolerance, though this could attenuate or abolish the positive effect upon fat oxidation, and thus endogenous CHO oxidation.

6.9 Limitations

Specific limitations to various aspects of the thesis are addressed where relevant, including methodological limitations which are also addressed in Chapter 2. However, there are a number of limitations common to all three experimental chapters which can be addressed here. Sample sizes of Chapters 3-5 are small (*n*= 8-11). In Chapter 3, this was due to the explorative nature of the study, as it was not clear whether there would be a difference in the primary outcome (exogenous CHO oxidation rates) between conditions. Therefore, a sample size comparable to previous investigations was chosen (O'Brien and Rowlands, 2011, Leijssen et al., 1995). For Chapter 4, a sample size calculation was performed based on the data from Chapter 3, to ensure that a difference in endogenous CHO oxidation could be detected, resulting in a required sample size of *n*=10. In Chapter 5, the required sample size was estimated to be 10, based on effect sizes observed with glycogen synthesis rates with sucrose or glucose ingestion (Fuchs et al., 2016). However, with hindsight it may be that the impact of ingesting two hepatically metabolised CHOs over ingesting one on liver glycogen synthesis, would not be expected to be as large as the impact of ingesting

one hepatically metabolised CHO, compared to none. However, it was not clear what alternative data could be used to estimate required sample sizes. The cost of the magnetic resonance methods was also high, and 10 participants was deemed feasible based on the available funding.

One further limitation surrounded the control of diets before study visits in all experimental chapters. In Chapters 3 and 4, participants were asked to replicate their diet, though this was not monitored, nor was adherence to the low ^{13}C CHO food requirements. Provision of food and meals to participants in the preceding days would have avoided the possibility of differences in diet impacting exercise metabolism or perceptual responses. However, background breath ^{13}C enrichment (which provides an indication of the ^{13}C enrichment of glycogen stores) was similar between the 5 conditions in Chapter 3. Furthermore, the shift in breath ^{13}C enrichment in the water trial was just 0.60‰, which is comparable to that seen in prior work (0.81-1.16 δ‰) even when a glycogen depleting bout was included prior to trial (Wallis et al., 2005, Rowlands et al., 2005). This suggests that the low ^{13}C CHO food requirements were followed well by participants. In Chapter 5, diets in the 48 hours before each trial were analysed, showing good, though not perfect, replication. There was considerable intra-individual variability in liver glycogen post-exercise, which could be attributable to differences in pre-exercise glycogen content, as the exercise bout was standardised.

6.10 Future work and conclusions

There is a variety of future work that is possible in follow up to the research conducted in Chapters 3-5. Although metabolic impacts of lactose ingestion in different settings have been investigated, the effects on exercise performance or capacity should be investigated. For example, whether lactose ingestion during exercise may lead to superior exercise performance or capacity could be examined to determine if the glycogen sparing observed is ergogenic. Similarly, lactose could be investigated alongside fructose ingestion to permit high CHO ingestion rates during exercise, which is possible with glucose and fructose ingestion. It may also be of relevance to investigate whether the additional fat oxidation observed with lactose ingestion during exercise is derived from intramuscular triglycerides or from adipose tissue. Further work could be done into understanding hepatic CHO metabolism, to try to understand the rate-limiting step for liver glycogen synthesis, which could explain the lack of an observed difference in liver glycogen synthesis with two hepatically metabolised CHOs, compared to one. Although the potential for lactose to act as a prebiotic was identified in Chapter 1, this thesis did not address this possible application. Future research could determine whether lactose ingestion could induce any of the positive effects that are observed with prebiotic supplementation, such as improved immune function (Parada Venegas et al., 2019).

To conclude, the broad aim of the thesis was to identify novel applications for lactose in human nutrition. This broad aim has been met by addressing the three specific aims of the thesis. Chapter 3 demonstrated that lactose does represent a viable energy source for exercise, by showing that exogenous CHO oxidation rates with lactose ingestion are comparable to sucrose. Chapter 4 aimed to further elucidate the impacts

of lactose ingestion during exercise, with the limited data giving an indication of the source of the observed glycogen sparing with lactose ingestion. Chapter 5 demonstrated that lactose can play a role in post-exercise liver glycogen resynthesis, capable of eliciting substantial liver glycogen repletion, despite not causing greater liver glycogen synthesis than sucrose in isolation.

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