Metabolism of exogenous ketones.

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I dedicate this Thesis to Mum, Dad, Torie and Geordie.

Thank you for believing in me.

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I declare that the work presented in this Thesis is my own including:

1. Study design (with supervision)
2. Ethical applications
3. Subject recruitment
4. Experimental trials (with supervision)
5. Data analysis
6. Data presentation

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7. The experiment investigating the effects of ketosis on pyruvate dehydrogenase flux in exercising skeletal muscle presented in Appendix E, was designed and performed in collaboration with Dr Kate Curtis (DPAG, University of Oxford).

Abstract

As metabolic substrates, ketone bodies provide an alternative to glucose in order to prolong survival during starvation. A low carbohydrate, high fat diet can be used to promote ketogenesis without fasting, but long-term compliance can be difficult. Dietary ketone bodies may be an alternative method to induce ketosis, so the aim of the work in this Thesis was to investigate the metabolism of exogenous ketones. In the first experimental Chapter, the effects of ketone ester and salt drinks on blood β-hydroxybutyrate (βHB), glucose, lipids, electrolytes and pH were determined in healthy humans at rest. Blood D-βHB levels were higher following ketone ester drinks, but it was found that total βHB levels with ketone salts were similar, as over 50% of βHB delivered in the salt was the L-isoform, which was only slowly removed from the blood. Circulating glucose and lipid concentrations fell following both ketone drinks. Blood pH fell following ketone ester consumption, but rose following ketone salt drinks, whilst both compounds raised blood sodium and chloride, and lowered potassium. Work in the second Chapter investigated the repeatability of ketone ester metabolism with food, successive drinks or continuous nasogastric (NG) infusion. Peak D-βHB levels were repeatable between- and within- subjects at rest but were lower after a meal, although blood acetoacetate, breath acetone and urine βHB were unaffected by feeding. βHB kinetic parameters were not altered by existing hyperketonemia from successive ketone ester drinks and total βHB uptake was identical when isovolumetric amounts of ketone ester were continuously infused through a NG tube. The third Chapter explored side-effects of ketone drinks: ketone ester drinks decreased appetite compared to isocaloric dextrose; which may have been linked to effects of βHB on enteroendocrine cells. Furthermore, both ester and salt drinks were found to be unpalatable, and to cause a few, mild gastro-intestinal effects that increased with intake. As exogenous ketones could be a performance enhancing supplement in sport, the fourth Chapter used a survey to investigate supplement use by endurance athletes. The results demonstrated widespread supplement use, which was highest at the elite level. In the final Chapter, the effect of glycogen levels on the oxidation of βHB was determined in isolated perfused rat hearts. Low cardiac glycogen levels decreased βHB oxidation and levels of the intermediates of glycolysis and the Krebs cycle, whilst increasing muscle amino acid levels, suggesting that low glycogen may have impaired anaplerosis. In conclusion, this work extends current understanding of the novel physiological ketosis that occurs following exogenous ketone consumption.

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Abbreviations

βHB	Beta-hydroxybutyrate	GLP-1	Glucagon-like peptide-1
AcAc	Acetoacetate	HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
ADP	Adenosine diphosphate	IV	Intra-venous
ANOVA	Analysis of variance	KH	Krebs-Henseleit
ATP	Adenosine triphosphate	LC-MS	Lipid chromatography-mass spectrometry
AUC	Area under the curve	LCHF	Low carbohydrate, high fat
BMI	Body mass index	MCT	Medium chain tryglyceride
C_{max}	Peak concentration	NAD	Nicotinamide adenine dinucleotide
CPT-1	Carnitine palmitoyltransferase I	NG	Nasogastric
DNA	Deoxyribonucleic acid	PDH	Pyruvate dehydrogenase
EDTA	Ethylenediaminetetraacetic acid	R_a	Rate of appearance
ELISA	Enzyme-linked immunosorbent assay	RS-BD	R,S- butanediol
FAD	Flavin adenine dinucleotide	SEM	Standard error of the mean
FDA	Food and Drug Administration	T_{max}	Time of peak concentration
FFA	Free fatty acids	TG	Triacylglycerol
GI	Gastro-intestinal		

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Chapter 1

Introduction

"Let food be thy medicine and medicine be thy food"

Hippocrates- Greek physician (460 BC - 377 BC)

1.1 Introduction

Diet is a vital determinant of health and physiological capacity. In the face of stressors such as disease or extreme exercise, diet becomes increasingly important as it not only provides substrates to sustain energy production, but also prompts cellular adaptations to better cope with the demands imposed by the environment. Scientific inquiry into the biological implications of nutrition has expanded in recent years to investigate how manipulation of the diet can optimize health and performance.

The key dietary macronutrients are carbohydrate, lipid and protein; the former two provide the key metabolic substrates for the conservation of energy in the form of ATP (adenosine triphosphate). Endogenously produced ketone bodies: acetoacetate (AcAc) and β-hydroxybutyrate (βHB), also act as a metabolic fuel although their role as a substrate is not widely appreciated as, whilst they are ubiquitously present at very low levels, they only accumulate during extreme physiological states (Table 1.1). The existence of ketone bodies in human biology was discovered in the late 19th century, when they were detected by clinicians in the urine of diabetic patients (Dreschfeld, 1886). This first impression has unfortunately persisted, defining the wider medical community's perception of ketones as a dangerous by-product of unbalanced metabolism (Vanitallie and Nufert, 2003). During diabetic crisis, concentration of ketones in the blood can exceed 20 mM. However, a distinction was drawn between this "pathological ketosis" and a "physiological ketosis" ($\approx$ 8 mM) by Krebs (1966). Physiological ketosis is a key adaptation that sustained human evolution (Cahill, 1970, 2006) and there is a growing body of literature suggesting that generating ketosis through nutritional interventions may be beneficial to health and performance (Veech, 2004; Veech et al., 2001).

	Condition	Ketosis (mM)	Reference
Endogenous ketosis	Fed	0.05 - 0.1	Balasse and Neef (1974)
	Starvation: 24 hours	0.3 - 0.5	Fery and Balasse (1983)
	Starvation: 10 days	3 - 6	Cahill (2006)
	Starvation: 4 weeks	5 - 8	Cahill (1970)
	LCHF diet	4 - 11	Gilbert et al. (2000)
Exogenous ketosis	Ketone salt	1 - 3	Plecko et al. (2002)
	MCT	0.5	Ohnuma et al. (2016)
	1,3- BD	0.2	Kies et al. (1973)
	Ketone Ester	3 - 6	Cox. et al. (2015)

Table 1.1: *Comparison of ketone levels attained in various conditions. BD = butanediol, LCHF = low-carbohydrate high-fat, MCT = medium chain triglyceride. Adapted from (Cox, 2012)*

This Chapter aims to describe the background and rationale for the investigation of the metabolism of exogenous ketones. Firstly it will introduce oxidative metabolism and the metabolic function of endogenous ketone bodies. Secondly, it will provide an overview of methods used to elicit endogenous ketone production and how these are currently applied in humans. Finally, this Chapter will describe the use of exogenous ketone supplements for nutritional ketosis.

1.2 Oxidative metabolism

"Metabolism" refers to the complex network of biochemical reactions that provide organisms with energy to sustain life (Figure 1.1). Stored energy contained in complex organic compounds obtained through the diet is made more accessible in the form of "high energy" phosphate groups (e.g. adenosine triphosphate (ATP), guanosine triphosphate (GTP)) and as hydride ion (electron) carriers (e.g. NADH, FADH) (Frayn, 2009).

The differing properties of dietary carbohydrate, lipids and proteins (amino acids) affect their storage and use within the body. Carbohydrates are highly water soluble and easy to transport through the body; furthermore they can be metabolised anaerobically to generate ATP when oxygen supply is limited. However, due to their solubility, they retain substantial

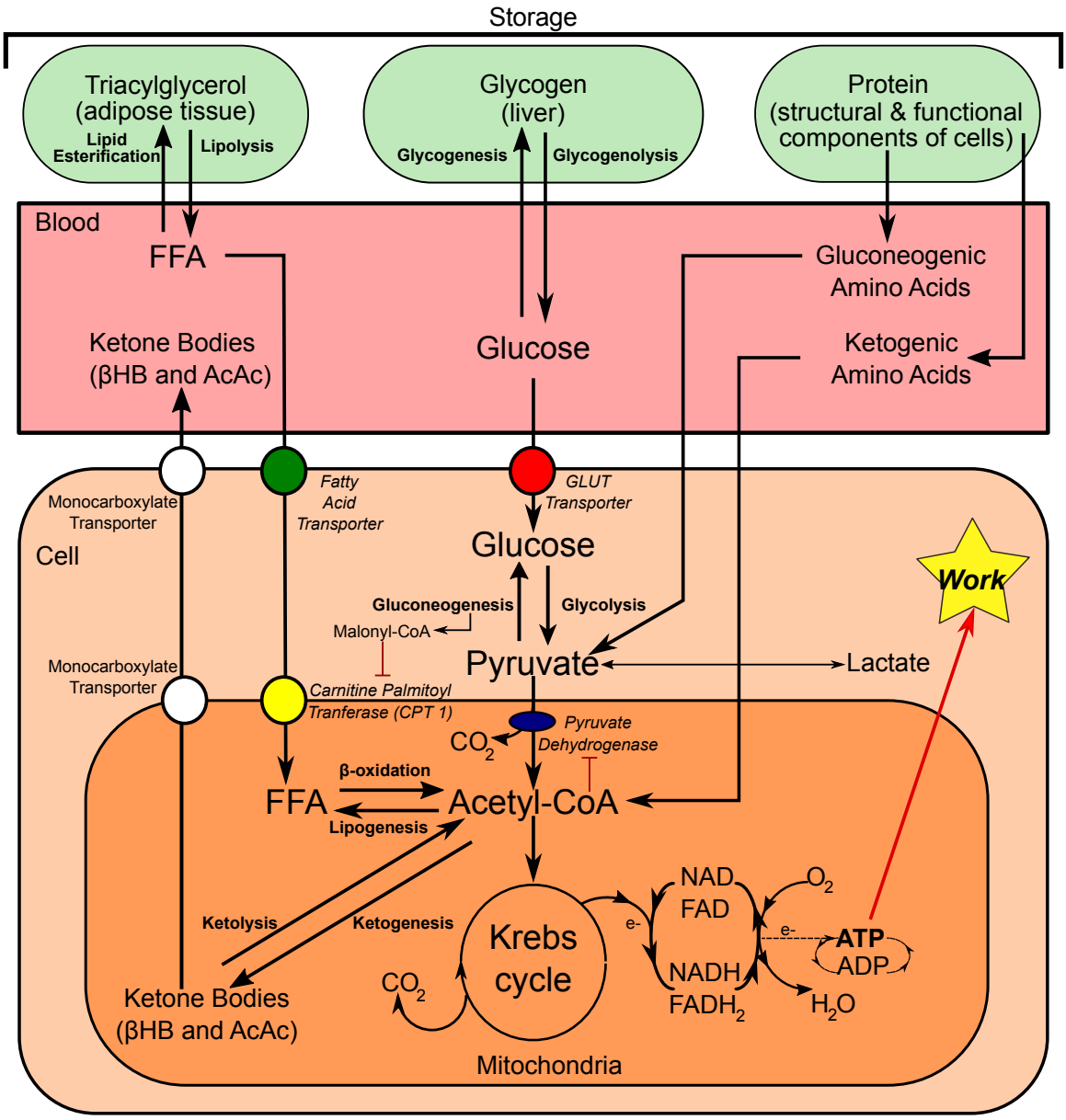

Figure 1.1: *Overview of oxidative metabolism. Large storage molecules are converted into intermediate substrates for transport to cells where they undergo conversion to acetyl-CoA. Following this they are transported to the mitochondria where they are terminally oxidised via the Krebs cycle. Lipogenesis and ketogenesis only take place in hepatocytes; hepatocytes are unable to re-convert ketone bodies to acetyl-CoA as they do not express acetoacetyl CoA thiolase. ADP = adeonsine diphosphate, ATP = adenosine triphosphate, βHB = β-hyrdoxybutyrate, e^- = electron, FAD = flavin adenine dinucleotide, NAD = nicotinamide adenine dinucleotide, FFA = non-esterified free fatty acid.*

amounts of water in their storage form (glycogen), so only limited amounts of glycogen are laid down. Additionally, they are partially oxidised and therefore do not contain as much potential energy as lipids. Lipids are highly reduced and energy dense compared to carbohydrates; however, they cannot provide energy anaerobically and their water insolubility makes them slow to mobilise and means they are unable to cross the blood-brain barrier and provide a substrate to the CNS. Proteins form the structural and functional components of cells and only contribute appreciably to oxidative metabolism during times of extreme substrate shortage via gluconeogensis (Figure 1.1).

The simple carbon skeletons derived from dietary carbohydrate, lipid and protein, undergo conversion to the common metabolic intermediate acetyl-CoA. This enters the Krebs cycle and is oxidised to $2CO_2$ molecules generating 3NADH, $1FADH_2$, and 1GTP or ATP molecule. The hydride ions resulting from the Krebs cycle enter the mitochondrial electron transport chain along with oxygen, and electrons travelling along the electron transport chain create potential energy that is used to drive proton diffusion through ATP synthase. This ultimately catalyses ATP formation from ADP and inorganic phosphate (Figure 1.1). The Krebs cycle is not a true "cycle," in fact it is better described as the "central hub" of metabolism as it provides not only the intermediates for energy generation also but for many biosynthetic reactions (Owen et al., 2002). Therefore, the removal (cataplerosis) of intermediates for bio-synthesis must be balanced by regeneration (anaplerosis) in order to maintain homeostasis (Owen et al., 2002).

The reciprocal relationship between the oxidation of non-esterified free fatty acids (FFA) and carbohydrate was described by Randle et al. (1963). At rest, when oxygen availability is high, lipid provides the bulk of the substrate for oxidative phosphorylation. The process of β-oxidation sequentially shortens FFA to produce acetyl-CoA and NADH, which accumulate in the mitochondria, inhibiting the enzyme pyruvate dehydrogenase (PDH), thus decreasing entry of pyruvate from glycolysis into the mitochondria (Randle et al., 1963) (Figure 1.1). Thus, lipid oxidation limits carbohydrate oxidation.

Carbohydrate oxidation is ultimately determined by flux through the glycolytic pathway (and PDH), and is tightly regulated by hormonal and metabolic signals, including insulin, glucagon, citrate, ATP, ADP and AMP (Randle et al., 1963). Glycolysis results in the anaerobic production of ATP through substrate-level phosphorylation, and the generation of pyruvate, which can enter the mitochondria and thence the Krebs cycle and ultimately lead to the production of ATP, CO_2 and water. In some cases, carbohydrate oxidation can also inhibit lipid metabolism. The mechanism underlying this observation was proposed by McGarry et al. (1977), who reasoned that plentiful carbohydrate stores act as a source of glycerol and cytosolic citrate for lipogenesis. A consequence of increased lipogenesis is an increase in cytosolic concentrations of malonyl-CoA, the first committed intermediate in the lipogenic pathway which inhibits the mitochondrial FFA transporter: carnitine palmotyl transferase-1 (CPT-1) (Figure 1.1). This prevents futile oxidation of newly formed FFA and promotes FFA esterification (Hue and Taegtmeyer, 2009).

1.3 Starvation ketosis

Ketones bodies are an endogenous substrate that can be metabolized in the Krebs cycle to sustain ATP production during dietary insufficiency. Early *homo sapiens* were faced with the problem of providing a constant substrate supply to the brain to sustain its function as it grew in size and became increasingly metabolically demanding. Despite only representing 2% of total body mass, the brain accounts for 25% of the basal metabolic rate (Pellerin, 2010); under normal conditions 87% of CO_2 production from the brain is accounted for by glucose, 11% from lactate and 1% from pyruvate (Himwich et al., 1937). FFA are unable to cross the blood brain barrier to act as a substrate (Frayn, 2009). Ketone production increases with prolonged starvation (Figure 1.2 A and Table 1.1) and βHB and AcAc can cross the blood brain barrier and provide a fuel for the brain that can account for up to 65% of cerebral metabolism (Figure 1.2 B).

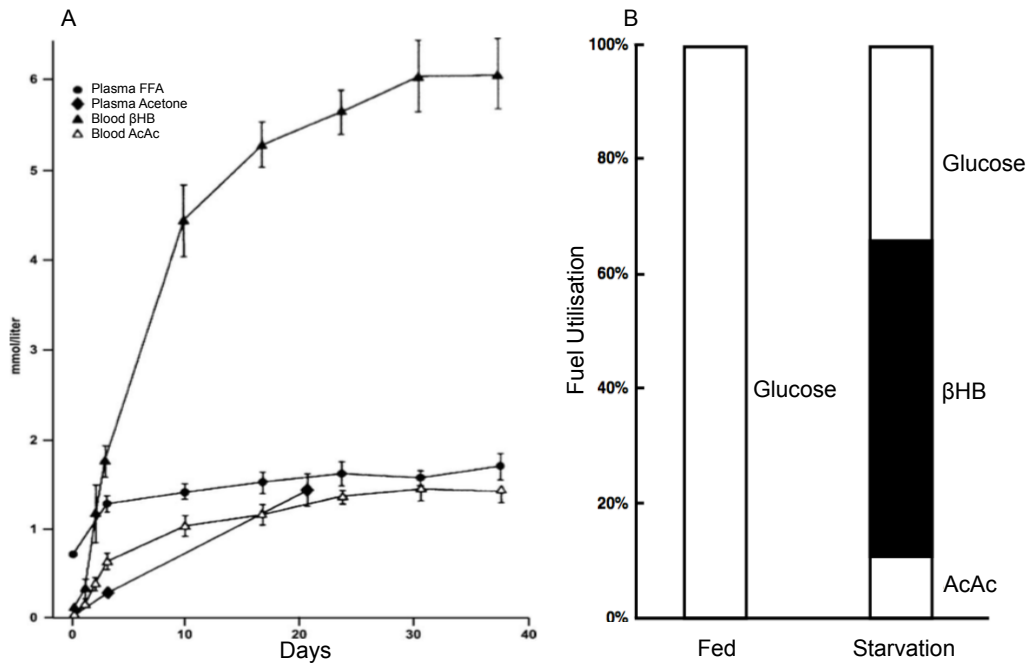

Figure 1.2: *A: Blood ketone levels accumulate slowly as starvation progresses and plateau after many weeks. B: Comparison of brain fuel utilisation during the fed state vs. starvation. In the fasted state ketone bodies can largely replace glucose as an oxidative fuel for the brain. Adapted from Cahill (2006)*

1.3.1 Endogenous ketone production

Starvation (specifically low levels of blood insulin and glucose) triggers ketone body production in the hepatocyte mitochondrial matrix (1.3). Two molecules of acetyl-CoA from β-oxidation of FFA are condensed via acetyl-CoA transferase to form acetoacetyl-CoA; a third is added to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) in a reaction catalysed by HMG-CoA synthase. HMG-CoA lyase then splits this to re-generate acetyl-CoA and form one molecule of AcAc (Frayn, 2009). βHB is formed from the NADH-dependent reduction of AcAc by βHB-dehydrogenase, and acetone results from spontaneous, non-enzymatic decarboxylation of AcAc. Acetone is a volatile molecule which is primarily excreted in the breath, although some evidence suggests that a small amount can be metabolised to pyruvate and oxidised (Kalapos, 2003).

Extra- and intra-hepatic signals are integrated to increase hepatic ketone body production in situations of decreased carbohydrate supply (Robinson and Williamson, 1980). Extra-hepatic control of ketogenesis is primarily mediated by changes in FFA release from adipose tissue (McGarry and Foster, 1977, 1972, 1979). In the fed state, insulin strongly inhibits hormone sensitive lipase on the surface of adipocytes (Stralfors et al., 1984). In the absence of dietary carbohydrate, levels of insulin are persistently low and greater concentrations of glucagon and cortisol enhance adipocyte lipolysis (McGarry and Foster, 1979). The resulting increase in circulating FFA results in greater delivery of ketogenic substrate to the liver (Keller et al., 1989; McGarry and Foster, 1979). Conversely, ketone bodies exert a powerful negative feedback on their own production: acting via the PUMA-G (nicotinic acid) G-protein coupled receptor to inhibit lipolysis and limit FFA availability (Taggart et al., 2005).

Intra-hepatic factors regulate the rate of ketone production from circulating FFA (Robinson and Williamson, 1980). This ensures that ketone production remains suppressed when lipolysis is elevated, but glycogen stores are replete (i.e. stress). Carbohydrate status of

the liver is the key intra-hepatic determinant of ketone production (Mayes and Felts, 1967). This was classically illustrated by an experiment that perfused fed and starved liver slices with equivalent amounts of FFA. Fed livers were found to re-esterify FFA to triacylglycerol, whereas starved livers converted FFA to ketone bodies (Mayes and Felts, 1967). In most circumstances hepatic lipogenesis and ketogenesis are inversely related, whilst gluconeogenesis and ketogenesis are directly related. This is due to operation of the Randle Cycle, as described earlier. In the post-absorptive state, abundant carbohydrate stores are permissive for lipogenesis, increasing in cytosolic levels of malonyl-CoA. The resulting inhibition of CPT1 prevents mitochondrial FFA uptake; thus limiting availability of acetyl-CoA in the matrix for ketogenesis (McGarry et al., 1977). Conversely, when fasted, oxaloacetate is shuttled from the mitochondria into the cytosol for gluconeogenesis due to increased activity of phosphoenolpyruvate carboxykinase (Hue and Taegtmeyer, 2009). If the fast is prolonged, hepatic glycogen stores become insufficient to act as an anaplerotic substrate and regenerate the intermediates of the Krebs cycle. In this situation, Krebs cycle oxaloacetate levels may be inadequate to condense with the high levels of acetyl-CoA resulting from increased lipid mobilization. Any accumulating acetyl-CoA is therefore re-routed towards synthesis of ketone bodies (Figure 1.3).

1.3.2 Ketone utilisation

Ketone bodies are oxidised in the mitochondria of all extra-hepatic tissues. The liver is unable to utilise ketones as a fuel because hepatocytes lack acetyl-CoA thiolase (Zammit et al., 1979), a key enzyme in the ketone oxidative pathway. As they cannot be oxidised by the hepatocyte, βHB and AcAc are released into the circulation, acting as a transportable form of lipid derived acetyl-CoA. Once in the blood stream, ketone bodies are predominantly confined to the fluid compartment as they are lipid-insoluble, this is illustrated by their low volume of distribution (0.1-0.2 L/kg body weight) (Keller et al., 1981). They leave the blood and enter cells via the monocarboxylate transporter family, in a proton-

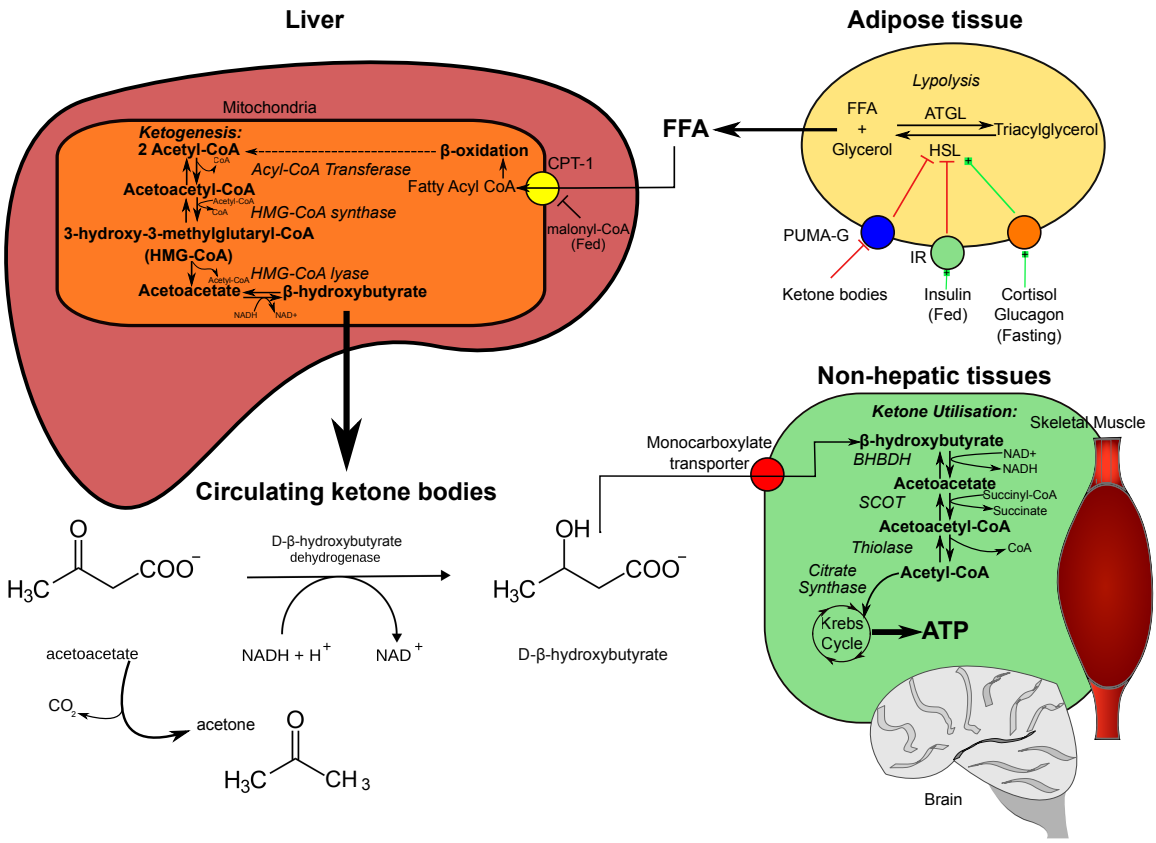

Figure 1.3: *Production and utilisation of endogenous ketone bodies. Ketone bodies are produced when the hormonal milieu promotes the release of non-esterified free fatty acids (FFA) from the adipose tissue. The liver converts FFA into ketone bodies when stored glycogen is low. Ketone bodies cannot be metabolised in the liver and are released into the circulation from whence they can provide a fuel for non-hepatic tissues such as the brain, heart and skeletal muscle. ATGL = adipose triglyceride lipase, ATP = adenosine triphosphate, BHBDH = β-hydroxybutyrate dehydrogenase, CPT-1 = carnitine palmitoyl transferase 1, HSL = hormone sensitive lipase, IR = insulin receptor, NAD = nicotinamide adenine dinucleotide, PUMA-G = G-protein coupled receptor, SCOT = succinyl-CoA transferase.*

linked, diffusion-driven process (Halestrap and Price, 1999). Organ-specific expression of monocarboxylate transporter isoforms with different kinetic properties is one factor that alters the rate of ketone utilisation in each tissue (Halestrap and Price, 1999).

The primary metabolic fate of ketone bodies is oxidation (described in: Frayn (2009) and Robinson and Williamson (1980)), consistent with their role as an alternative fuel source during starvation. βHB enters the mitochondria through a monocarboxylate transporter, undergoes conversion to acetoacetate by βHB dehydrogenase and then addition of a CoA group from succinyl-CoA by 3-oxo-acid transferase. The resulting acetoacetyl-CoA acts a substrate for the formation of two molecules of acetyl-CoA in a reaction catalysed by acetyl-CoA thiolase. Acetyl-CoA is then available to condense with oxaloacetate and enter the Krebs cycle. βHB may also be stored as lipid if it undergoes conversion to butyryl-CoA in the cytosol (Figure 1.3).

Ketone bodies are a highly efficient oxidative fuel. Experiments carried out in isolated perfused rat hearts showed that provision of ketones decreased oxygen consumption but also increased hydraulic work, resulting in an overall increase in cardiac efficiency of 28% (Sato et al., 1995). The mechanism underlying this was postulated to be an improvement in mitochondrial energetics. As ketones are more chemically reduced than pyruvate, they reduce the NAD^+ couple and oxidise the Co-Q couple: site I and II of the mitochondrial electron transport chain (Sato et al., 1995). Increased redox span between sites I and II of the electron transport chain results in a greater energy released by electrons travelling across this span, according to:

$$\Delta G' = -nF\Delta E_{\frac{SiteII}{SiteI}}$$

where G represents the Gibbs free energy, n the number of electrons, F the Faraday constant and E the difference in redox potential between sites I and II (Sato et al., 1995). ATP releases energy stored within the terminal phosphate bond when it is hydrolysed to ADP. The theoretical "standard" value for free energy released as a result of ATP hydrolysis

is fixed; however, the actual cellular free energy of ATP hydrolysis can vary with changing intra-cellular conditions; if the concentration of products or reactants change. Hence, when the electrons of the electron transport chain release more energy, the protons diffusing through ATP synthase also increase in energy, which increases the free energy of ATP hydrolysis (Veech et al., 2001).

Another consideration in the relative metabolic efficiency of substrates is their inherent combustion enthalpy, which refers to the energy stored in the bonds of the molecule itself. βHB has a higher combustion enthalpy per C2 (-243.6 kCal/mol C2) compared to pyruvate (-185.7 kCal/mol C2) (Veech, 2004). This means βHB provides more potential energy to the electron transport chain. However, palmitate (resulting from β-oxidation of FFA) has a higher combustion enthalpy than both βHB and pyruvate (-298 kCal/mol C2) (Veech, 2004). Despite this, palmitate metabolism does not provide equivalent mitochondrial efficiency, as it donates 50% of the electrons resulting from β-oxidation via $FADH^+$ and the flavoprotien site at complex II, which is downstream of the site of electron donation by $NADH^+$ resulting from βHB and pyruvate oxidation (Veech, 2004). Therefore, the potential ATP yield resulting from palmitate oxidation is decreased.

1.3.3 Non-metabolic roles of ketone bodies

Ketone bodies can function as a cellular signal to provide a link between the environment (diet), enzymatic activity and gene expression via chromatin remodelling (Newman and Verdin, 2014). These signals drive manifold physiological adaptations that enhance survival capacity, such as improved tolerance of oxidative stress (Newman and Verdin, 2014). βHB acts as an endogenous histone-deacetylase inhibitor *in vitro*, producing dose-dependent hyper-acetylation of histones (the structural protein of DNA), and other protein targets (Shimazu et al., 2013). Furthermore, ketones increase mitochondrial biogenesis and up-regulate the expression of uncoupling proteins in several tissues including brown adipose tissue (Srivastava et al., 2012) and the hippocampus (Sullivan et al., 2004). This

may increase resting energy expenditure or decrease production of harmful mitochondrial reactive oxygen species. Finally, elevated levels of ketone bodies decrease several markers of systemic inflammation *in vivo* (Paoli et al., 2015; Ruskin et al., 2009). As experimental techniques develop, it is possible that further signalling roles for βHB will be uncovered.

1.3.4 Summary: starvation ketosis

Ketone bodies fulfil a vital evolutionary role as a highly efficient metabolic substrate. The production of ketones is coupled to carbohydrate availability, providing an alternative fuel for the brain and protecting body protein stores to extend survival during periods of starvation. Furthermore, the signalling effects of ketone bodies may also have implications for health and disease.

1.4 Nutritional ketosis

1.4.1 History of the ketogenic diet

Fasting has been used to alleviate symptoms of disease since the ancient Greeks, with Hippocrates writing that "to eat when you are sick, is to feed your sickness". The importance of ketosis itself in the success of fasting for disease management was unknown, as ketone bodies were only relatively recently discovered (Wheless, 2004). Furthermore, accurate techniques to quantify ketones in the blood were only discovered in the last century. As the benefits of ketosis became more widely appreciated, methods were developed that manipulated food intake to recreate the physiological conditions necessary to trigger endogenous ketogenesis without the need for starvation.

"Ketogenic" diets elicit ketosis by minimizing carbohydrate intake to $<$50 g/day and providing the bulk of the daily energy requirement as fat (70%) and to a lesser extent protein (15%) (Kossoff and Rho, 2009; Westman et al., 2003). The resulting low levels of

insulin, but ample dietary fat, drive a shift towards β-oxidation and create conditions that favour ketogenesis without calorie restriction. Many in the scientific community view low-carbohydrate, high-fat (LCHF) ketogenic diets with scepticism, as it has long been believed that carbohydrate is an essential nutrient for human health and performance (Christensen and Hansen, 1939; Phinney, 2004; Burke and Kiens, 2006). Contrary to this view, a LCHF ketogenic diet not only supports life, but may improve general health relative to high carbohydrate diets (Forsythe et al., 2008; Phinney, 2004). The extent to which this is attributable to the macronutrient balance of the diet, or due to ketone bodies themselves remains unclear in many cases.

Before the development of agriculture, evidence suggests the human race thrived for thousands of years as hunters, with a diet heavily reliant on animal products (Fiorenza et al., 2011). This is demonstrated by cultures that remained isolated from the Western world until recently, such as the Inuit of the Arctic Circle. Their diet was described in the early 20th century by an anthropologist, Vilhjalmur Stefansson, to consist solely of the spoils of hunting and fishing (Stefansson, 1946). He noticed that the Inuit were careful to limit their protein intake, giving the leanest meat to their dogs and eating the fatty cuts themselves, suggesting that their diet was richer in fat than protein as is widely believed. Stefansson recreated the Inuit diet himself under scientific observation, causing consternation by surviving for 12 months in apparent good health (McClellan, 1930a,b). Other arctic explorers unintentionally illustrated the ability of a LCHF diet to sustain physical and cognitive performance; several expeditions became stranded without rations and walked hundreds of miles to safety sustained solely by hunting (Schwatka and Stackpole, 1965).

To many a LCHF diet is counter-intuitive approach to maintain health, due to the widespread fear of dietary fat and its supposed link to obesity and its associated complications (Hegsted et al., 1965; Keys et al., 1950). In 1953 Ancel Keys, an American biochemist, published an epidemiological study that claimed that dietary fat was a key risk factor in the development of heart disease (Keys, 1953). This “diet-heart hypothesis” pro-

posed that blood LDL and cholesterol derived from dietary fat accelerated the development of atherosclerotic plaques. As a result of his work, global food policy and public practice were radically changed. The USADA 1977 Dietary Goals for Americans (McGovern, 1977) recommended a decrease in dietary fat intake, and a diet based around grains and cereals. Subsequently, both total calories consumed and the relative amount of calories from carbohydrate increased; this was most pronounced in women, whose total intake increased by 21.7% and carbohydrate intake increased by 38.4% (Hite et al., 2010). Keys' "diet-heart" hypothesis was not supported by clinical evidence; and subsequent large trials including the Framingham Study (Anderson et al., 1987) and Women's Health Initiative Randomized Controlled Dietary Modification Trial (Howard et al., 2006) failed to show that decreasing dietary fat lowered the risk of heart disease. The incidence of obesity rose dramatically following the adoption of the USDA guidelines and some investigators, including most famously John Yudkin, hypothesised that the increased dietary carbohydrate was responsible for the developing health crisis in his book, first published in 1972 'Pure White and Deadl' (Revised version: Yudkin and Lustig (2013)). However, the role of sugar and starch was overlooked due to popular concerns over dietary fat.

The notion that carbohydrate rich diets may be worse for health and weight control than diets high in fat long pre-dated Yudkin's work. The earliest advocate for the low carbohydrate diet was Anthleme Brillat-Savarin, a French lawyer, physician and epicure who practiced in the 1820's. However, the British undertaker, William Banting, is widely credited to be the founder of the LCHF diet. In 1862, Banting published his "Letter on Corpulence, Addressed to the Public" which described how a diet stripped of "farinaceous" starchy foods, and high in fat had been effective in his struggle with obesity (Banting, 1863). His modern counterpart, whose career carried him from fame to infamy, was Dr. Robert Atkins. He brought his version of the ketogenic LCHF diet to the masses in the 1972 book: Dr. Atkins' Diet Revolution (Atkins et al., 1972). The Atkins diet aimed to dramatically reduce dietary carbohydrate ($<$20 g/day; $<$10% of daily energy requirement)

before gradually increasing intake to find the critical carbohydrate level where ketosis could be maintained. The majority of dietary energy comes from fat (60-70% of daily energy requirement) and protein (20-30% of daily energy requirement) (Atkins et al., 1972). It is estimated that Atkins treated over 60,000 patients for obesity and related conditions using this diet over 40 years of practice. However, no clinical studies were performed at that time to validate the benefits of the diet and there were reports of unpalatable side effects of the induction phase ("Atkins Flu") including fatigue, weakness, dizziness, headache and nausea. Finally, cultural biases against fat in the diet were at their most fervent when Atkins was practicing; his recommendations were in stark contrast to those promoted by the US government.

Following Atkins' death in 2003, others took up the mantle of promoting the LCHF diet for health. Recently a group of scientists including Volek, Phinney and Westman, were funded by the Atkins Foundation to formally study the effects of the Atkins diet. They found that it largely outperformed the diet based on the 1977 USDA guidelines with respect to measured coronary risk factors including decreased low density lipoprotein-cholesterol and total blood saturated FFA alongside increased high density lipoprotein cholesterol (Forsythe et al., 2008). This may be due to the decrease in carbohydrate and concomitant changes in the hormonal milieu, or due to effects of ketone bodies on substrate metabolism (Paoli et al., 2012, 2015). Their work came as the pendulum of public perception begun to swing in favour of dietary fat, thanks to the emergence of popular writers and speakers such as Taubes (2008), Teicholz (2014) and Lustig (2009). These writers exposed flaws in the work of Ancel Keys, his "diet-heart hypothesis," and the corruption in the political decisions that resulted in the last 40 years of vilification of dietary fat. Furthermore, they highlighted the clinical, epidemiological and mechanistic work illustrating the role of high dietary carbohydrate in the development of obesity and diabetes and set the stage for a revived interest in ketogenic LCHF diets.

1.4.2 Applications of ketogenic diets

The LCHF diet has failed to widely replace the modern carbohydrate-centred paradigm for the general public, due to its low palatability and issues with compliance. However, it has been used as an effective, non-pharmacological therapy for the treatment of chronic metabolic disorders (Reviewed in (Branco et al., 2016)) and is gaining popularity as a strategy to optimise health and physical performance outside of the clinic (Volek et al., 2008a; Volek and Phinney, 2011). The underlying mechanism for its efficacy in specific diseases has been the subject of detailed reviews and is thought to be due to a synergistic effect of altering fuel dietary provision and the direct effects of ketone bodies. Exploration of the full mechanistic details is beyond the scope of this Thesis, but an outline of current uses of ketogenic diets in humans for disease and performance is presented below:

1.4.2.1 Neurological conditions

In the early 20th century fasting was linked to improved seizure control (Wilder, 1921), and since then ketogenic diets have been used to treat several conditions characterized by drug-resistant convulsions. Adherence to a LCHF diet significantly improves clinical outcomes in infantile spasms, Lennon Gastasut Syndrome and other inherited metabolic disorders such as GLUT 1 deficiency (Kang et al., 2007b,a; Kossoff et al., 2003, 2006; Ramm-Pettersen et al., 2013). In some cases the efficacy of the diet was particularly striking: one clinical study reported that the incidence of seizures was decreased by $>$90% in 46% of patients (Numis et al., 2011). However, dietary compliance can be an issue in young patients and if ketosis falls as a result of consumption of too great an amount of carbohydrate or protein then seizures can return (Amari et al., 1995).

The underlying mechanism of the anticonvulsant effect of ketogenic diets is the subject of much debate and several hypotheses have been proposed (reviewed in (Branco et al., 2016)). These include: decreasing reliance on blood glucose for metabolism,inhibition of

excitatory glutamatergic synaptic transmission, inhibition of the mTOR pathway, which has a pathophysiological role in different seizure types and activation of ATP-sensitive potassium channels generating a hyperpolarizing current and decreasing neuronal excitability. Regardless of the mechanism, the transformative power of ketosis in many patients with seizures cannot be ignored.

The neuro-protective effects of a ketogenic diet go beyond epilepsy. Ketogenic diets have undergone preliminary trials as a therapy for neurodegenerative diseases including Parkinson's disease (Vanitallie et al., 2005) and Alzheimer's disease (Henderson et al., 2009). In a feasibility study of 7 patients with Parkinson's disease, 28 days of a ketogenic diet decreased the severity of the clinical symptoms of the disease (Vanitallie et al., 2005). Similarly, in Alzheimer's disease, studies have shown acute improvements in cognitive performance when ketosis was induced by a medicinal food containing a mixture of ready precursors for ketone synthesis (medium chain triglycerides - MCTs) (Reger et al., 2003; Henderson et al., 2009). However, the evidence is inconclusive, as another more recent trial did not observe any effect cognitive improvements of MCT supplements (Ohnuma et al., 2016). *In vitro* and animal work has shown promising results, (Blesa et al., 2012; Kashiwaya et al., 2000) but these are yet to be translated clinically. There are several mechanisms postulated for the beneficial effects of ketogenic diets on Alzheimer's disease and Parkinson's disease, mainly focusing on ketone bodies as an alternative energy source, bypassing faulty steps in metabolism (Veech et al., 2001). The aetiology of both diseases is complex, and ketones may exert a therapeutic effect independently of their role as a fuel, such as decreasing free radical production or decreasing neuronal excitability.

1.4.2.2 Weight-loss

The LCHF diet has experienced mixed popularity as a weight loss strategy since Banting described it in 1825. Compliance with a LCHF diet has been associated with greater reduction in body fat compared to a calorie-matched diet with a higher % of energy from

carbohydrate (Hall et al., 2015).

Weight loss during a LCHF diet may be attributed to the changes in macro-nutrient composition of the diet (i.e. lower carbohydrate, higher fat or protein). Decreased carbohydrate consumption results in lower levels of insulin secretion, increasing lipolysis and decreasing lipogenesis and lipid storage, and thus favourably altering body fat mass (Dyson et al., 2007; Volek et al., 2002). Higher fat consumption results in a decreased resting respiratory quotient and thus greater reliance on lipid oxidation (Paoli et al., 2012). Furthermore, high levels of dietary fat may increase basal metabolic rate due to decreased mitochondrial efficiency through increased expression of uncoupling proteins (Cole et al., 2011). Although protein content of a LCHF diet is restricted, a relative increase in dietary protein, which is highly satiating (Veldhorst et al., 2008), may result in decreased voluntary calorie consumption. Furthermore, there is an increased metabolic cost of gluconeogenesis represented by the thermic effect of proteins (Fine and Feinman, 2004).

Increased satiety is believed to be a key factor in the success of the LCHF diet in weight loss (Feinman et al., 2015). Ketosis itself may directly affect satiety and substrate metabolism (Gibson et al., 2015). Appetite is regulated by an interplay between peripheral and central inputs: ketone bodies could affect either of these factors. For example, ketogenic diets have been shown to favourably alter the levels of appetite-regulating hormones such as ghrelin and leptin (Sumithran et al., 2013) which act as peripheral signals to regulate food intake. Additionally, βHB may act centrally on the hypothalamus itself and decrease food intake (Arase et al., 1988; Scharrer, 1999).

1.4.2.3 Metabolic conditions

Prior to the discovery of insulin, dietary carbohydrate restriction was the preferred therapeutic approach for diabetes mellitus (Feinman et al., 2015). The rationale was that the most salient feature of both type 1 and type 2 diabetes is hyperglycaemia and that a decrease in dietary carbohydrate may alleviate this symptom. Recently diabetics have not

needed to adopt a low carbohydrate diet, as other therapies such as insulin have become readily available to control blood glucose levels. However, side effects of dietary intervention are insignificant in comparison to those of intensive insulin therapy (Feinman et al., 2015; Gerstein et al., 2008). A large study examining outcomes of insulin therapy, (Action to Control Cardiovascular Disease), was prematurely ended due to an increased mortality from CVD in the intensively insulin treated group vs. the standard treatment group (253 vs. 203 deaths, hazard ratio 1.22 P= 0.04) (Gerstein et al., 2008). In the absence of a full randomized-controlled trial into the efficacy of the LCHF diet in diabetes, there has been a call to re-evaluate the available evidence for its use as a first approach in diabetes management (Feinman et al., 2015). LCHF diets have been shown to improve long term control of blood glucose, illustrated by a decrease in the amount of glycated haemaglobin (HbA_{1c}) (Hussain et al., 2012). Levels of HbA_{1c} are accepted to be a reliable predictor of micro- and macro-vascular complications in type 2 diabetes, thus the reliable decrease in HbA_{1c} during a LCHF would be of significant clinical value.

Restriction of dietary carbohydrate has benefits beyond glucose control for the management of metabolic conditions. A LCHF diet can facilitate weight loss (discussed above), decrease the amount of medication used by type 1 and type 2 diabetics (Yancy et al., 2005), and decrease the features of the metabolic syndrome (Volek et al., 2008a,b). Furthermore, ketogenic diets have also been used to treat other peripheral disorders of metabolism including deficits in glycolysis or glycogen storage disorders, for example Cori's and Lafora's diseases (Cheng et al., 2007; Valayannopoulos et al., 2011). In these rare diseases provision of ketone bodies bypasses the defective steps in metabolism and can transform the quality of life of some individuals.

Finally, there is a growing appreciation that cancer is a metabolic disease (Warburg, 1956), and an interest in therapies that exploit differences in the metabolism of tumors and healthy cells. The central rationale for these therapies centres on "The Warburg Effect": the metabolic shift seen in tumours from oxidative respiration to energy production through

glycolysis (Warburg, 1956). Ketogenic diets decrease glucose availability and ketone bodies have an inhibitory effect on glycolysis, thus the diet may selectively starve cancer cells as they lack the mitochondrial capacity and metabolic flexibility to oxidise ketone bodies. Ketogenic diets are currently undergoing preliminary trials as an adjuvant to conventional therapy in cancer patients (Nebeling et al., 1995; Zuccoli et al., 2010; Schmidt et al., 2011) and Nebeling et al. (1995) showed promising effects of the LCHF diet as an anti-tumour agent. The patients, with advanced stage glioma, both achieved ketosis and displayed a significant decrease in tumour growth and an improvement in their clinical symptoms.

1.4.2.4 Endurance Sport

The current paradigm for fuelling endurance exercise performance emphasizes the key role of carbohydrate availability on determining time to exhaustion and power output (Bergstrom et al., 1967). Recently the LCHF diet has been proposed as an alternative dietary strategy for athletes to enhance sub-maximal endurance exercise performance, improve recovery from exercise and to optimize body composition (Volek et al., 2015; Volek and Phinney, 2012). Once habituated to a LCHF diet, athletes have a greatly enhanced capacity for lipid oxidation (Volek et al., 2016; Burke et al., 2016) and reduced reliance on glucose oxidation (Webster et al., 2016). This could theoretically improve endurance performance due to the relatively greater amount of energy stored as lipid compared to carbohydrate. LCHF-adapted athletes following a ketogenic diet have elevated resting levels of βHB (Volek et al., 2016); therefore βHB may act as an important oxidative substrate for these athletes during exercise. Furthermore, combining a ketogenic diet with resistance training results in increased lean mass gain compared to a low fat diet (Volek et al., 2002), and a decrease in body fat mass. This improves an athlete's power to weight ratio, which could benefit performance in many sports, including cycling, triathlon, gymnastics and athletics.

However, the use of LCHF diets in athletes has been harshly criticised by some sports scientists (Burke and Kiens, 2006; Burke, 2015). This is because, despite the theoretical

benefits, no studies have yet shown an improvement in performance with a LCHF diet (reviewed in Burke (2015)). In fact, LCHF diets may even be detrimental to performance as glycolytic metabolism can be compromised (Stellingwerff et al., 2006). Even in endurance events where the majority of the effort is sub-maximal, such as stage racing in cycling, the outcome can be dependent on a high intensity (glycolytic) effort at a crucial moment. This concern was supported by a study that attempted to mimic a competition scenario saw impairment in 1km (but not 4km) sprint performance (Havemann et al., 2006). A further possible drawback of a LCHF diet for athletes, is an increase in mitochondrial uncoupling protein expression and a corresponding decrease in oxygen efficiency, which has been linked to a decrease in physical and cognitive performance in humans and rodents (Edwards et al., 2011; Murray et al., 2009; Burke et al., 2016).

Other dietary strategies have been developed in attempts to harness the beneficial metabolic adaptations of the LCHF diet and avoid any detrimental adaptations. One example is following a period of LCHF diet by carbohydrate loading, but whilst the improvements in the capacity for lipid oxidation seen with a LCHF diet persisted following carbohydrate feeding (Burke et al., 2002), this intervention did not ultimately improve performance (Carey et al., 2001). Therefore, whilst there is anecdotal and circumstantial evidence for use of LCHF diets in athletes, a beneficial effect on endurance performance has yet to be demonstrated.

1.4.3 Summary: nutritional ketosis

It is well known that the macronutrient composition of the diet can be manipulated to promote ketogenesis without the need to fast. Variants of the ketogenic diet have come and gone over the years and have proven a consistent source of controversy. It is now becoming apparent that a ketogenic LCHF diet does not pose an undue risk to health; it may, in fact, even be advantageous. Ketogenic diets have been trialled as a therapy in several clinical conditions, and evidence is accumulating from animal studies and *in vitro* work hinting at further applications. In many cases it is unclear if benefits seen as a result of a LCHF diet

are purely due to changes in macronutrient consumption, or metabolic effects of ketone themselves.

The difficulties experienced by patients and their carers in maintaining long-term compliance with a ketogenic diet cannot be over-stated. The diet is perceived as unpalatable and can cause several short-term side effects such as constipation, acidosis, hypoglycaemia, dehydration and lethargy (Branco et al., 2016). Maintaining ketosis can be difficult as excess protein or even a small amount of carbohydrate consumed can inhibit endogenous ketone production (Amari et al., 1995). Finally, increasing ketone production and adaptations favouring peripheral ketone utilisation can take weeks; during which time unpleasant hypoglycaemic symptoms may be experienced (Zhang et al., 2013). Therefore, there is a need for alternative strategies to induce a sustained therapeutic ketosis, in order to harness the broad benefits of ketone metabolism.

1.5 Exogenous ketosis

There are no naturally occurring sources of dietary ketone bodies; however, synthetic ketones (or ketone precursors) are a potential alternative to dietary manipulation to achieve ketosis. Exogenous ketone supplementation creates a novel physiological state, where ketone bodies reach a level usually seen after prolonged fasting, but with no shortage of other dietary macronutrients. This section will introduce the forms of exogenous ketone supplements that have been used to this point to create "fed ketosis."

1.5.1 Ketone salts

Several investigators have used intra-venous (IV) infusions of ketone salts, such as sodium hydroxybutyrate or sodium acetoacetate, to artificially elevate blood ketone levels (Balasse and Ooms, 1968; Sherwin and Felig, 1975; Fery and Balasse, 1988; Keller et al., 1988; Mikkelsen et al., 2015). However, whilst their results give us important insights into the

interaction between ketones and other metabolic fuels, the IV method of administration is impractical outside of a research or hospital setting. Ketone salts can also be consumed orally, although they have not achieved widespread use in this form. A small number of clinical studies that gave oral ketone salts have been conducted in children with inborn defects in metabolism (Plecko et al., 2002; Van Hove et al., 2003; Valayannopoulos et al., 2011). In these studies, levels of ketosis (0.4 - 2.5 mM) were lower than in 'starvation ketosis' (up to 8 mM); however, this may be because of the existence of optical isoforms of βHB (D- and L- βHB), with only the D- isoform detectable by standard enzymatic assay.

The chiral nature of βHB may have implications for the use of ketone salts. The phenomenon of optical isomerism (chirality) means that the two isoforms of βHB are not super-imposable onto their mirror image (Cotton, 2008). Optical isoforms do not necessarily interact equivalently with their surroundings (i.e. enzymes or substrates). A natural consequence of the synthetic chemistry that produces ketone salts is that the reactions yield a 50:50 mix of two optical isoforms of the ketone compound: D- and L- (or R and S). D-βHB is the primary isoform produced by endogenous ketogenesis and metabolised by the peripheral tissues; however, the metabolism of L-βHB is poorly understood. Whilst the consensus is that L-βHB is not readily oxidised by mammalian tissues (Webber and Edmond, 1977; Desrochers et al., 1992), an enzyme that could produce L-βHB was isolated from rat liver (Reed and Ozand, 1980) and in further experiments it was shown that L-βHB could be converted to physiological ketone bodies (D-βHB and AcAc) (Lincoln et al., 1987; Desrochers et al., 1992), or used for sterol bio-synthesis (Webber and Edmond, 1977).

A further potential issue with exogenous ketone salts is the concomitant administration of high amounts of inorganic cations, such as sodium (Na^+), potassium (K^+) or calcium (Ca^{2+}). The metabolism of βHB may leave a excess of residual alkali (cations), which can cause an acute metabolic alkalosis up to pH 7.6 (Cahill, 2006; Fery and Balasse, 1988; Muller et al., 1984). Furthermore, long term consumption of ketone salts and of high levels of inorganic ions may have harmful side-effects. A connection between dietary Na^+ and

hypertension has long been embedded in medical dogma; even ancient Chinese clinicians wrote that: "if too much salt is used in food, the pulse hardens" (Veith and Barnes, 2015). Over the years, it was widely believed that there was a direct, linear relationship between increasing salt consumption and high blood pressure (Dahl, 1960). This resulted in recommendations to limit dietary Na^+ (World Health Organisation, 2003; Institute of Medicine, 2005) but although several meta-analyses found that long-term reductions in Na^+ intake lowered the incidence of cardiovascular events (He and MacGregor, 2002; Strazzullo et al., 2009; Aburto et al., 2013), this has been challenged by recent investigators (Graudal et al., 2012; DiNicolantonio et al., 2013; Mente et al., 2016). Aside from increasing cardiovascular risks, recent evidence suggested that high Na^+ intake could also be linked to gastric cancer (D'Elia et al., 2012), osteoporosis (He and MacGregor, 2008) and urinary stones (Massey, 1995). Combining sodium-hydroxybutyrate with alternative βHB organic salts (i.e. K^+, Ca^{2+}, lysine, arginine) for ketone body delivery is a strategy that could decrease the impact of high sodium consumption, and may counter some of these unwanted side-effects (Beylot et al., 1994).

Despite the uncertainty surrounding the health implications of ketone salt administration, the convenience of oral ketones compared to infusions, and a growing interest in therapeutic ketosis has led to a resurgence in the investigation of oral ketone salts. There is a growing range of such products recently marketed as a nutritional supplement for athletes and followers of the ketogenic diet. Research in rodents has shown that these salts can raise blood ketone levels to 2 - 3 mM when given in combination with MCTs, but failed to raise blood ketone levels when given alone (Kesl et al., 2016). Therefore, further work to both develop alternative ketone salt compounds and investigate the physiological effects of existing ketone salts will shed light on the translational potential of these compounds as a source of exogenous ketone bodies.

1.5.2 Precursors for ketogenesis

Molecules that readily undergo conversion to ketone bodies can be given as dietary supplements to artificially produce ketosis without dietary manipulation. Two such precursors are MCTs and butanediol. MCTs act as a ready substrate for ketogenesis as they travel directly to the liver in the portal blood and are oxidised to form acetyl-CoA, which acts as a precursor for hepatic ketogenesis (Traul et al., 2000). MCT supplementation has been shown to raise blood ketone levels to 4-6 mM in rodents (Kesl et al., 2016) and 2.5 mM in humans (Henderson et al., 2009). The ketogenic properties of MCTs have lead to their inclusion as the primary ingredient in medicinal foods undergoing trials for neurodegenerative diseases (see above) (Henderson et al., 2009; Ohnuma et al., 2016). However, high levels of MCT consumption can cause severe gastro-intestinal (GI) distress (Henderson et al., 2009; Ivy et al., 1980), providing a significant obstacle in the use of MCT supplementation for ketosis in many people.

The butyl-alcohol RS-1,3-butanediol (RS-BD) can be consumed to undergo hepatic metabolism into βHB and create a non-fasted ketosis. "RS" refers to the dual presence of the two optical isomers of 1,3-BD, which is metabolised to give the two isoforms of βHB: L- and D-βHB. It was shown *in vitro* (Desrochers et al., 1992), rodents (Tobin et al., 1978), and humans (Kies et al., 1973) that intra-gastric RS-BD delivery elevates D- βHB levels up to 15 mM. However, animal studies have shown lethargy and sedation following RS-BD at high concentrations (Desrochers et al., 1995b). If elevation of blood RS-BD were to have the same effects in humans, this may manifest as cognitive and neuromotor symptoms similar to alcohol intoxication. While chronic consumption of RS-BD as a food additive has been deemed to be safe by the Food and Drug Administration (FDA) (Dymsza, 1975), high doses of 50 g of RS-BD may be required to sufficiently elevate blood ketone bodies. Therefore, the risk of side-effects mean that RS-BD supplementation alone is unlikely to be an acceptable strategy to achieve nutritional ketosis.

1.5.3 Ketone Esters

Administration of exogenous ketones as a synthetic ester compound offers an alternative method to elevate blood ketones, without a concomitant large cation or alcohol load. Ketone ester compounds are made up of a ketone body (either βHB or AcAc), joined by an ester bond to a molecule such as glycerol or BD; allowing delivery of ketone bodies to the circulation via the gastrointestinal route. The first published use of a ketone ester, was an ester of glycerol and acetoacetate, given parenterally to rats by Birkhahn and Border (1978) and Birkhahn et al. (1979). In 1995, Desrochers et al. (1995a) investigated parenteral and oral administration of RS-1,3-butanediol-acetoacetate mono- and di-ester compounds in pigs for their potential as a nutritional agent; these compounds elevated blood ketone levels up to 5 mM.

Currently, two different ketone esters are undergoing investigation in rodents and in humans, and have been shown to rapidly, predictably and significantly elevate blood ketone levels up to 7 mM (Clarke et al., 2012a,b; Murray et al., 2016; Kesl et al., 2016; Viggiano et al., 2016). Our group in Oxford has developed one of these ketone ester compounds: R-1,3-butanediol-R-3-hydroxybutyrate. This ketone ester is produced via trans-esterification of pure R-1,3-Butanediol (R-BD) to D-βHB using enzymatic catalysis in a process that avoids production of the 'non-physiological' L-βHB isoform (Figure 1.4). When consumed orally, the ester bonds are hydrolysed by ubiquitously expressed gut esterase enzymes (Van Gelder et al., 2000), releasing R-BD and D-βHB into the blood stream. R-BD is metabolised by hepatic alcohol and aldehyde dehydrogenase enzymes to form D-βHB (Figure 1.4). Both molecules of resultant βHB reach the systemic circulation, as the liver is unable to oxidise ketone bodies. Consumption of this ketone ester has been shown to reliably elevate blood ketone levels in humans with very few GI and systemic side effects (Clarke et al., 2012a,b; Shivva et al., 2016). The second ketone ester compound is under investigation at the University of South Florida. This is a di-ester of acetoacetate and RS-

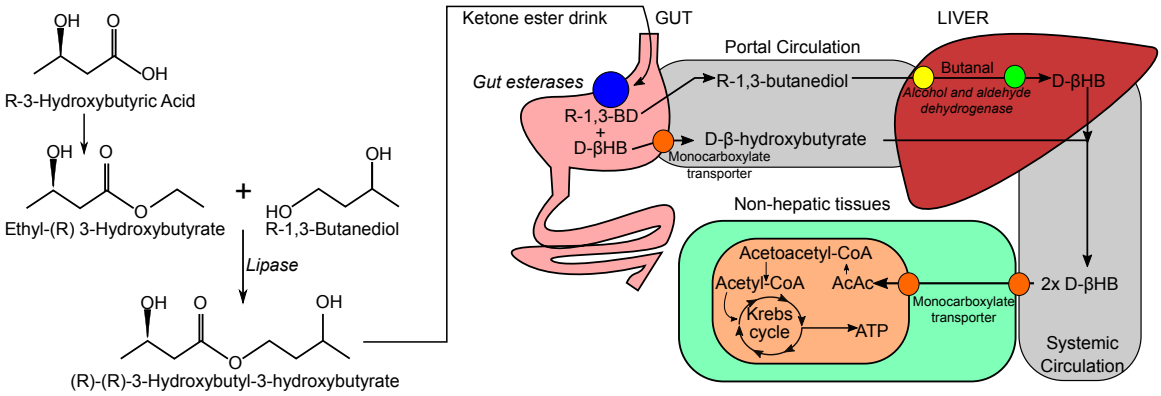

Figure 1.4: *Synthesis and metabolism of R-1,3-butanediol-R-3-hydroxybutyrate. R-1,3-butanediol and R-3-hydroxybutyric acid are esterified using lipase. This forms a ketone ester that can be consumed in a drink. The ester bond is cleaved by non specific gut esterase enzymes and the resulting R-1,3-BD and D-βHB are released into the portal circulation. Hepatic processing of R-1,3-BD results in a further molecule of D-βHB, which is released into the systemic circulation. AcAc= acetoacetate, ATP = adenosine triphosphate, D-βHB = D-β-hyrdoxybutyrate, R-1,3-BD= R-1,3 butanediol.*

BD (RS-BD-$AcAc_2$); in rodents this ketone ester raises blood βHB to 1 - 4 mM and blood AcAc to up to 5 mM (D'Agostino et al., 2013; Poff et al., 2014; Kesl et al., 2016; Viggiano et al., 2016). Published work has yet to demonstrate the use of this compound in human subjects.

Oral ketone ester consumption may be an ideal solution to achieve nutritional ketosis, being convenient and alleviating the need for an unpalatable diet. Furthermore, they circumvent the risks associated with high levels of salt consumption, and the GI distress caused by MCT administration. The presence of elevated ketone bodies, alongside normal post-absorptive levels of other substrates and hormones creates a novel physiological state that may afford the benefits of ketone metabolism in synergy with carbohydrate and lipid metabolism. Therefore, ketone esters could be both a translational and experimental tool for metabolic physiologists and professionals who are interested in studying and optimising human physiology.

The potential for ketone esters has been demonstrated by recent work that fed ketone

esters to rodents and humans and demonstrated an improvement endurance performance (Murray et al., 2016; Cox et al., 2016b). Ketone ester drinks, consumed with carbohydrate, resulted in a 2% improvement in endurance performance alongside a decreased reliance on carbohydrate stores and increased intra-muscular fat oxidation (Cox et al., 2016b). Therefore, ketone esters and other exogenous ketone bodies may avoid some of the issues of a LCHF diet for athletes, and could provide some of the benefits of ketone metabolism without compromising high intensity, glycolytic capacity.

1.5.4 Summary

Ketone supplements are not widely used outside of research, primarily due to concerns surrounding acute and chronic effects of high levels of salt or alcohol resulting from ketone salt or RS-BD consumption. MCT supplementation gives rise to a modest ketosis, but levels attained are limited by the dose of MCTs that can be consumed without GI distress. Ketone esters largely avoid these problems and have the potential for application in both clinical and sporting settings, as ketone metabolism may be a key factor in the benefits described as a result of a ketogenic LCHF diet.

1.6 Thesis context and outline

Ketone bodies provide a natural alternative metabolic fuel to the conventional substrates of carbohydrates, lipid and protein. They fulfil a crucial evolutionary role to protect the brain during dietary carbohydrate restriction, and are intrinsically a highly efficient oxidisable fuel. The widespread metabolic effects of ketone metabolism may be exploited to regulate and optimise human physiology in health and disease through direct effects of ketone bodies, their interactions with other substrates and modification of cellular signalling pathways (Veech et al., 2001). It is not known to what extent exogenous ketone supplementation will elicit the same effects as endogenous ketosis, as the physiological milieu of “fed keto-

sis" is radically different to the fasted or low carbohydrate state. Furthermore, exogenous ketone compounds may be metabolised differently by the body. Before the translational potential of ketosis induced by ketone drinks can be investigated, it is essential to understand the metabolic and physiological effects of the currently available exogenous ketone compounds. The work in this Thesis aimed to:

1. Study the metabolic effects of exogenous ketone ester and ketone salts.

2. Investigate factors that may alter the metabolic effects of the ketone ester: R-1,3-butanediol-R-3-hydroxybutyrate. This included characterisation of the repeatability of blood ketone and metabolite kinetics and the effect of food and of existing hyperketonemia on ketone ester uptake and elimination.

3. Describe the side-effects of ketone drinks, including changes in appetite and the associated GI and systemic symptoms.

4. Examine the current use of nutritional supplements in athletes in order to provide the context for use of ketone drinks as a performance-enhancing supplement in sport.

5. As individuals consuming ketone drinks may have replete carbohydrate (glycogen) stores, the effect of glycogen levels on ketone oxidation was assessed in the isolated perfused heart.

Chapter 2

General Methods

2.1 Participants and screening

The studies in Chapter 3, 4 and 5, received ethical approval following review by external NHS Research Ethics Committees: London Queen's Square Research Ethics Committee (14/LO/0288) and South West (Frenchay) Research Ethics Committee (15/SW/0244).

All participants were provided with detailed study information, protocol description and study time-line more than 24 h before the consent process, and signed a consent form prior to any study procedures. Participants were free to withdraw from further participation at any time. They also completed a confidential questionnaire and medical screening form. Participants were healthy, aged 18 - 60, non-smokers, not on regular relevant medication and with no history of major illness. Body composition was assessed using a Bioelectrical Impedance Body Composition Analyser (Bodystat ® 1500, Bodystat, Isle of Man, UK).

2.2 Randomisation, pre-visit and general visit procedures

Visit order was randomly allocated prior to commencement of each study. Study participants were asked to refrain from alcohol for the previous 24 h, to consume a similar pre-testing meal the night before each visit, and to arrive following an overnight (12 h) fast. Testing started between 0800-0830 h each day with a minimum interval of 72 h between each study visit. Fasting blood samples were collected prior to any study intervention. Following drink consumption, sampling points occurred at regular intervals. Water was permitted *ad-libitum*. Participants remained sedentary at the test facility and collected any urine that they passed during the study.

2.3 Study drinks

Each participants body weight was recorded at their first study visit and used to calculate the content of each drink, details of which are given in the relevant Chapter. Body weight

was monitored at each visit to check that no significant changes had occurred. Participants were given a maximum of 5 min to consume each drink to reduce bias in gut absorption.

2.4 Blood sampling and analysis

Blood samples (2 - 4 ml) were obtained using a 22 G venous catheter inserted percutaneously into an antecubital vein and immediately stored in vaccutainers coated with EDTA and/or lithium heparin (BD, New Jersey, USA). D-βHB was measured on whole blood using a handheld monitor and reagent strips (Precision Xtra, Abbott Diabetes Care, UK). Samples were then immediately stored on ice, centrifuged (1000-2000 xg for 10 min) and duplicate plasma aliqouts stored at -80°C until further analysis. Urine passed during the study was collected in plastic containers (Simport, Quebec, Canada), total volume was recorded and 1 ml aliquots were frozen and retained for analysis.

Plasma glucose, non-esterified fatty acids (FFA) and triacylglycerol (TG) and urinary D-βHB were assayed using a commercial semi-automated benchtop analyzer (ABX Pentra, Montpellier, France). Insulin was measured using a commercially available ELISA assay (Mercodia, Uppsala, Sweden).

2.5 Data processing and statistical methods

The kinetic parameters of βHB were calculated using the same procedures for each study. Area under the curve (AUC) of plasma βHB for each participant in each condition was calculated using the trapezium rule. The initial rate of appearance of βHB in the first 30 min of each experiment was calculated using measured D-βHB levels at baseline and at 30 min for each individual. D-βHB excretion was calculated using the urine D-βHB concentration and total volume.

Statistical analysis of all data used Prism 6^{TM} computer software. Significance was taken at $p < 0.05$ and all results are expressed as mean ± SEM, unless stated otherwise.

Initial data exploration was undertaken and normality was tested using D'Agostino and Pearson's test, if data did not satisfy this test, then non-parametric tests were used. For parametric tests sphericity (equal difference between the variance between groups) was not assumed when values were collected from the same subject in different conditions and so a Geisser-Greenhouse's correction was applied. If data were normally distributed and two means were compared, a student's t-test with equal SD was used. If data were not found to be normaly distributed, then a Wilcoxon matched-pairs signed rank test was used. Repeated measures one-way ANOVA were used to compare study conditions (3 or more means), or change in the level of a single variable, where data were normally distributed. Post-hoc Tukey corrections for multiple comparisons were performed as part of the ANOVA. If data were not normally distributed, Freidman's non-parametric test for repeated measures was used to with a post-hoc Dunn's test to correct for multiple comparisons. Two-way repeated measures ANOVA was performed to compare the change in a given variable both over time and between conditions. Tukey corrections were applied to determine the difference between conditions and Dunnett's corrections were applied when the row means (time) were compared to a single control row (time- baseline). Linear regressions were performed using Prism to give R square values for goodness of fit and correlations calculated separately for significance.

Chapter 3

Ketone ester and salt drinks for nutritional ketosis

3.1 Abstract

Ketone esters and ketone salts are two sources of ketones that may be included in the diet to raise blood βHB levels. Both compounds deliver the molecule βHB, but accompanied by a different chemical moiety. Therefore, they may have different kinetic parameters and disparate effects on ion flux, acid-base balance and metabolism. The work in this Chapter aimed to investigate the effects of equimolar βHB given as either a ketone ester (R-1,3-butanediol-R-3-hydroxybutyrate) or ketone salt of sodium and potassium βHB, on blood levels of βHB, FFA, TG, glucose, electrolytes (Na^+, K^+, Cl^-, HCO_3^-), and blood pH, as well as urinary βHB excretion. The ketone ester resulted in 2-fold higher peak concentrations of blood D-βHB and up to 2-fold higher total D-βHB appearance than the salt. In attempting to determine the reason for the considerable differences in blood D-βHB, it was found that the ketone salt was racemic, in that it elevated both D- and L-βHB. When the L- isoform was accounted for, the total βHB levels were similar between compounds, but the L-isoform was only slowly removed from the blood and remained elevated, despite a fall in the level of D-βHB. Plasma glucose, FFA and TG concentrations fell significantly, and to a similar extent following consumption of all ketone drinks. The salt contained a large ion load, and caused a rise in blood Na^+; however, the ester also caused a rise in Na^+, along with a fall in K^+. To investigate this unexpected effect, a second study was undertaken and revealed that the ester drink caused a transient drop in blood pH to 7.31, whilst the salt raised pH to 7.42, although in this study the overall changes in electrolyte levels were similar between conditions. In conclusion, ketone ester and salt drinks both raised total blood βHB levels but with striking differences in the isoforms delivered. These ketone compounds had similar effects on metabolism and electrolyte levels, but disparate effects on acid-base balance.

3.2 Introduction

There are no substantial sources of ketone bodies found naturally in the diet, but infusions of ketone salts have been used to rapidly elevate levels of blood βHB in studies of ketone metabolism (Keller et al., 1988; Balasse and Ooms, 1968; Fery and Balasse, 1988). Such infusions are invasive, cumbersome and require clinical supervision to ensure blood ketone levels remain stable. Dietary ketone compounds have recently become available as nutritional supplements in the form of ketone salts and ketone esters, but their uptake, kinetics and effects on human physiology and metabolism have not been fully characterised.

'Dietary' ketone salts have been included in parenteral feeds in a small number of clinical studies (Plecko et al., 2002; Van Hove et al., 2003; Valayannopoulos et al., 2011); however, concerns surrounding high levels of salt consumption have prevented their wider use. Increasing sodium chloride consumption from 5 - 6 g per day to 9 - 12 g per day has been linked to several health complications (He and MacGregor, 2008), including hypertension, increased risk of cardiovascular disease (Strazzullo et al., 2009) and gastric cancer (D'Elia et al., 2012). The health implications of ketone salt consumption are speculative, as no studies have yet characterised the acute effects of ketone salt consumption on levels of βHB or levels of metabolites and electrolytes.

Ketone monoester and diester compounds that deliver βHB or acetoacetate accompanied by a ketone precursor, such as butanediol, may circumvent some of the problems of inorganic ion consumption. The first oral ketone ester compounds (1,3-butanediol-acetoacetate mono- and di-esters) effectively raised blood ketone levels to 7 mM in pigs (Desrochers et al., 1995a,b). More recently, Clarke et al. (2012b) described the efficacy of the first ketone ester for human consumption: (R)-3-hydroxybutyl (R)-3-hydroxybutyrate. Although this ester could be safely consumed to achieve levels of ketosis up to 3 - 4 mM (Clarke et al., 2012a,b), the short-term physiological and metabolic effects of its consumption have not been fully examined.

A well-known metabolic complication of endogenous ketosis is acidosis (Laffel, 1999; Keene, 2006). Normally, pH is tightly regulated to remain within the range of pH 7.35 - 7.45, and the systemic effects of large deviations (for example pH less than 7.1 or above 7.6) are well known (Orchard and Cingolani, 1994). There are two traditional approaches to physiological acid-base buffering mechanisms. Firstly, the Henderson-Hasselbalch approach, which centres on the inter-conversion of bicarbonate, carbon dioxide, water and protons as the primary buffering system in the blood (Henderson, 1908). Secondly, the Stewart approach, which considers that blood pH changes (by water dissociation) in response to changes in fully dissociated electrolytes ('Strong Ion Difference,' Stewart (1978)). Both are likely to be pertinent in complex biological systems, in which multiple factors influence dissociation and hence proton concentration. Previous investigators have demonstrated a slight increase in blood pH with ketone salt infusions (up to pH 7.48) (Muller et al., 1984; Fery and Balasse, 1988), indicating that there may be differences between endogenous ketosis (causing acidaemia) and exogenous ketosis (causing alkalaemia). Therefore, the work undertaken in this Chapter aimed to investigate the effects of two oral exogenous ketone compounds, an ester and a salt, on levels of circulating βHB, metabolites, electrolytes and on acid-base homeostasis.

3.3 Methods

3.3.1 Participants and study design

The study was approved by South West (Frenchay) Research Ethics Committee (15/SW/0244), and participants were recruited as described in Chapter 2.1. Firstly, a randomised, four-arm, cross-over study of n = 15 participants was undertaken to compare ketone levels following consumption of equimolar amounts of 'βHB equivalents' (i.e. the βHB anion or butanediol from ketone ester hydrolysis) in ketone ester or ketone salt drinks (Figure 3.1). Pre-test procedures and randomisation were as described in Chapter 2.2. Following an overnight

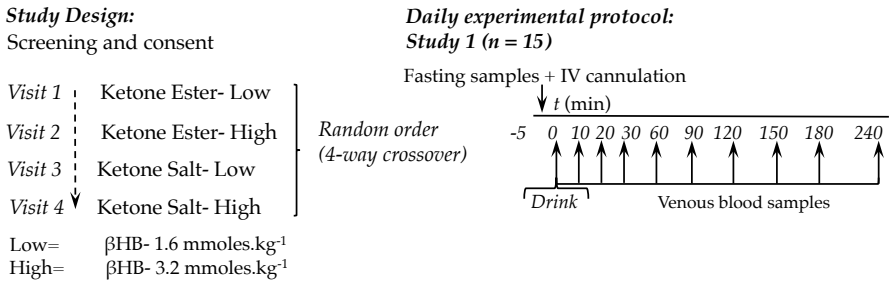

Figure 3.1: *Schematic illustrating Study 1 design and sampling schedule for each study visit.*

fast, participants consumed either 1.6 mmoles.kg $^{-1}$ of βHB (low dose), or 3.2 mmoles.kg $^{-1}$ of βHB (high dose) in the form of a ketone ester or a ketone salt (details below). Blood pressure was monitored at the start of each visit, otherwise each visit followed the pre-visit and general visit procedures described in Chapter 2.2-4. Venous blood samples were collected as shown in Figure 3.1.

Following analysis of the cross-over study results, a further group of participants (n = 7 for each drink) completed a second study to investigate the short-term effects of the ketone ester or salt drinks on blood electrolyte levels and on acid-base balance (Figure 3.2). Participants consumed 3.2 mmoles.kg^{-1} of βHB, with blood sample collection from a retrograde cannula in a dorsal vein of a gently heated hand. Samples were collected before the drink and then at regular intervals for 120 min; blood collected in this way is representative of arterial blood ('arterialised') and thus can be used for immediate measurement of blood gases and pH (Forster et al., 1972).

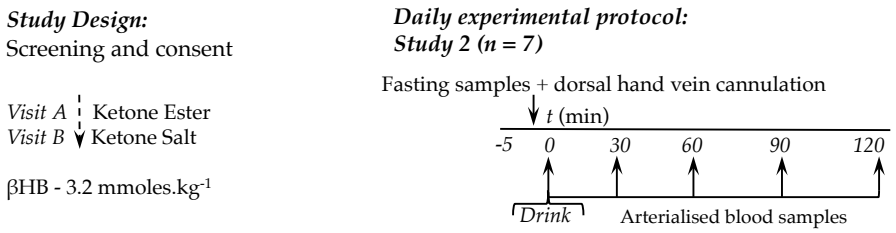

Figure 3.2: *Schematic illustrating Study 2 design and sampling schedule for each visit.*

Table 3.1: *Calculation of βHB moles in the suggested serving size of the ketone salt (PrototypeNutrition., 2016), and of volume of ketone ester required to deliver equimolar βHB. βHB M_r = 104 * = every 1 mole of ester delivers the equivalent of 2 moles of βHB. † = ketone ester M_r = 176 and density = 1.073 g/ml. ‡ standardised based on a 70 kg person consuming 30 or 60 ml of the ketone salt.*

	Salt		Ester	
	Low	High	Low	High
Drink volume (ml)	30	60	9.2 †	18.4 †
βHB (g)	11.7	23.4	11.7	23.4
βHB (moles)	0.11	0.22	0.11*	0.22*
βHB (mmoles.kg^{-1}) ‡	1.6	3.2	1.6	3.2
Na^+ (g)	1.6	3.2	0	0
K^+ (g)	1.6	3.2	0	0

3.3.2 Study drinks

The total βHB (mmoles.kg^{-1}) was calculated for a 70 kg person consuming 1x (30 ml), and 2x (60 ml) the manufacturers' suggested volume of the ketone salt, Ketoforce (PrototypeNutrition, Illinois, USA). This resulted in either 11.7 g (0.11 moles) or 23.4 g (0.22 moles) of βHB for each dose and a final weight adjusted amount of 1.6 mmoles.kg^{-1} or 3.2 mmoles.kg^{-1}. The drinks contained either $\approx$ 3.2 or 6.4 g of sodium and potassium combined (Table 3.1). The pH of the liquid salt was 10.5.

An equimolar amount of ketone ester was calculated, assuming that 1 mole of the ester (R-1,3-butanediol-R-3-hydroxybutyrate) delivered 2 moles of D-βHB, as hydrolysis of one mole of ester released 1 mole of D-βHB and one 1 mole of 1,3-butanediol, which underwent hepatic metabolism to D-βHB (Table 3.1). The pH of the liquid ester was 4.6.

Both drinks were diluted using water to a final volume of 300 ml and 6 g of an artificial flavouring (Symrise, Holzminden, Germany) was added to each drink, which contained 19 kCal with 4 g of carbohydrate (0.3 g sugars) (Nutritional data in Appendix A). Participants

were blinded to their drink allocation and drinks were colour, but not taste matched.

3.3.3 Sample collection and analysis

During the cross over study, blood samples were collected, stored and analysed as described in Chapter 2.4. Urinary pH was measured on all samples taken from the final volume after 4 hours of collection. Na^+ and K^+ were analysed at baseline and on 60 min plasma samples (as this time point represented peak levels of ketosis) using a clinical chemistry analyser with an indirect ion sensitive electrode (Abbott, Illinois, USA). Chiral analysis for plasma D-βHB and L-βHB was undertaken using gas-chromatography coupled mass spectrometry (LC-MS) on a subset of plasma samples (baseline, 30, 60, 90, 120, 180, 240 min) collected from a randomly chosen cohort of subjects (n = 5) who had consumed the high dose salt drink drink. This analysis was carried out by IPOS (Huddersfield, UK. See Appendix A).

During the second study, arterialised blood samples obtained from a heated hand vein were collected into heparinised blood gas syringes (Pico100: Radiometer, Copenhagen, Denmark) that included an anticoagulant pellet. Samples were stored on ice and analysed within 10 min for βHB, blood gases, pH and electrolytes using a clinical blood gas analyser (ABL: Radiometer, Copenhagen, Denmark).

3.3.4 Data processing and statistical methods

Statistical analysis was undertaken as described in Chapter 2.5. Detailed results of statistical comparisons are reported in Appendix A. The amount of L-βHB present in the blood following ketone salt drinks was calculated based on the enzymatically measured D-βHB concentration and the ratio of D/L βHB peak areas obtained through LC-MS for each sample. Four subjects were excluded from the urine analysis as they did not provide samples.

3.4 Results

3.4.1 Participants

Anthropometric measurements of study participants for the four-arm cross-over study and the two-arm pH study are shown in Table 3.2.

Table 3.2: *Anthropometric characteristics of study participants in the four-arm cross-over study (Study 1, n = 15) and the two-armed pH study (Study 2, n = 7) Values are mean and range.*

Characteristic	Study 1 n = 15	Study 2 n = 7
Age (y)	22 (20 - 34)	26 (22 - 34)
Height (m)	1.8 (1.6 - 2.1)	1.8 (1.6 - 2.1)
Weight (kg)	71 (57 - 110)	72 (60 - 110)
BMI (kg/m^2)	23 (20 - 29)	23 (20 - 25)
Sex (M/F)	9 / 6	3 / 4

3.4.2 Blood and urine ketone levels

Ketone ester consumption increased blood D-βHB concentrations to a C_{max} of 2.8 ± 0.2 mM at the high dose and to 1.4 ± 0.2 mM at the low dose. Ketone salt consumption gave a D-βHB C_{max} of βHB of 1.0 ± 0.1 mM at the higher dose and 0.8 ± 0.1 mM at the lower dose. Once the D-βHB C_{max} was reached, levels declined steadily over the remainder of the 3 h protocol (Figure 3.3 A). Ketone ester drinks resulted in higher D-βHB AUC than salt drinks at both matched doses (Figure 3.3 B, values in Table 3.3). Kinetic parameters of D-βHB and differences between arms following ketone drinks are summarised in Table 3.3 and Appendix A, Table A.1. βHB rate of appearance (R_a) was faster for both ketone ester drinks than ketone salt drinks. βHB R_a increased with dose following ketone ester consumption, but was identical for the low and high doses of ketone salt. The time taken

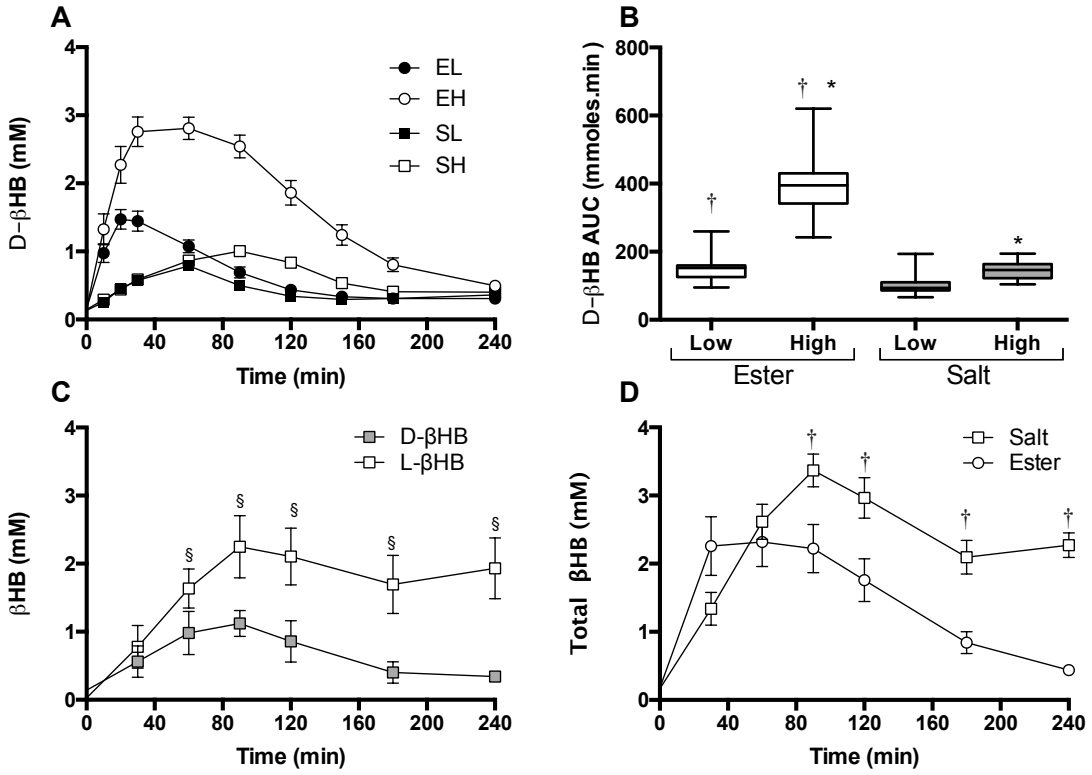

Figure 3.3: *Blood βHB following mole-matched ketone ester (KE) and ketone salt (KS) drinks at two doses in 15 subjects at rest. Values are mean ± SEM. A: D-βHB plotted over time. B: Box plot showing the mean, range and inter-quartile range of the D-βHB AUC for each drink. C: Measures of both D-βHB and L-βHB in a sub-sample of 5 subjects consuming the high dose of the ketone salt. D: Total (D+L) βHB in the same 5 participants consuming ketone ester or salt drinks. Abbreviations: EH, ketone ester high; EL, ketone ester low; SH, ketone salt high; SL, ketone salt low. † = $p < 0.05$ between ketone ester and ketone salt equivalent dose. * = $p < 0.05$ between low and high dose of same drink. § = $p < 0.05$ between D- and L-βHB isoforms.*

Table 3.3: *Summary of D-βHB kinetic parameters following equimolar ketone ester and ketone salt drinks at two doses in subjects at rest (n = 15). Abbreviations: R_a, Rate of appearance in 30 min following drink. † = p <0.05 between ketone ester and ketone salt equivalent dose. * = p <0.05 between high and low dose of same drink. Values are mean ± SEM*

		D-βHB R_a ($mmoles.min^{-1}$)	D-βHB T_{max} (min)	D-βHB AUC (mmoles.min)
Ester	Low	0.05 ± 0.01 † *	30 ± 4 † *	153 ± 11 † *
	High	0.09 ± 0.01 †	56 ± 7 †	397 ± 25 †
Salt	Low	0.02 ± 0.01	56 ± 4 *	101 ± 8 *
	High	0.02 ± 0.01	92 ± 5	147 ± 7

to reach maximal blood D-βHB levels (T_{max}) was significantly shorter for the ketone ester (high dose = 51 ± 4 min) than the salt (high dose = 92 ± 6 min) (p <0.001).

On finding that blood D-βHB concentration was ≈ 50% lower with the ketone salt compared to the ester, the pure salt and liquid ester were analysed using a chiral column for D- and L- βHB isoforms. It was found that the ketone ester compound consisted of purely the D- isoforms of βHB and butanediol, whereas the ketone salt contained both D- and L- βHB isoforms (Table 3.4). Plasma samples from subjects who had consumed the ketone salt then underwent the same analysis, and both isoforms were found to be significantly elevated after these drinks. Levels of the L- isoform were significantly higher in plasma than the D- isoform, reaching a level of ≈ 2.3 mM vs. 1.0 mM of D-βHB. Plasma levels of L-βHB remained significantly elevated for the duration of the experiment (p <0.001 to 240 min) (Figure 3.3 D). When the levels of D- and L- βHB were combined, from 90 min onwards the total βHB concentration was significantly higher (for these 5 subjects) following the salt compared to the ester, peaking at 3.4 ± 0.2 mM.

Table 3.4: *Chiral purity of pure liquid ketone ester and ketone salt*

	D-βHB (%)	L-βHB (%)
Ketone Ester	100	0
Ketone Salt	49	51

Table 3.5: *Urinary D-βHB excretion (g) and urinary pH in resting subjects (n = 15) following equimolar ketone ester and salt drinks at two doses. † = p <0.05 between ketone ester and ketone salt equivalent dose. * = p <0.05 between low and high dose of same drink. Values are mean ± SEM*

		Urinary D-βHB excretion (g)	Urine pH	Urine vs Blood βHB
Ester	Low	0.05 ± 0.01 *	6.37 ± 0.21	r = 0.67 *
	High	0.33 ± 0.08	5.80 ± 0.14	r = 0.64*
Salt	Low	0.08 ± 0.01 *	8.42 ± 0.05 †	r = 0.55*
	High	0.25 ± 0.03	8.48 ± 0.04 †	r = 0.45

Urinary D-βHB excretion was significantly greater with the higher dose of both ketone drinks; however, there were no significant differences in excretion between the two drinks at matched doses (Table 3.5 and Appendix A, Table A.2). D-βHB excretion represented less than 1.5 % of the total D-βHB consumed for both: ≈ 0.3 g was excreted following the high dose drinks which had contained over 20 g of βHB. Urine D-βHB was correlated to D-βHB AUC in 3/4 conditions 3.5.

Baseline urinary pH measured on a subset (n = 7) of participants prior to drink consumption was 5.7 ± 0.1. Urinary pH was more alkaline following ketone salt drinks, although pH was the same for both salt doses (Tables 3.5). By comparison, following ketone ester drinks, urine pH was similar to baseline (Table 3.5 and Appendix A, Table A.2).

3.4.3 Plasma metabolite levels

Ingestion of both ketone compounds significantly altered the levels of circulating metabolites seen during the study protocol (Appendix A, Table A.3). Plasma levels of FFA and TG fell significantly below their baseline value after consumption of both ketone ester and salt drinks (Figure 3.4 A and B). At 90 min following a ketone drink, FFA fell from the values seen at baseline to 0.24 ± 0.05 mM, and TG to 0.7 ± 0.1 mM. There were no differences between plasma FFA or TG concentrations following either study drink at either dose.

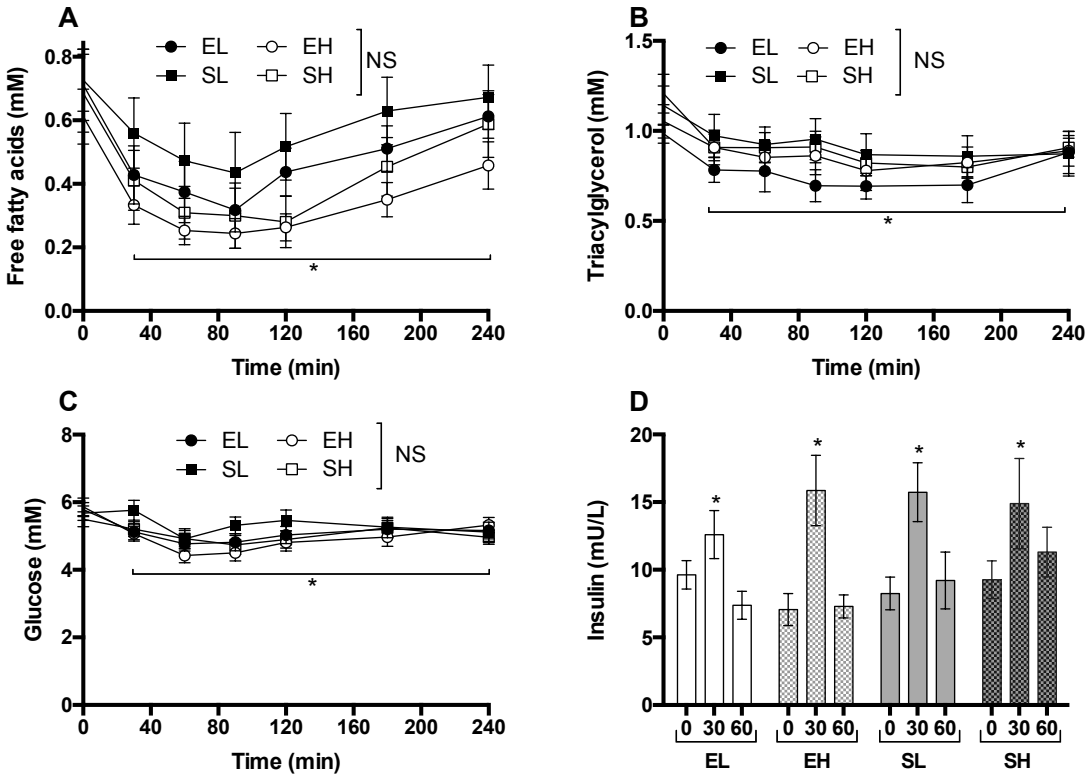

Figure 3.4: *Levels of plasma metabolites (non-esterified fatty acids, triacylglycerol and glucose) and insulin following equimolar ketone ester and ketone salt drinks at two doses in n = 15 subjects at rest. Values are mean ± SEM. A: Plasma FFA plotted over time for each condition. B: Plasma TG plotted over time for each condition. C: Glucose plotted over time for each condition. D: Plasma insulin measured before, 30 min post- and 60 min post- each study drink. Abbreviations: EH, ketone ester high; EL, ketone ester low; SH, ketone salt high; SL, ketone salt low. * = $p < 0.05$ effect of time. NS = no significant difference between study drinks.*

Plasma glucose decreased significantly following ketone drink consumption to reach a minimum value of 4.4 ± 0.2 mM after 90 min (Figure 3.4 C). There were no differences between plasma glucose levels following any study drink. All ketone compounds significantly increased plasma insulin at 30 min compared to baseline levels, although there were no differences between the levels reached for each compound or dose (Figure 3.4 D). Following a transient rise, insulin returned to basal levels by 60 min for all drinks.

3.4.4 Plasma electrolyte levels

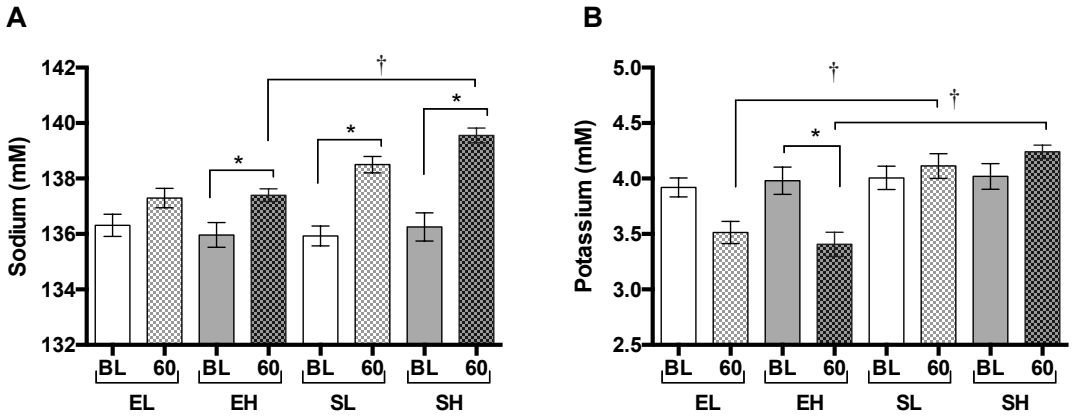

Figure 3.5: *Plasma sodium and potassium levels were measured at baseline and 60 min after consuming a ketone ester or ketone salt drink n = 15 subjects at rest. Values are shown as mean ± SEM. A: Mean plasma Na^+ prior to the ketone drink and 60 min post-drink; B: Mean plasma K^+ prior to ketone drink and 60 min post-drink. Abbreviations: BL, baseline; EH, ketone ester, high; EL, ketone ester, low; SH, ketone salt, high; SL, ketone salt, low. † = p <0.05 difference between E and S at equivalent time point. * = p <0.05 change from baseline with same ketone drink.*

Ketone salts increased plasma sodium levels 60 min after the drink by 3.3 mM and 2.6 mM after the high and low dose respectively ($p < 0.001$) (Figure 3.5 A and Appendix A, Table A.4). There was no increase in plasma potassium seen following both the high and low dose salt drinks ($p = 0.29$) (Figure 3.5 B and Appendix A, Table A.4). Despite containing no inorganic ions, ketone ester drinks significantly increased plasma sodium levels by 1.4 mM at 60 min after the high dose drink ($p = 0.005$) (Figure 3.5 A). Furthermore,

ketone ester ingestion significantly decreased plasma potassium by 0.4 mM with the low dose drink and 0.5 mM with the higher dose drink, reaching a minimum value of 3.5 $\pm$ 0.1 mM at 60 min ($p < 0.001$) (Figure 3.5 B). Whilst these changes were significantly different from baseline, both electrolytes remained within the normal reference range of 135 - 145 mM for sodium and 3.5 - 4.5 mM for potassium (Longmore et al., 2014)

3.4.5 Effects of ketone ester drinks on blood electrolytes and pH

In order to explain the observed differences in electrolyte levels, the experiment was repeated to allow repeated measurement of blood electrolytes and pH. Blood D-βHB rose following both drinks as seen previously, reaching a C_{max} of 1.1 $\pm$ 0.1 mM with the ketone salt and 2.4 $\pm$ 0.1 mM with the ketone ester. Following the ketone ester drink, there was a significant drop in blood pH to 7.31 $\pm$ 0.02, 60 min after the drink, representing acidaemia (Longmore et al., 2014) ($p = 0.002$) (Figure 3.6 A and B, Appendix Table A.5). There was an inverse relationship between blood pH and blood βHB ($r = -0.5$, $p = 0.02$) (Figure 3.6 C). Following the ketone salt drink, a significant increase in blood pH was seen between 60 - 90 min (90 min = pH 7.42 $\pm$ 0.1; $p = 0.006$), following the time course of the D-βHB appearance (Figure 3.6 A and B), although pH remained within the normal range. There was a direct relationship between blood pH and blood D-βHB ($r = +0.5$, $p = 0.02$) (Figure 3.6 D). Blood pH was significantly different between ketone drinks at all time points (effect of compound: $p < 0.001$; Appendix Table A.5).

Blood bicarbonate fell significantly from $\approx$ 23 mM to 18.0 $\pm$ 0.9 mM 30 min after ketone ester ingestion ($p = 0.002$) (Figure 3.6 E) and remained significantly lower than baseline until 120 min after the drink. Following the ketone salt drink, bicarbonate fell after 30 min to 20.6 $\pm$ 0.4 mM ($p = 0.035$), but then returned to baseline. From 30 min, bicarbonate levels were significantly higher following the ketone salt drink compared to the ketone ester (effect of compound: $p < 0.001$, Appendix Table A.5).

Similarly to the observations of Study 1, in Study 2 there were significant changes in

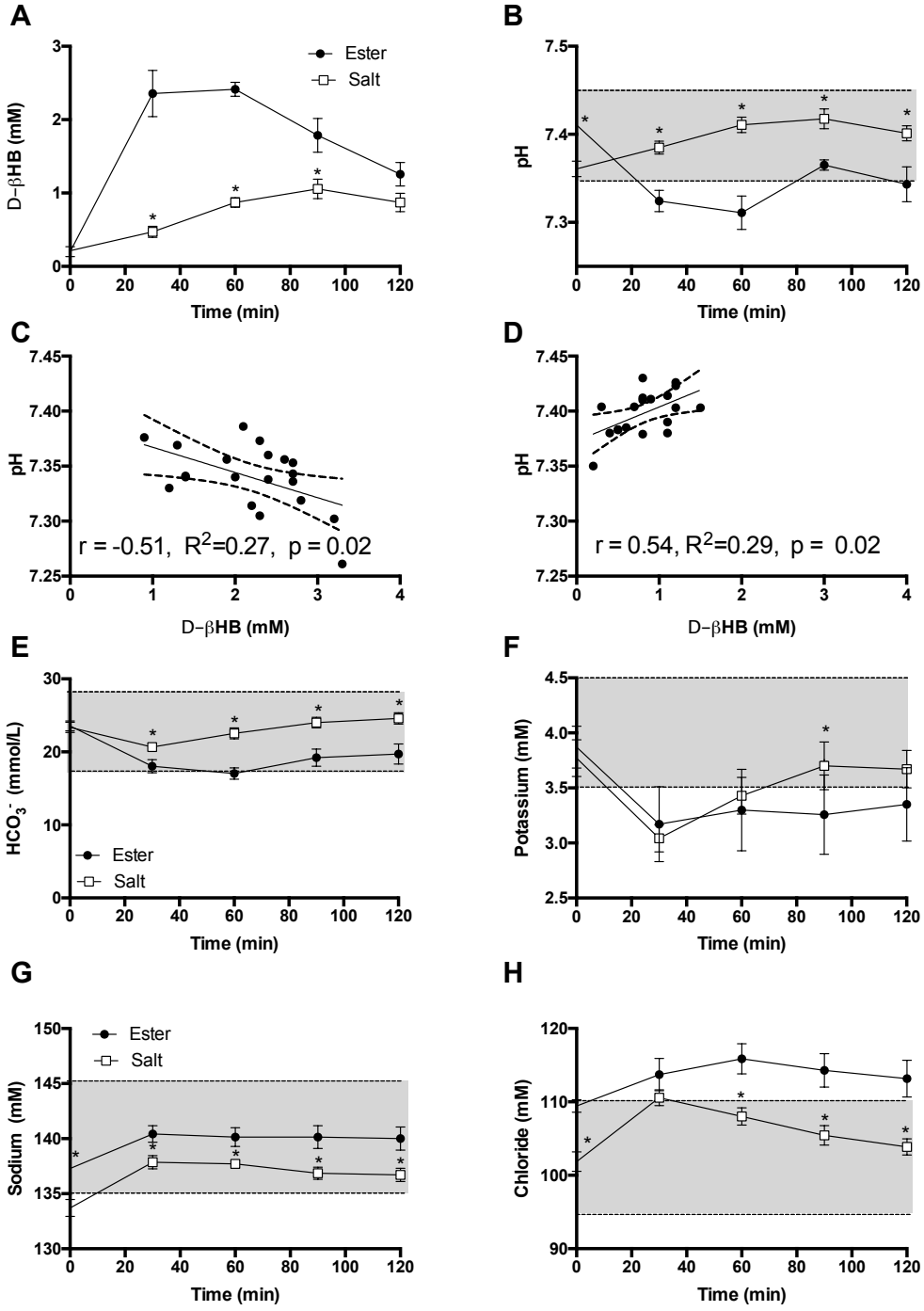

Figure 3.6: *D-βHB, pH, bicarbonate (HCO_3^-) and electrolytes were measured in arterialised blood samples from resting subjects (n = 7 for each condition) following a ketone ester or salt drink containing 3.2 mmoles.kg^{-1} of βHB. Values are means ± SEM. Shaded areas represent the normal reference range. A: D-βHB. B: Blood pH. C: Blood pH plotted against blood D-βHB for each individual in the first 60 min after the ketone ester drink (95% confidence limits shown). D: Blood pH plotted against blood D-βHB for each individual in the first 60 min after the ketone salt drink (95% confidence limits shown). E: Blood bicarbonate. F: Blood potassium. G: Blood sodium. H: Blood chloride.* = p <0.05 ester vs. salt.*

blood levels of potassium and sodium following ketone ester consumption (Figure 3.6 F and G, Appendix Table A.5). Potassium fell to 3.2 $\pm$ 0.3 mM (p = 0.003) after 30 min, and remained low for the duration of the experiment (Figure 3.6 F). Ketone ester consumption increased blood sodium levels from 136 mM at baseline to 140 $\pm$ 1 mM (p = 0.019) after 90 min and did not fall before the end of the study (Figure 3.6 G). Levels of chloride, the major blood anion, also increased following the ketone ester drink from 109 $\pm$ 1 mM to 114 $\pm$ 2 mM 90 min after the ketone ester (p = 0.018)(Figure 3.6 H).

Changes in blood electrolytes following a ketone salt drink were also as previously seen in Study 1. Blood sodium significantly increased to 138 $\pm$ 0.4 mM (p = 0.02) at 60 min, but potassium initially decreased to 3.04 $\pm$ 0.1 mM at 30 min(p = 0.045), before rising to baseline level at 60 min (3.43 $\pm$ 0.1 mM, p = 0.82)(Figure 3.6 F and G; Appendix Table A.5). Chloride also increased by $\approx$ 9 mM to 111 $\pm$ 1 mM ($p<0.001$) (Figure 3.6 H).

3.5 Discussion

3.5.1 Main findings

Both ketone ester and ketone salts elevated total blood βHB levels. The ketone ester delivered purely D-βHB, but the salt elevated blood levels of both D- and L-βHB, with the L- isoform being more slowly removed than the D- isoform. Both ketone compounds had similar effects on blood metabolites, significantly lowering plasma levels of FFA, TG and glucose and also on blood electrolyte levels. However, they differed in their effect on acid-base balance, with ketone ester consumption causing an acute acidosis, but ketone salt ingestion causing alkalosis (Summary Figure 3.7).

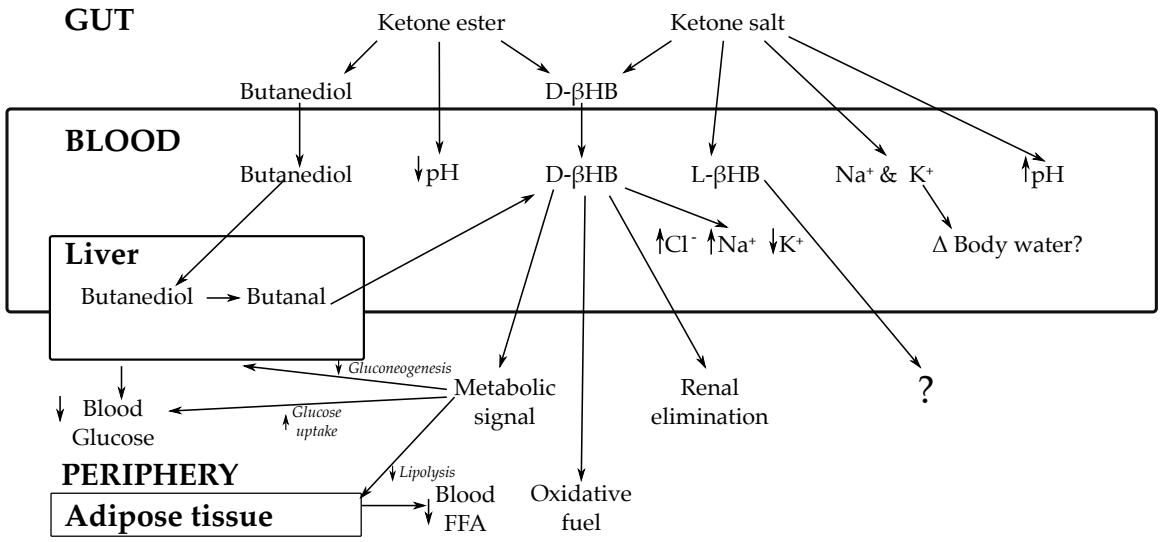

Figure 3.7: *Summary of the acute metabolic effects of ketone ester and ketone salt drinks.*

3.5.2 Blood βHB after ketone drinks

In agreement with previous investigation of this ketone ester compound (Shivva et al., 2016; Clarke et al., 2012b), blood D- βHB rapidly increased following consumption and exhibited non-linear (both first order and Michaelis-Menten elimination kinetics), as D-βHB was used as a metabolic fuel. When only D-βHB was measured it appeared that ketone salts did not achieve similar levels of ketosis; however, when the level of L-βHB was taken into consideration, total ketosis was similar for the two compounds. In fact, total βHB was higher following ketone salts, although this may have been due to accumulation of L-βHB whereas D-βHB was continually removed. Therefore, although the study drinks were equimolar with respect to total βHB, it remains unknown if blood D-βHB kinetic parameters for each compound would be similar if matched amounts of D-βHB were given in ester or salt drinks.

Following the salt drink, blood D- βHB increased slowly, before falling by the end of the study. However, L-βHB levels remained elevated for at least 4 hours, suggesting a low rate of removal. This is supported by the work of Webber and Edmond (1977), who suggested

that the L- isoform was less readily oxidised than the D- isoform and instead was preferentially used for cholesterol synthesis and *de novo* lipogenesis (Figure 3.8). Most previous work examining L-βHB metabolism was carried out in rodent models, so its metabolic fate in human tissue was unknown. This study raises some questions surrounding the utility and safety of L-βHB supplementation: is it a relevant oxidative fuel, and could it accumulate to dangerous levels with repeated ketone salt drinks? For these reasons, delivery of pure D-βHB may be preferable to racemic mixtures. This could be achieved through ketone ester compounds, which provide only D-βHB, or through purification of the racemic salt to increase the proportion of D-βHB delivered.

Whilst it appears that L-βHB is not rapidly removed from the blood for use as a fuel, it is unclear if it has other metabolic effects. The presence of high levels of L-βHB could alter the handling of D-βHB by competition for uptake via the monocarboxylate transporter, or by inhibiting βHB dehydrogenase, thereby slowing D-βHB oxidation. Furthermore, whilst it is known that D-βHB acts as both an intracellular and extracellular signalling metabolite, it is unclear if L-βHB would have equivalent effects. Some investigators have proposed that L-βHB may be more physiologically abundant than previously believed, and fulfill an intracellular signalling role to modulate the metabolic effects of D-βHB (Tsai et al., 2006). These investigators found a 2:1 ratio of D:L βHB in rodent heart homogenates, which increased to 8:1 in homogenates from diabetic animals. This shift was suggested to contribute to a reduced glucose utilization by the diabetic cardiomyocytes. Many questions about L-βHB remain unanswered and the metabolic effects of L-βHB in humans must be investigated to understand any risks.

The speed of initial D-βHB uptake differed between the ketone ester and salt (as indicated by the R_a and T_{max}), and was not affected by dose for the salt. Clearly, this may have been due to the presence of unmeasured L-βHB in the salt. However, differences may also have arisen as the hydrolysis of the ester bond of the ketone ester gave equimolar amounts of D-βHB and butanediol. Butanediol is a small, uncharged, polar molecule that can freely

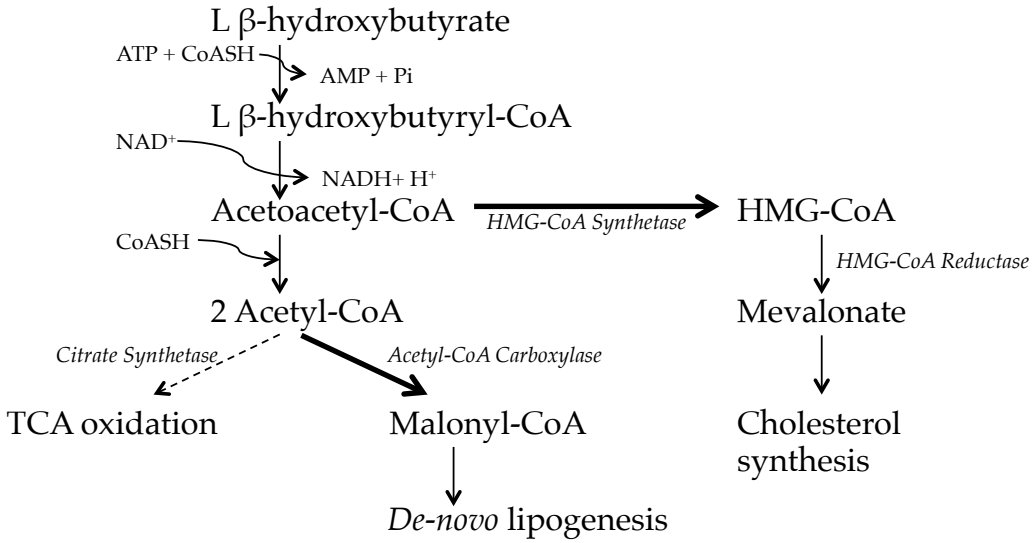

Figure 3.8: *Current understanding of the metabolic fate of L-βHB. Primary fate in rat tissue was conversion to sterols or use as a substrate for de-novo lipogeneis (Webber and Edmond, 1977), although a small amount may be oxidised (Desrochers et al., 1992). HMG-CoA = 3-hydroxy-3-methylglutaryl-coenzyme A.*

cross the gut epithelium into the portal bloodstream (Holford, 1987), and the rapid uptake and metabolism of butanediol may have resulted in a faster increase in βHB with the ketone ester than the salt. A further possibility is that diffusion of solutes (such as βHB) out of the ketone salt solution was slow, due to the high osmolarity of the salt compound in solution in comparison to the that of the ester.

3.5.3 Effect of ketone drinks on blood pH and electrolytes

The effects of the two ketone compounds on blood acid-base homeostasis were consistent with the pH of each solution, with the ketone ester being an acidic solution that caused acidosis, and the salt being an alkaline solution that caused alkalosis. The underlying mechanism may be explained by the different chemical moiety accompanying βHB in each ketone compound. The ketone salt provided βHB^- along with an inorganic cation (Na^+ or K^+), which would be fully dissociated in solution. When βHB^- was removed from the blood there may have been an electrolyte imbalance that resulted in the mild alkalinisation

of the blood, as seen previously with salt infusions (Muller et al., 1984; Fery and Balasse, 1988). In contrast, ketone ester hydrolysis provided one mole of βHB^- along with one mole of butanediol. Butanediol subsequently underwent hepatic metabolism to form the complete keto-acid (along with a proton), which may have lowered the blood pH (Figure 3.9). Other mechanisms for the changes in blood pH were considered, but did not adequately explain the divergence seen. Taking a 'Stewart' approach (Stewart, 1978) involved calculation of the 'strong ion difference' (*Strong ion difference* = ($[Na^+]$ + $[K^+]$ + $[Mg^{2+}]$) - ($[Cl^-]$); however, this was not different between drinks (data not shown). Whilst blood bicarbonate levels were different between drinks, as levels remained within the clinical reference range the changes were not of a sufficient magnitude to evoke carbonic acid (Henderson-Hasselbalch (Henderson, 1908)) as a mechanism. A final possibility was that a shift in body water occurred and altered pH (for example, a 'contraction' alkalosis or a 'dilutional' acidosis); however, the speed of the change in pH and similarities in electrolyte levels following both compounds suggest that this was not the case.

The changes in electrolyte levels following ingestion of both ketone compounds were similar in the second experiment, suggesting a shared mechanism. The factor common to both compounds was the rapid provision of βHB^-; the electrolyte shifts observed could have occurred as a result of βHB^- uptake and metabolism within cells (Figure 3.9). βHB^- is transported into the cell in a proton-linked manner via the monocarboxylate transporters (Halestrap and Price, 1999), resulting in a fall in intracellular pH, and increased proton and chloride efflux via sodium-proton exchange and/or sodium-bicarbonate-chloride transport. These processes could have caused the influx of sodium, which would be effluxed via the sodium-potassium pump (Palmer, 2014). The ultimate result would be the movement of sodium and chloride out of the cell and of potassium inwards. A similar decrease in blood potassium, with an increase in blood sodium, can occur during endogenous metabolic acidosis (Palmer, 2014); however, it is unclear if the acute shifts seen here would persist with chronic exogenous ketosis or if compensation would occur.

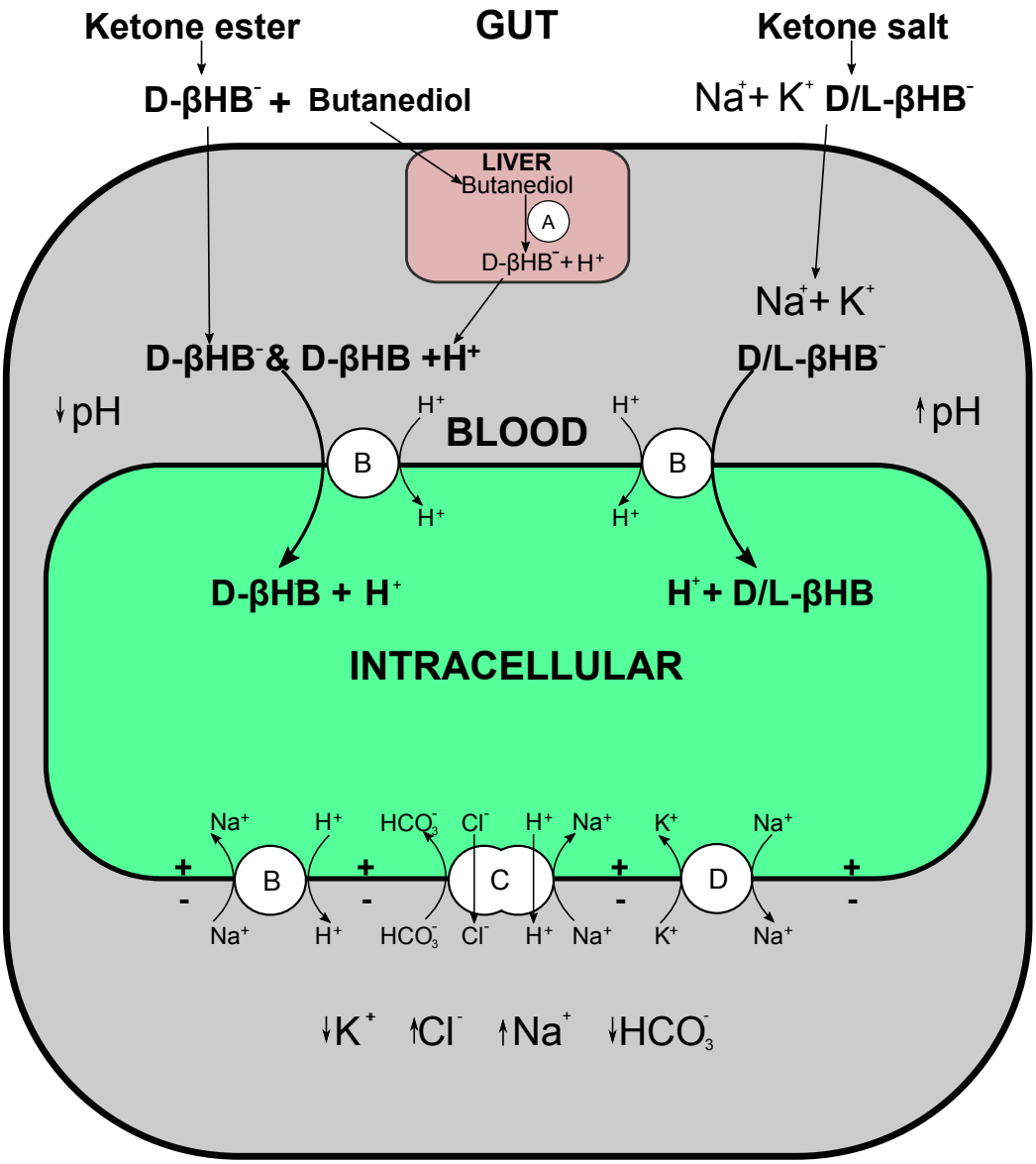

Figure 3.9: *Proposed mechanism for rapid changes in blood pH and electrolytes following ketone ester and salt drinks. A: Butanediol from ketone ester hydrolysis was metabolised to the complete keto-acid βHB + H^+, decreasing blood pH. B: D/L βHB^- were taken up into the intracellular compartment along with a proton through the monocarboxylate transporter, decreasing intracellular pH. This resulted in a electrolyte imbalance with the ketone salt, raising blood pH. C: The rise in intracellular protons resulted in proton efflux, in exchange for sodium influx via sodium-proton exchange. D: Further protons were extruded through sodium-proton exchange, linked to uptake of bicarbonate and chloride release. This raised extracellular chloride levels and decreased extracellular bicarbonate levels. E: As intracellular sodium was maintained by C and D, activity of the sodium-potassium ATPase (E) was maintained, giving an increase in extracellular sodium and decrease in extracellular potassium.*

It appears that the effects on blood electrolytes and blood pH observed here are a result of complex interactions between metabolic chemistry and the resultant physiological responses, manifested as ion fluxes. A likely source of variability is the use of 'arterialised' blood sampling in place of direct arterial samples. Amongst other things, arterial and venous blood differ in terms of their pH, partial pressure of CO_2 and bicarbonate levels (Longmore et al., 2014), therefore, in order to further explore the underlying mechanisms, future work should obtain direct arterial blood samples.

At the doses of ketone compound used in this study, changes in blood pH were closely related to changes in D-βHB levels. This suggests that there may be an advisable upper limit to ketone ester or salt ingestion, and that care should be taken using exogenous ketones in patients who may have a reduced ability to compensate. If either drink was consumed at high doses for an extended time, the resulting chronic perturbations to acid-base balance may cause medical complications, although alkalosis is generally considered to be more benign than acidosis (Kraut and Madias, 2010; Palmer and Alpern, 1997). Therefore, the effects of increasing doses, or prolonged consumption of ketone drinks on acid-base balance should be investigated and monitored to avoid any adverse effects.

A further risk of long-term use of ketone salt drinks is the physiological stress of high levels of inorganic ion consumption. Even though there were no significant changes to blood electrolyte levels following ketone salt drinks, the low levels of D-βHB resulting from the salt at both low and high doses may necessitate multiple salt drinks, or higher salt doses to bring blood D-βHB over 0.5 mM for a sustained time, which would further increase sodium and potassium consumption. Three 30 ml ketone salt drinks (the lower dose studied here) per day would result in the ingestion of a total of 19.2 g of Na^+ plus K^+ (PrototypeNutrition., 2016). The cumulative effect of high levels of ion consumption on the kidney may be damaging: elevating blood pressure, increasing the amount of protein in the urine and increasing the risk of kidney stones (du Cailar et al., 2002). A solution to the high sodium and potassium load associated with the ketone salt drink may be to produce a salt

of βHB$^-$ conjugated to an organic salt forming cation, such as arginine, lysine or histidine (Beylot et al., 1994).

3.5.4 Effect of ketone drinks on blood metabolites

Endogenous ketone bodies inhibit their own production through suppression of lipolysis, mediated by agonism of the PUMA-G nicotinic acid receptor in adipose tissue (Taggart et al., 2005). This anti-lipolytic effect is conserved with exogenous ketone bodies as infusions of ketone salts caused a fall in blood FFA and TG during ketosis (Balasse and Ooms, 1968). Here, a similar effect was seen with ketone drink consumption. Interestingly, there was no significant dose:response relationship between blood D-βHB and the changes in plasma FFA or TG, suggesting that inhibition of lipolysis did not increase with increasing D-βHB concentrations. It is unknown if L-βHB also acted as an anti-lipolytic signal. Maximal ketone-mediated inhibition of lipolysis may have occurred at low levels of D-βHB and not increased further with rising blood D-βHB levels.

The results of this study are in agreement with previous work that showed a fall in blood glucose as a result of ketone salt infusions (Balasse and Ooms, 1968; Mikkelsen et al., 2015). The underlying mechanism could be attributed to suppression of hepatic glucose output (Miles et al., 1981), a direct effect of ketone bodies on the release of gluconeogenic precursors from peripheral tissues (Sherwin and Felig, 1975), a ketone-mediated increase in insulin secretion (Biden and Taylor, 1983; Beylot et al., 1994) or through ketone bodies increasing peripheral glucose uptake (Kashiwaya et al., 1997). Preservation of hepatic carbohydrate and of gluconeogenic amino acids is in keeping with the evolutionary role of ketones: through provision of an alternative cerebral metabolic substrate and reducing the need for proteolysis to provide gluconeogenic substrate (Cahill, 1970).

Finally, consumption of all ketone drinks elevated plasma insulin levels. The small increase may have been due to the $\approx$ 0.3 g of sugar from the sweetener (4 g carbohydrate total); however, the relationship between ketone bodies and insulin secretion is not well

understood. Isolated pancreatic islets stimulated by ketones *in vitro* secreted insulin (Biden and Taylor, 1983) and insulin secretion may occur following exposure to exogenous ketones *in vivo* (Madison et al., 1964; Miles et al., 1981). However, Balasse and Ooms (1968) failed to reproduce these findings, although it was not a major end point of their experiments. Given the small amount of carbohydrate in the sweetener, it is impossible to confirm or exclude the possibility of an insulinotropic effect of exogenous ketone bodies.

3.6 Conclusion

In conclusion, ketone esters and salts consumed in drinks can raise levels of blood βHB in non-starving humans without the need for dietary manipulation. The ketone ester rapidly increased blood D-βHB, which was subsequently metabolised. By comparison, the ketone salt elevated both D- and L-βHB isoforms, with the latter only slowly removed from the blood. Due to molecular differences between ketone compounds, a disparate effect on acid-base homeostasis was observed. However, both ketone compounds had similar effects on levels of blood electrolytes and substrates. Therefore, it appears that not all exogenous ketone compounds are 'equal' in their metabolic and physiological effects, and this may have implications for their use in dietary ketosis.

Chapter 4

Repeatability of ketone and metabolite kinetics following ketone ester consumption

4.1 Abstract

The ketone ester, R-1,3-butanediol-R-3-hydroxybutyrate, is a novel dietary tool to elevate circulating D-βHB levels in humans, however it is unknown how much variability in ketone ester metabolism exists both within- and between individuals, and whether pre-existing exogenous ketosis alters ketone handling. Characterising the kinetics and repeatability of βHB levels following ester consumption in different states, such as following a meal or following previous ketone drinks, may provide insights into exogenous ketone body metabolism. The work in this Chapter aimed to study the variability of ketone kinetics and metabolite levels when ketone ester drinks were consumed following a standard meal or after an overnight fast. It also aimed to study ketone metabolism after feeding equimolar, isovolumetric amounts of the ketone ester as three consecutive drinks compared to continuous infusion through a nasogastric (NG) tube. Ketone ester drinks elevated blood βHB levels with a high degree of within- individual repeatability in both the fed and fasted state, although 1.4- fold more βHB appeared in the blood and peak βHB increased by 1 mM when fasted. Plasma AcAc, breath acetone and urinary βHB increased following the ketone drinks, but were the same if drinks were taken when fed or fasted. Plasma FFA, TG and glucose significantly fell and to a similar extent following all ketone ester drinks, irrespective of meal feeding. Finally, both serial ketone ester drinks and continual NG feeding of the ketone ester achieved a prolonged nutritional ketosis with no changes in ketone appearance or elimination with pre-existing ketosis and the same total uptake with both administration methods. In conclusion, these results provide an insight into factors which may affect the metabolism of exogenous ketone compounds.

4.2 Introduction

The kinetics of βHB following consumption of the ketone ester, R-1,3-butanediol-R-3-hydroxybutyrate, depends on the rate of βHB appearance due to hydrolysis, absorption and hepatic conversion of butanediol into βHB, and βHB disappearance through metabolism or excretion. These processes may be affected by anthropometric or environmental factors that result in differences in blood βHB profiles between and within individuals, despite equivalent amounts being consumed. As the results of Chapter 3 illustrated that ketone ester consumption raised D-βHB in a dose dependant fashion, the objective of this study was to investigate whether this compound could deliver reproducible levels of βHB under a range of conditions and in a range of subjects, as this has implications for its use in nutritional ketosis.

Lean body mass and sex are two anthropometric characteristics that are important co-variables altering blood βHB levels following ketone ester drinks (Shivva et al., 2016). Further variation between individuals may occur with expression of the different isoforms of gut esterases (Liederer and Borchardt, 2006), monocarboxylate transporters (Halestrap and Price, 1999), and alcohol and aldehyde dehydrogenases (Crabb et al., 2004). Each has distinctive kinetic properties that may influence the absorption and metabolism of the ketone ester, or its downstream metabolites. The degree of non-specific, random inter-individual variance in βHB kinetics following ketone ester drinks has yet to be quantified.

A key environmental factor which could influence ketone kinetics is meal ingestion. Even with a strictly standardised meal protocol, gastric emptying is variable, and may have a marked effect on small molecule absorption, particularly in the proximal small bowel (Brophy et al., 1986; Goo et al., 1987). As supplemental ketones are intended to bypass the need for starvation to achieve ketosis, ketone esters may be consumed together with normal meals, therefore it is vital to understand the effect of food in the gut on βHB kinetics.

The ratios between the physiological ketone bodies of βHB, AcAc and acetone that are

seen during endogenous ketosis may differ with exogenous ketosis. During endogenous ketosis where blood ketone levels are low, βHB is converted to AcAc in a 1:1 ratio (Laffel, 1999) and acetoacetate undergoes spontaneous decarboxylation to acetone at a rate proportional to its concentration (Reichard Jr et al., 1979). However, if a large amount of βHB is delivered alone, then equilibrium between the physiological ketone bodies may not be reached acutely. These ratios could be further altered if the metabolic and hormonal milieu is changed by the nutrient bolus of a mixed meal.

Metabolism of endogenous ketone bodies changes as ketosis increases and is prolonged, with ketones being increasingly used by the brain but decreasingly used in peripheral tissues (Cahill, 1970). It appears that the decrease in peripheral ketone use may be directly related to the levels of ketosis, rather than the manifold physiological changes seen during starvation, as ketone salt infusions illustrated that the metabolic clearance of ketones fell with increasing ketosis (above 8 mM) (Fery and Balasse, 1985). It is uncertain whether ketone kinetic profiles following a ketone ester drink would be changed if it were to be consumed on top of a pre-existing exogenous ketosis (between 1 - 3 mM), for example if administered in regular boluses or continuously, such as through a NG tube. Previous work using this ketone ester gave three milkshake based drinks a day for five days at three doses, to study the incidence of GI symptoms, without any adverse effects. However, this study did not record the kinetic profiles of blood βHB following the three drinks (Clarke et al., 2012b). As the ester is taken up through the gut rather than infused directly into the blood, any of the processes involved in its uptake or metabolism might also be altered by previous drinks. Ultimately, it is important to know if this ketone ester can maintain blood ketone body concentrations at an appreciable level (for example- >1 mM) without changes in ketone metabolism that could affect βHB appearance in the blood or lead to its accumulation.

By investigating the repeatability of delivered ketosis, the effects of a meal on ketone kinetics, and piloting two dosing methods to maintain ketosis, this study aimed to further

the understanding of physiological factors that affect the metabolism of exogenous ketone bodies, and to provide practical information that could inform the future use of the ketone ester.

4.3 Methods

4.3.1 Participants and study design

The study was approved by London Queen's Square Research Ethics Committee (14/LO/0288), and participants were recruited as described in Chapter 2.1. A randomised, five-arm cross-over study was undertaken to compare inter and intra-participant repeatability of ketosis following ingestion of identical ketone ester drinks in the fed and fasted state (n = 16) (Figure 4.1). Pre-test procedures and randomisation were as described in Chapter 2.2. Following an overnight fast, participants consumed one ketone ester drink (2.2 mmoles.kg $^{-1}$ of ketone ester, representing 4.4 mmoles.kg $^{-1}$ of 'βHB equivalents') for four of the study arms, twice whilst fasted, and twice following a standardised meal. For one study arm the ketone ester drink was replaced by an isocaloric dextrose drink consumed whilst fasted (Table 4.1). Venous blood samples, urine samples and respired gas samples were collected as shown in Figure 4.1.

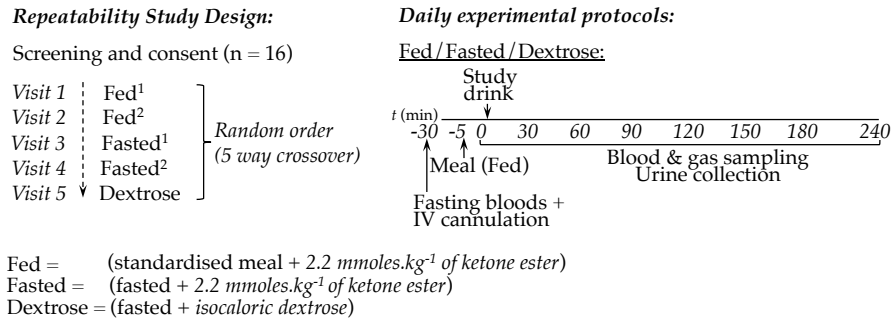

Figure 4.1: *Schematic illustrating the overall study design and the sampling schedule for each of the study visits (fed and fasted kinetics and repeatability).*

Additionally, a two-armed observational study was undertaken to compare ketosis following equivalent amounts of ketone ester given via serial ketone ester drinks or continuous NG infusion. These visits also followed the general procedures described in Chapter 2. 2 - 4, with venous blood samples collected as shown in Figure 4.2. Following an overnight fast, study participants (n = 12) consumed 6.6 mmoles.kg^{-1} of ketone ester total over nine hours, divided equally between three drink boluses (2.2 mmoles.kg^{-1}) spaced three hours apart. A further subset of participants (n = 4) were given 6.6 mmoles.kg^{-1} total of ketone ester through an NG tube over nine hours, also following an overnight fast. The NG tube (8 Fr- Corpack MedSystems, London, UK) was inserted into the stomach and its position confirmed using a thoracic radiograph, before infusion commenced. One third of the total ester volume was given as a bolus at the start of the protocol; infusion of the remaining ketone ester began after one hour and was administered at a continuous rate ($\approx$ 120 ml.h^{-1}) to ensure delivery of $\approx$ 0.55 mmoles.kg.h^{-1} of ketone ester for a further eight hours.

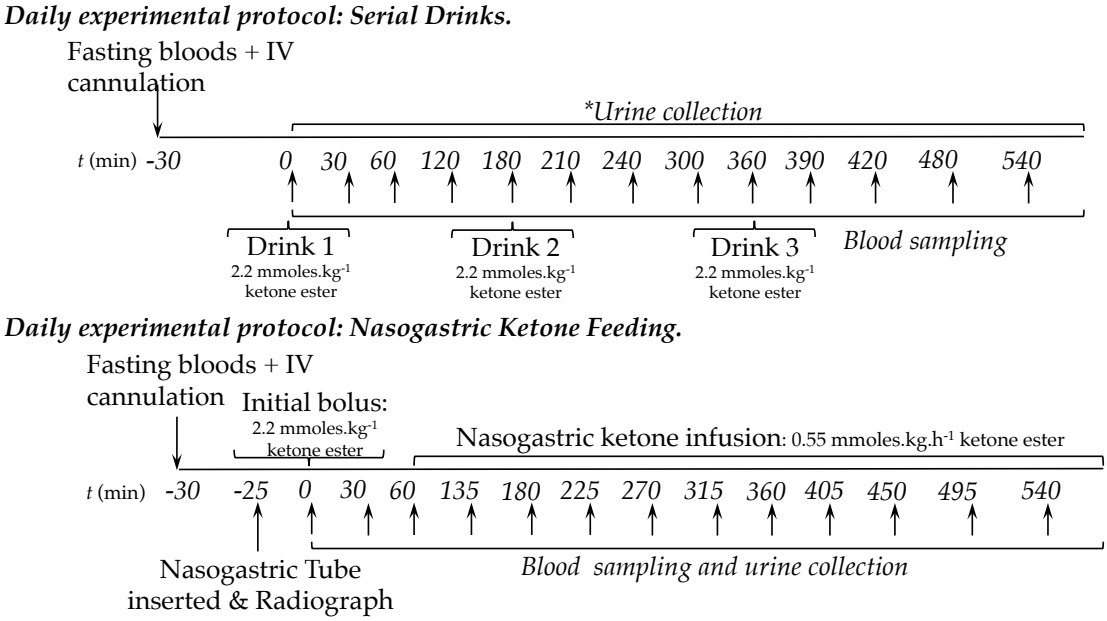

Figure 4.2: *Schematic illustrating the overall study design and the sampling schedule for each of the study visits (Serial drinks and NG infusion).*

4.3.2 Study drinks and infusions

In the cross-over study, the amount of ketone ester ingested by each participant was calculated as 2.2 mmoles.kg^{-1}. The ketone ester was diluted to a final volume of 500 ml using a commercially available citrus flavoured drink (Glaceau, New York, USA), which contained 65 kCal from 15 g of sugar (Nutritional data in Appendix B, Figure B.1). The energy content (kCal) of each participant's ketone ester drink was used to calculate the weight of powdered dextrose in their dextrose drink (Table 4.1). The drinks were colour and volume matched and participants were blinded to taste by addition of a bitterness additive to the dextrose drink. Investigators asked participants to identify their drink following consumption to assess the efficacy of the blinding process.

Each drink in the "serial drinks" visit contained 2.2 mmoles.kg^{-1} of ketone ester diluted to 500 ml with the same citrus flavoured drink as used before. For the bolus given at the start of the "NG feeding" visit, 2.2 mmoles.kg^{-1} of ketone ester was diluted to 500 ml with citrus water and administered through the NG tube using a syringe in under 5 min. A volume of ketone ester equating to 4.4 mmoles.kg^{-1} was diluted into 1 L with citrus drink, and 60 min after the initial bolus, infusion commenced using a clinical infusion pump (Alaris, New Jersey, USA) at 120 ml.h^{-1} via a nasogastric tube.

4.3.3 Standardised meals

During each 'fed' visit, participants consumed a standard meal immediately before the ketone drink (Table 4.1). The FDA guidelines for Food Effect Bioavailability studies (FDA, 2002) were used to design the standard meal, which consisted of porridge oats (54 g), semi-skimmed milk (360 ml) and banana (120 g), which gave 600 kCal with the macro-nutrient ratio: (Carbohydrate: Protein: Fat) 2:1:1.

Table 4.1: *Breakdown of calories in each study arm in study drinks and standard meal, ketone calories calculated for a 70 kg participant.*

	Fed	Fasted	Dextrose
Study Drink:			
Ketone ($kCal.kg^{-1}$)	1.9	1.9	-
Dextrose ($kCal.kg^{-1}$)	-	-	1.9
Sports Water ($kCal.kg^{-1}$)	65	65	65
Standard Meal:			
Carbohydrate (kCal)	300	-	-
Protien (kCal)	150	-	-
Fat (kCal)	150	-	-
Total	798	198	198

4.3.4 Sample collection and analysis

Blood samples were collected, stored and analysed as described in the Chapter 2.4. Acetoacetate was assayed on the 'fed' and 'fasted' plasma samples using enzymatic methods (Bergmeyer, 1965). In the 'fed', 'fasted' and 'dextrose' visits, respired gases were analysed for acetone at the time of blood sampling using a handheld electrochemical device (NTT DOCOMO, Tokyo Japan) (Tsuguyoshi et al., 2013).

4.3.5 Data processing and statistical methods

Statistical analysis was undertaken as described in Chapter 2.5, detailed results of statistical comparisons are reported in Appendix B. For the serial drinks experiment, the amount of βHB eliminated was approximated by calculating the area under the curve (AUC) from 60 min after the drink to 180 min after each drink, with βHB concentrations normalised to the value at 180 min.

Additionally, a linear mixed effects model was constructed in collaboration with a

statistician to estimate partitions of variance in R, using the lme4 and blme packages (Bates D, 2015; Chung et al., 2013). Linear mixed effects models represent generalized forms of repeated measures ANOVA, and allow for richer covariance structures (such as the presence of missing data, or correlated observations) than traditional approaches. Feeding state and visit number were considered to be fixed effects in this model, and inter-participant variability was considered as a random effect. Inter-participant variability was calculated according to the adjusted generalized R2 metric (as proposed by Nakagawa and Schielzeth (2013)), to partition variance between the fixed effects of feeding, inter-participant variability, and residual variability. The coefficient of variation for βHB C_{max} and AUC were calculated using the method of Vangel (1996). Following the initial analysis of βHB variability within participants (ie. visit 1 vs. visit 2), all subsequent results (including metabolites) are presented as the mean value for both visits in the same condition.

4.4 Results

4.4.1 Participants

Anthropometric characteristics of study participants are shown in Table 4.2.

4.4.2 Repeatability of blood βHB kinetics and effect a of meal

Consumption of a ketone ester drink elevated blood βHB concentrations with a high degree of between- and within-participant repeatability, however meal consumption significantly decreased the βHB C_{max} and AUC ($p < 0.001$ in both cases). βHB kinetic parameters following ketone ester ingestion are summarised in Table 4.3. In both conditions blood βHB rapidly rose and plateaued between 30 - 120 min with subsequent elimination following a non-linear pattern, consistent with previous work (Clarke et al., 2012a,b; Shivva et al., 2016) (Figure 4.3). Feeding significantly altered several aspects of βHB kinetics: the mean

Table 4.2: *Anthropometric characteristics of study participants who completed the five armed cross over experiment (Study 1, n = 16), the serial drinks experiment (Study 2, n = 12) and the NG feeding experiment (Study 3, n = 4). As some participants completed more than one experiment, the total n = 18. Values are mean and range. ND = not done.*

Characteristic	Study 1: mean (range)	Study 2: mean (range)	Study 3: mean (range)
Age (y)	28 (21-42)	28 (21-42)	26 (23-56)
Height (m)	1.8 (1.5-2.1)	1.8 (1.5-1.9)	1.8 (1.7-1.9)
Weight (kg)	73 (54-111)	70 (55-86)	66 (57-79)
BMI (kg/m^2)	23 (19-28)	23 (19-28)	21 (19-23)
Body Fat (%)	16 (4-33)	16 (4-33)	ND
Sex (M/F)	10/ 6	7/5	3/1

βHB C_{max} and AUC were decreased by 45 % and 37% respectively with concomitant meal ingestion (Figure 4.4 A and B) and the time course of BHB appearance (drink x time interaction) was significantly altered by feeding state (Appendix B, Table B.1). βHB C_{max} and AUC increased proportionally and were strongly correlated in both condition (Spearmans R: -0.6). The coefficient of variation of both βHB C_{max} and AUC was constant at $\approx$ 25%. There was no increase in blood βHB following the dextrose drink.

Table 4.3: *Kinetic parameters of D-βHB when n = 16 participants consumed equimolar ketone ester drinks, twice whilst fasted and twice following a meal. Mean values ± SEM * p <0.001 fasted vs. fed.*

		Fed			Fasted	
Parameter	Visit 1	Visit 2	**Mean**	Visit 1	Visit 2	**Mean**
C_{max} (mM)	2.2±0.2	2.2±0.2	**2.2±0.1**	3.2±0.2	3.3±0.2	**3.3±0.1***
T_{max} (min)	73±8	73±10	**73±6**	64±7	68±5	**66±4**
AUC (mmoles.min)	344±28	370±16	**357±16**	478±31	502±23	**490±19***

The βHB C_{max} in each condition had a similar between-subject range when fed (1.3 - 3.5 mM) and fasted (2.3 - 4.7 mM). The greatest difference in within-subject peak βHB

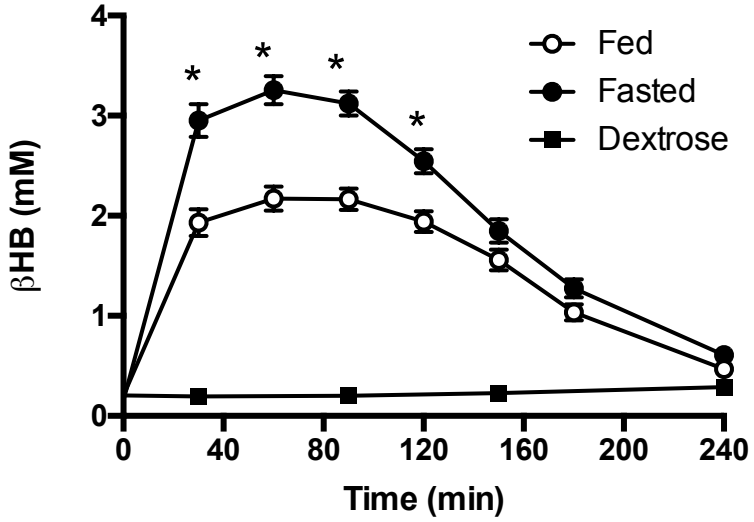

Figure 4.3: *Blood βHB following mole-matched ketone ester or isocaloric dextrose drinks in the fed and fasted state in n = 16 subjects at rest. Values are mean ± SEM. Significance denoted by: * p <0.001 fed vs. fasted.*

was 1.2 mM when fed and 1.9 mM when fasted (Figure 4.5). Approximately 61% of the variation in the data was attributable to feeding (fed vs. fasted), <1% to visit order, 16% to inter-participant variability, and the residual 24% variability explained by non-specific random effects. Therefore, the data strongly indicate that variability between participants was less than that within the population, and that accurate individual prediction of βHB C_{max} following a weight-adjusted ketone ester drink can be achieved.

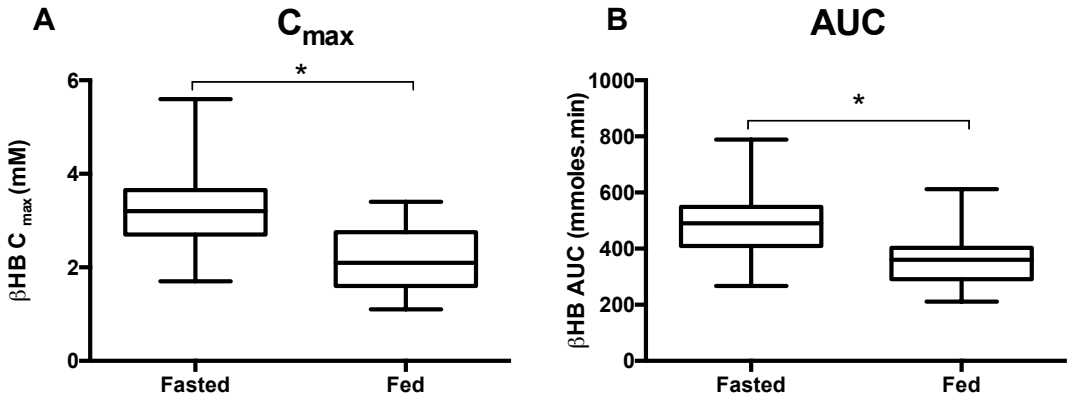

Figure 4.4: *Blood D-βHB following mole-matched ketone ester drinks in the fed and fasted state in n = 16 subjects at rest. A: Box plot showing mean, range and inter-quartile range of D-βHB C_{max} in each condition. B: Box plot showing mean, range and inter-quartile range of D-βHB AUC in each condition. Significance denoted by: * p <0.001 fed vs. fasted.*

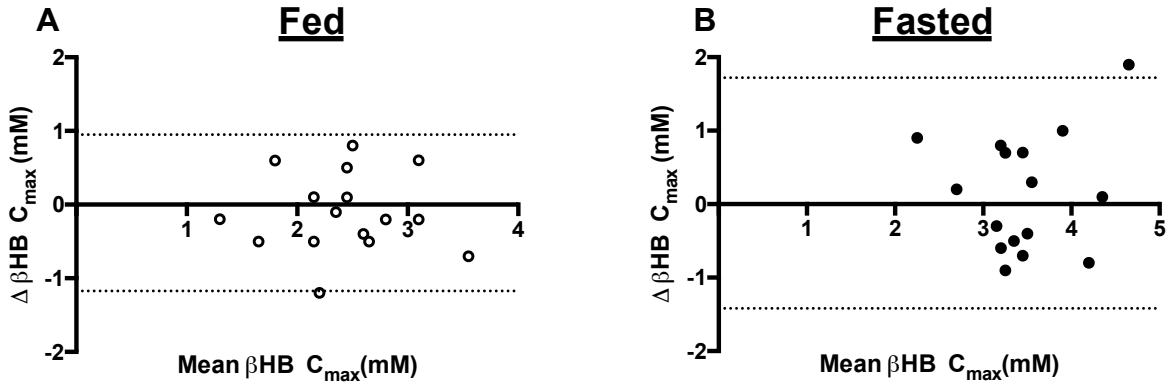

Figure 4.5: *Variability in βHB C_{max} levels when n = 16 subjects separately consumed two ketone ester drinks in both the fed and fasted state. A: Mean βHB C_{max} and difference between βHB C_{max} over two visits in fed condition (open circles). B:βHB C_{max} and difference between βHB C_{max} over two visits in fasted condition (closed circles). X axis = mean βHB C_{max} of the 2 visits (mM), Y axis = difference between βHB C_{max} in each visit. 95% confidence limits are shown.*

4.4.3 Plasma AcAc, breath acetone and urinary βHB excretion

Despite the disparity in βHB kinetics, consumption of the ketone ester significantly raised plasma acetoacetate, breath acetone levels and urinary βHB levels to a similar extent with no differences between the fed and fasted conditions (Figure 4.6 and Appendix B, Table B.1). Plasma AcAc appearance and elimination followed a similar time course to βHB overall, with the initial rapid rise followed by a plateau between 30-90 min at $\approx$ 0.76 mM in each condition, before steadily decreasing over the remainder of the protocol (Figure 4.6 A). There were no differences in AcAc levels between conditions. The ratio of βHB:AcAc was more reduced (i.e greater amount of βHB, the reduced form of ketone body) when fasted (6:1) compared to when fed (4:1) (p = 0.004) (Figure 4.6 B).

Breath acetone concentrations increased at a much slower rate than that of blood ketone bodies, reaching a plateau of approximately 65 $\pm$ 4 ppm at 120 min following ketone ester consumption in both conditions (Figure 4.6 C). Breath acetone did not decrease from this plateau and remained significantly elevated to the end of the protocol. There was no increase in breath acetone following the dextrose drink.

A small amount of βHB was excreted in the urine following the ketone ester in both conditions. The total amount of βHB eliminated in the urine was not altered by meal consumption (fed = 0.50 $\pm$ 0.6 g, fasted = 0.49 $\pm$ 0.6 g, p = 0.9) (Figure 4.6 D) and represented $\approx$ 1-2% of the ingested βHB. Urine βHB excretion was correlated to βHB AUC in both the fed (r =0.39, p = 0.05) and fasted (r = 0.62, p = 0.002) conditions.

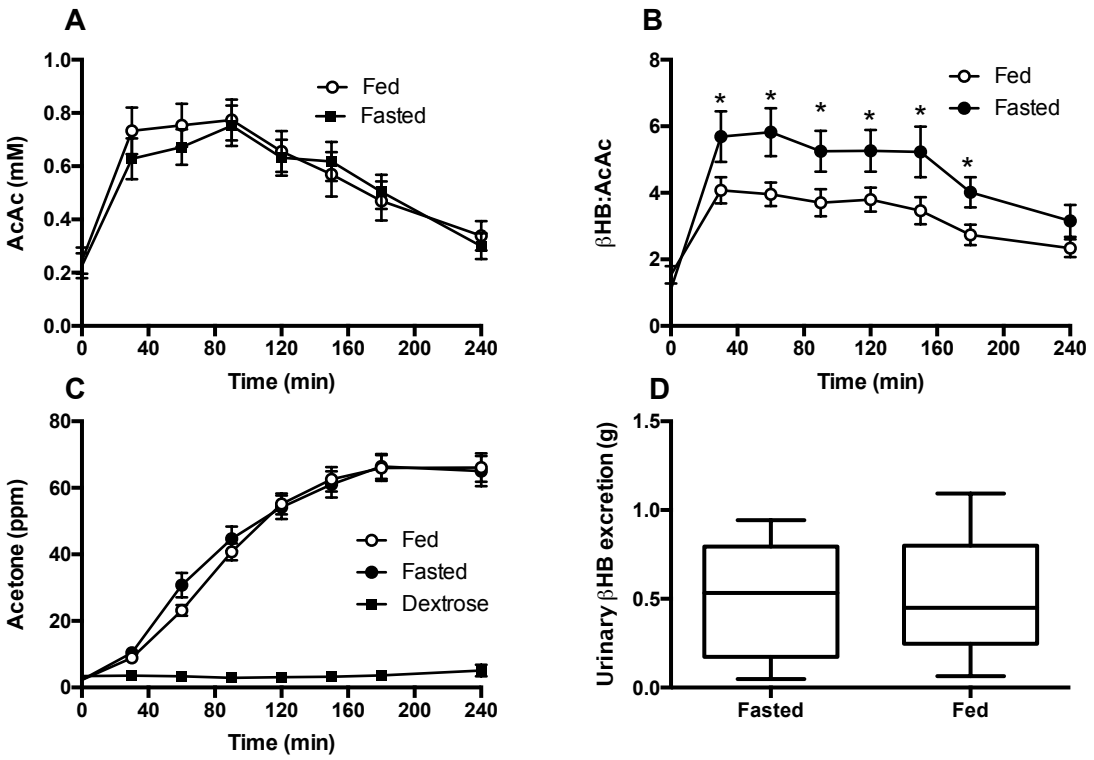

Figure 4.6: *Plasma acetoacetate (AcAc), breath acetone and urinary βHB excretion following mole-matched ketone ester (KE) or isocaloric dextrose drinks in the fed and fasted state in n = 16 subjects at rest. Values are mean ± SEM. A: Measured AcAc over time. B: Calculated ratio of βHB: AcAc over time. C: Measured breath acetone (ppm = parts per million) over time. D: Box plot showing mean, range and inter-quartile range of urinary βHB excretion in each condition. Significance denoted by: * p <0.05 fed vs. fasted.*

4.4.4 Plasma metabolite levels

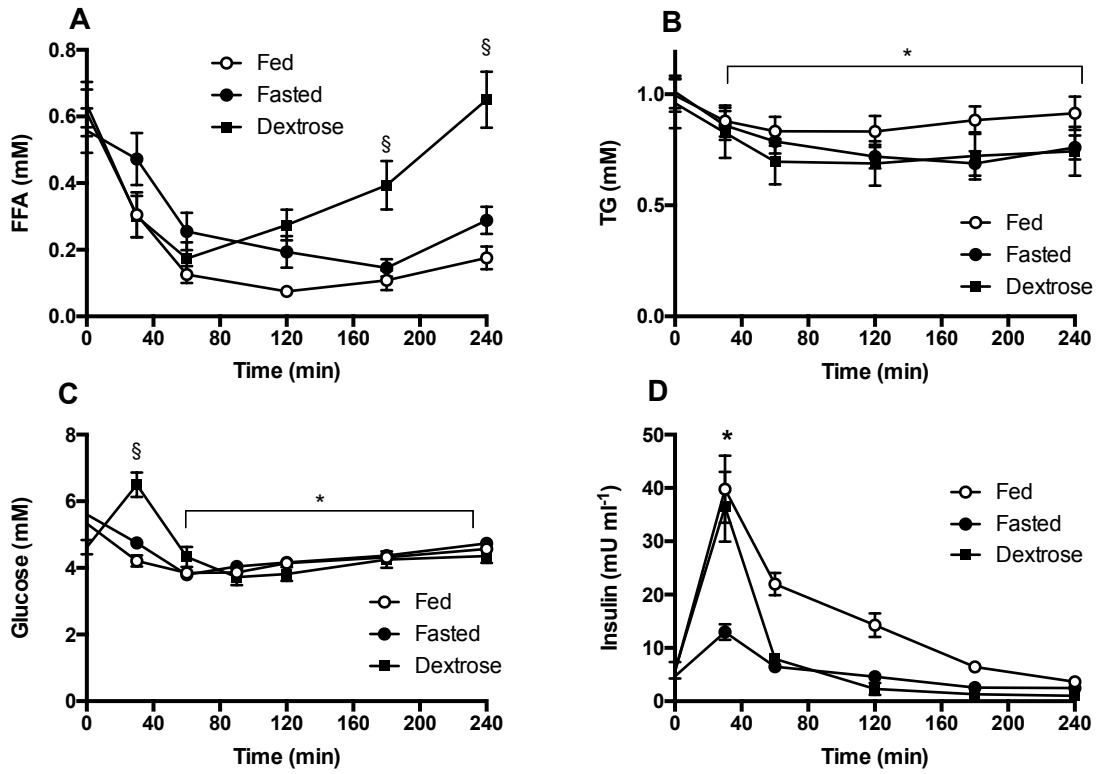

Figure 4.7: *Plasma metabolite levels following mole-matched ketone ester or isocaloric dextrose drinks when fed or fasted in n = 16 subjects at rest. Values = mean ± SEM. A: Plasma FFA over time, B: Plasma TG over time. C: Plasma glucose over time. D: Plasma insulin over time. Significance denoted: § = p <0.05 dextrose vs. KE; * = p <0.05 vs. baseline. NS = not significant.*

Consumption of all study drinks caused a significant fall in plasma concentrations of FFA, TG and glucose during the study (Figure 4.7 and Appendix, B Table B.2). Plasma FFA levels were significantly different between study conditions (p = 0.007) (Figure 4.7 A), being higher after the dextrose drink than following ketone drinks at 180 and 240 min (p <0.001) but otherwise levels were not different at any time point between ketone drinks in the fed and fasted condition. In contrast, the fall in TG seen following study drink consumption was not significantly different between any condition (p = 0.13) (Figure 4.7 B). The dextrose drink caused a transient rise in glucose, however the overall tendency

was that study drink consumption caused plasma glucose to fall by a similar amount in all conditions (p = 0.6) (Figure 4.7 C). Plasma insulin rose to similar levels after both the meal and the dextrose drink, increasing 8- fold, from 5.5 $\pm$ 0.5 mU ml^{-1} to 39.8 $\pm$ 6.3 mU ml^{-1} after the meal (p = 0.003) (Figure 4.7 D and Appendix B, Table B.2). Insulin also increased 3-fold from 4.7 $\pm$ 0.3 mU ml^{-1} to 13.0 $\pm$ 1.5 mU ml^{-1} when the ketone ester was consumed whilst fasted.

4.4.5 Blood βHB with equimolar & isovolumetric drinks or infusion

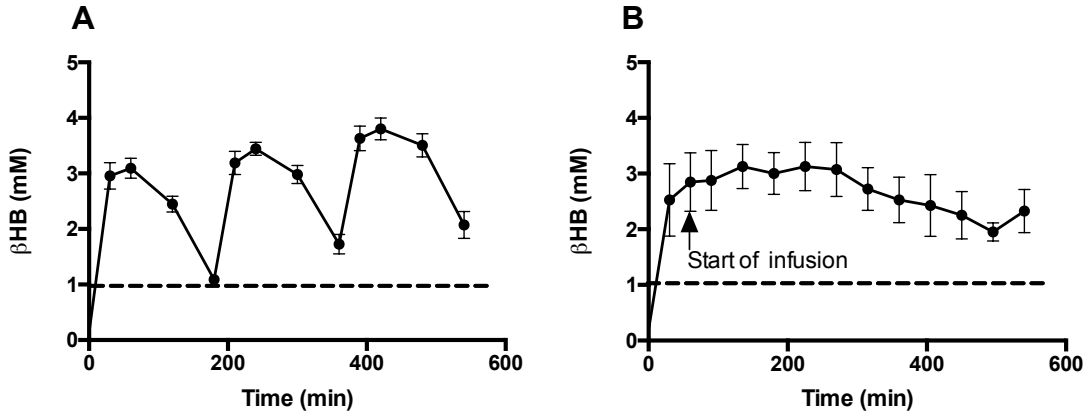

Figure 4.8: *Blood βHB following 3 ketone ester drinks consumed following a fast, by resting subjects (n =12) (A), or with NG ketone ester feeding (n = 4) (B); both methods maintained blood βHB levels above 1 mM (dotted lines shown)*

Both methods of ketone ester administration maintained levels of blood βHB above 1 mM for the nine hour protocol (Figure 4.8 A and B). When three drinks were consumed in series, blood βHB levels fluctuated regularly following each bolus (Figure 4.8 A). After each drink, the βHB C_{max} increased as βHB levels had not returned to baseline between each drink, however there were no significant differences in βHB C_{max} between drinks (Table 4.4 and Appendix B, Table B.3). There was a tendency for both the total rise in βHB and the rate of βHB appearance (R_a) over 30 min to decrease with successive drinks, with βHB R_a being significantly higher following the first drink compared to the second and third drinks

(p = 0.01 1 vs. 2; p = 0.03 1 vs. 3) (Table 4.4 and Appendix B, Table B.3). The amount of βHB eliminated between 60 - 180 min after each drink were similar for each bolus, and the minimum βHB level after the second and third drinks were not significantly different (p = 0.08) (Table 4.5 and Appendix B, Table B.3)).

Table 4.4: *βHB kinetic parameters following three equimolar ketone ester drinks at 3 h intervals. C_{max} was taken directly from the data. Rise in βHB was calculated by subtracting the baseline (or minimum) βHB concentration from the C_{max}. βHB R_a was calculated from the change in βHB in the 30 min directly following each drink. * = p <0.05 Drink 1 vs. 2; ‡ = p <0.05 Drink 1 vs. 3*

	C_{max} (mM)			Rise in βHB (mM)			βHB R_a (mmoles.min^{-1})		
	Drink 1	Drink 2	Drink 3	Drink 1	Drink 2	Drink 3	Drink 1	Drink 2	Drink 3
Mean	3.1	3.4	3.8 ‡	2.8	2.5	2.3	0.10*	0.07	0.06‡
SEM	0.2	0.1	0.2	0.8	0.5	0.7	0.01	0.01	0.01

Table 4.5: *βHB kinetic parameters following three equimolar ketone ester drinks at three hour intervals. Estimated elimination (Elim.) was calculated as the area under the curve from 60 min to 180 min after each drink, normalised to the minimum level of βHB. Minimum (Min.) values were taken 180' after each drink. * = p <0.05 Drink 1 vs. 2; ‡ = p <0.05 Drink 1 vs. 3*

	Elim. (mmoles.min)			Min. βHB (mM)		
	Drink 1	Drink 2	Drink 3	Drink 1	Drink 2	Drink 3
Mean	142	127	122	1.1*	1.7	1.9‡
SEM	37	45	54	0.1	0.2	0.3

Table 4.6: *βHB AUC with equimolar, isovolumetric ketone ester as drinks (n=12) or infusion (n=4)*

	AUC (mmoles.min)
Serial Drinks	1394
Infusion	1305

There were no fluctuations in blood βHB when given by a continuous infusion into the stomach (Figure 4.8 B). The initial bolus of ketone ester 60 min before the start of the NG ketone infusion resulted in a βHB C_{max} of 2.9 $\pm$ 0.5 mM. Once infusion began, βHB levels plateaued until 270 min, remaining between 2 - 3 mM before they slowly decreased towards the end of the protocol. The total βHB uptake, as indicated by the AUC, was identical if equimolar and isovolumetric amounts of ketone ester were given in serial boluses or as a constant infusion (Table 4.6)

4.5 Discussion

4.5.1 Main Findings

As ketone ester consumption was found to be an effective strategy for raising blood D-βHB levels in Chapter 3, its kinetic properties and metabolic effects were studied further here. This work reports that ketone ester drinks repeatably elevated blood βHB levels when consumed by subjects in both the fed and fasted state. Meal consumption decreased the peak βHB level attained and the amount that appeared in the blood following equimolar drinks. However, the changes in blood metabolites and levels of blood AcAc, urinary βHB excretion and breath acetone, were not altered by feeding. Finally, it appeared that ketone uptake and elimination from successive drinks was not altered by existing exogenous ketosis at the levels administered in this study, and that equivalent ketone delivery could be achieved with equivalent amounts given as serial drinks or a continuous infusion.

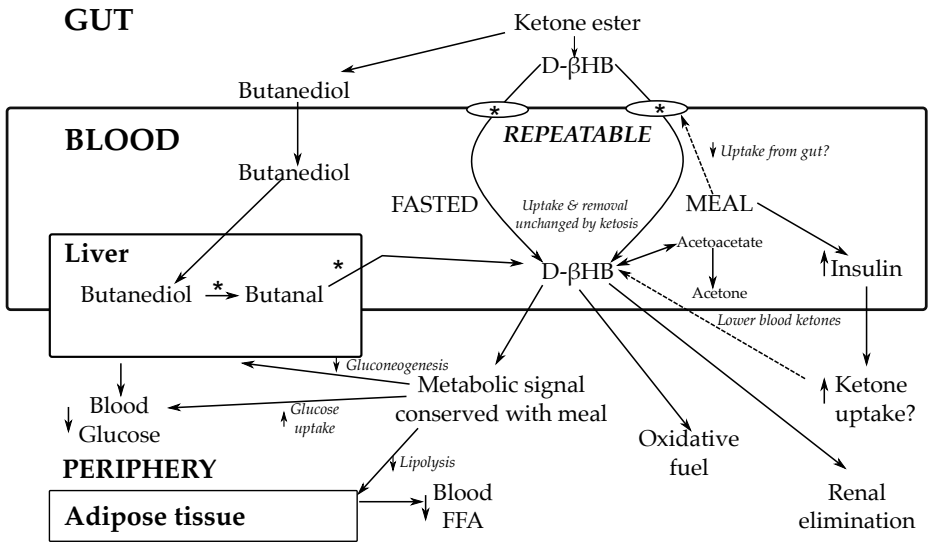

Figure 4.9: *Summary of the factors which affect ketone ester metabolism. * indicates a site where genotypic differences may affect βHB metabolism.*

4.5.2 Repeatability of βHB levels after ketone ester drinks

Ketone ester drinks effectively elevated ketone levels in all subjects with only small variations in pharmacokinetic parameters between and within individuals. However, despite being small, these differences may still be physiologically relevant. With this degree of variation between individuals, ketosis following ketone ester drinks should be monitored to verify that levels attained are adequate at lower doses and controlled at higher doses. The study population was relatively homogeneous (healthy young Caucasians), therefore it cannot be ascertained whether variation may increase with other factors such as age, concurrent pathophysiology, genotypic differences in enzyme expression, body habitus and lifestyle (Liederer and Borchardt, 2006). This may be of particular importance with disease (for example, liver disease), where metabolism may be compromised, or if the targeted level of ketosis is particularly high or low.

There was also a small amount of variability within each individual consuming identical drinks in the same condition. This intra-participant variability in βHB kinetics was most

likely attributable to normal day-to-day changes in GI function, including gastric emptying, portal blood flow and intestinal transit time. Maximal intra-participant variability in peak βHB was lower following a meal, suggesting that food may have resulted in a more consistent gastric emptying time than in the fasted condition and thus more repeatable rate of βHB delivery into the blood.

4.5.3 Ketone kinetics following a meal

The presence of food in the gut has a well documented effect on GI handling of small hydrophilic hydrocarbons, such as βHB, through slowing or preventing their uptake (Fraser et al., 1995; Melander, 1978; Horowitz et al., 1989). Therefore, the finding that a meal lowered blood βHB levels observed following ketone ester ingestion was not surprising. A further possibility is that the increase in blood insulin following a meal increased the clearance of βHB from the blood (Keller et al., 1988), thus resulting in lower levels of βHB.

Despite higher blood βHB levels in the fasted state, there were no differences in plasma AcAc levels with or without a meal. A similar increase in the βHB:AcAc ratio at higher levels of βHB has been reported during both endogenous ketosis (Owen et al., 1967) and during exogenous ketone infusions (Beylot et al., 1986; Mikkelsen et al., 2015). This suggests that the rate of conversion of βHB to AcAc may not be able to match the rate of appearance of βHB following ketone ester consumption. Alternatively, as the conversion of βHB to AcAc is redox linked (dependant on the ratios of NAD^+:NADH) the ratio may have been altered by changes in hepatic redox potential, which has been suggested to play a role determining blood ketone body ratios (Desrochers et al., 1992; Laffel, 1999). Hepatic redox potential may have changed due to changes in glucose availability, insulin action or the synchronous oxidation of butanediol from KE ingestion (Wilson et al., 1974; Desrochers et al., 1992, 1995a,b).

It has been suggested that acetone production from the spontaneous decarboxylation of

AcAc occurs in direct proportion to plasma AcAc concentration (Koorevaar and Van Stekelenburg, 1976). Acetone then appears in the breath at levels related to its concentration in the blood. As with plasma AcAc, levels of breath acetone were unaffected by meal consumption and therefore a direct relationship between these two ketone bodies cannot be excluded by this work. The kinetic profiles of breath acetone seen following the ketone ester drink did not parallel the levels of blood βHB or AcAc. Levels rose more slowly, suggesting that the distribution of acetone through the body compartments was not the same as for AcAc and βHB. Acetone is a more fat-soluble molecule, and therefore may have been sequestered into the lipid compartment and subsequently released slowly. The physiological and metabolic effects of elevated levels of acetone are largely speculative, partially as it is highly volatile and therefore difficult to measure. It may act as an oxidative fuel, but may also have other potential side-effects, such as central nervous system toxicity (Kalapos, 2003). Breath acetone measurements provide a non-invasive method to approximate blood ketosis, which avoid repeated blood sampling. However, it did not appear that breath acetone measurements would accurately reflect rapid changes in blood βHB concentration arising from the ketone ester drink and therefore blood sampling should remain the preferred method for monitoring βHB levels.

Measurement of urinary ketone levels is another method used to estimate the concentration of blood ketone bodies (Martin and Wick, 1943; Taboulet et al., 2007). As there were no differences in urinary elimination of βHB between conditions this method did not accurately reflect blood βHB levels following the ketone ester drink. Furthermore, the lack of difference in urinary βHB suggested that altered renal handling did not play an important role in the differences in βHB kinetics between conditions. Following the ketone ester drinks the amount of βHB excreted ($\approx$ 0.5 g) represented a small proportion of the total $\approx$ 17g consumed. Therefore, it appears that the major fate of the hydrolysis products of the ketone ester was uptake into peripheral tissues, presumably for oxidative metabolism, rather than renal excretion.

4.5.4 Blood metabolites following ketone ester drinks

The effects of ketone ester drinks on metabolite levels were similar in the fed and fasted condition. This demonstrates that the signalling effects of exogenous ketones were maintained in the presence of other nutrients. The expected metabolic consequence of a nutrient bolus would be a rapid increase in blood glucose and insulin, with the latter mediating a fall in circulating FFA and TG. This would be followed by a slow return of metabolites to basal levels as the insulin-mediated inhibition of lipolysis and hepatic glucose release was alleviated (Dahl, 1960). This metabolic pattern was observed here following the dextrose drink. However, following ketone ester drinks, FFA and glucose levels fell and remained similarly low in both conditions, despite higher insulin levels throughout the fed arm. This suggested that there was no extra synergistic effect of insulin and ketone bodies on the decrease in FFA, and that inhibition of lipolysis by ketone bodies was similar to insulin-mediated inhibition.

Elevation of blood ketones when fasted caused a similar fall in plasma glucose as seen following the control drink, which had triggered a substantial insulin release. It has been proposed that the hypoglycemic effect of ketone bodies is attributable to decreased hepatic glucose output (Mikkelsen et al., 2015), increased glucose uptake into tissues (Kashiwaya et al., 1997) or increased insulin secretion (Gerlach and Hiby, 1974). As discussed in Chapter 3, the presence of sugar in the drink diluent ($\approx$ 15 g per drink, Table 4.1) means that an insulinotropic effect of ketones cannot be proven. However, the increase in insulin seen in the fed and dextrose conditions were identical, thus it appears that nutritional ketosis did not inhibit normal insulin release.

4.5.5 βHB metabolism with hyperketonemia

βHB uptake and clearance were not altered by hyperketonemia of 1 -3 mM that resulted from previous ketone ester consumption. The level of blood ketosis may have an impact on

ketone clearance, as suggested by previous work using ketone infusions which showed that above 8 mM ketone clearance by peripheral tissues fell (Balasse and Fery, 1989; Hall et al., 1984; Wastney et al., 1984). However, it is possible that the progressive decline in ketone clearance described by these investigators was in fact caused by the physiological changes of starvation rather than the level of ketones themselves, per se. Here, pharmacokinetic parameters indicative of βHB uptake and elimination remained largely identical with three successive drinks, and it appeared that a pseudo-steady state in blood ketone levels may have been reached if further identical boluses were given at the same intervals. However, it is unclear if this pattern would continue should higher doses be consumed or if the interval between drinks were to be shortened so that blood ketone concentration increased to the levels reached in the infusion studies. When ketone bodies were given at a constant rate through a NG tube blood ketone levels began to fall towards the end of the study, suggesting that ketone clearance may even have increased over time. Therefore, it appears that the ability to metabolise the ketone ester was not altered by elevated blood βHB at these concentrations with either serial drinks or continual ketone feeding.

Serial ketone ester drinks resulted in fluctuating blood βHB levels, with a rapid rise and decline seen in each three hour interval. This pattern would necessitate regular ketone ester consumption to maintain ketosis at >1 mM. To minimize these fluctuations, it may be useful to modify the formulation of the ketone ester to slow ketone uptake. Two different mixtures of ketone ester have been studied milkshake vs. sports water and shown to significantly alter the resulting βHB kinetics (Shivva et al., 2016). NG administration of this ketone ester could also be effectively used to deliver nutritional ketosis without the fluctuations in βHB seen following bolus feeding, although this would only be practical in a clinical setting.

4.6 Conclusion

In summary, ketone kinetic profiles following ketone ester drinks were predictable between and within individuals when standardised drinks were consumed, demonstrating that metabolism of this exogenous ketone ester varies little within a healthy population. This predictability was maintained in the presence of a meal, although consumption of the ketone ester along with food altered overall βHB kinetics compared to when fasted. Blood AcAc, breath acetone and urinary βHB were not reliable measures of blood ketosis as they did not reflect the differences in βHB levels seen between the fed and fasted state. Finally, isovolumetric amounts of ketone ester as serial drinks or NG infusion were equally effective in maintaining ketosis >1 mM over nine hours with no significant effect of existing ketosis on ketone ester metabolism at the doses studied. In conclusion, these results broaden the current understanding of the anthropometric and environmental factors that can influence the metabolism of exogenous ketones such as this ketone ester.

Chapter 5

Systemic effects of exogenous ketones

5.1 Abstract

Achieving ketosis through dietary manipulation has systemic effects beyond changes to substrate provision. LCHF diets have been linked to appetite suppression and gastrointestinal (GI) upsets, but the role of βHB in these effects is unclear (Gibson et al., 2015; Kossoff et al., 2006). βHB in ester and salt drinks enters the body via the gut, and is distributed throughout the body by the blood, so these drinks could cause similar effects to a LCHF diet. The work in this Chapter aimed to examine the effects of ketone drinks on 'appetite' and gut hormone levels and to document the GI side-effects of ketone drinks consumed under different conditions.

Subjects rated their appetite on a visual analogue scale following drinks containing ketone ester, ketone salt or dextrose, and their plasma levels of the hormone ghrelin were measured. All ketone drinks decreased appetite, and from one hour after ketone ester consumption, appetite was ≈15% lower and plasma ghrelin was >30% lower compared to an isocaloric dextrose control drink. A second experiment used a murine enteroendocrine cell line (GLUTag) to examine the effects of βHB on the secretion of glucagon-like peptide-1 (GLP-1), which is linked to satiety. The addition of D-βHB potentiated GLUTag cell GLP-1 secretion by >30% in response to glucose stimulation. Therefore, ketone drinks may exert an effect on appetite through the enteroendocrine system via ghrelin and GLP-1.

The incidence and severity of GI symptoms following ketone ester and salt drinks was measured using a questionnaire. Many participants reported no symptoms following the drinks, and those that were reported were predominantly (69 -75%) 'mild' in severity. Symptoms increased with increasing dose of both compounds, were significantly greater with the salt than the ester at the high dose, but were lower when the ketone ester was consumed during exercise compared to rest. Therefore, ketone drinks are well tolerated as a method for dietary ketosis in most individuals.

5.2 Introduction

Endogenous ketosis that accompanies starvation or low-carbohydrate high-fat (LCHF) diets develops over many days, and is accompanied by hormonal and metabolic shifts (Cahill et al., 1966). This makes it difficult to ascertain which changes are attributable to ketone bodies and which are caused by the diet's nutrient composition. Commonly reported side-effects of LCHF diets include a decrease in appetite (Gibson et al., 2015) and an increase in incidence of systemic (e.g nausea) and GI (e.g diarrhoea) symptoms (Hallbook et al., 2015). Earlier Chapters (3 and 4) demonstrated the efficacy of exogenous ketone ester and salt drinks to raise blood levels of βHB without dietary manipulation. Therefore, if ketone drinks are to offer an alternative to a LCHF diet for ketosis, it is important to determine whether they have similar effects.

5.2.1 Effects of ketosis on appetite and gut hormone secretion

Appetite regulation plays a crucial role in determining body weight and is controlled by a network of nuclei in the hypothalamus (Morton et al., 2006). Parts of the hypothalamus lack an effective blood brain barrier, being where the brain can 'sample' the composition of the blood (Ballabh et al., 2004). This allows integration of orexigenic and anorexigenic inputs, which promote food intake and discourage food intake, respectively. Inputs can be neural, hormonal or metabolic in nature and originate from the blood, adipose tissue, peripheral sensory receptors, or the gut (Morton et al., 2006).

The hormonal response to a meal has a powerful effect on feeding behaviour. The physical and chemical properties of a meal trigger the release of a plethora of hormones from specialised enteroendocrine cells in the gut. Many of these hormones act as neuropeptides and modulate the activities of the peripheral and central nervous systems and thus alter food intake (Morton et al., 2006; Carneiro et al., 2016). Two examples of such gut hormones are ghrelin and GLP-1. Following a meal, ghrelin secretion is suppressed, and levels then rise

over time to increase 'hunger' and initiate feeding (Nakazato et al., 2001). GLP-1 secretion by gut L-cells is triggered by nutrients in the gut to potentiate insulin secretion. As well as their peripheral actions, both GLP-1 and insulin have an anorexigenic effect in the central nervous system (Keller et al., 1988; Turton et al., 1996).

Several studies have shown that adherence to a LCHF diet can decrease appetite, leading to lower food intake and weight-loss (Gibson et al., 2015; Sumithran et al., 2013; Johnstone et al., 2008). Decreased appetite may be caused by either ketone bodies in the blood or by an increase in satiating dietary macro-nutrients, such as protein (Poppitt et al., 1998). It is unknown the extent to which ketosis decreases appetite through a direct effect on the central nervous system or in the periphery, by altering hormone secretion, there being evidence to support both. In favour of a central effect, intracerebral infusions of βHB decrease food intake in rodents (Arase et al., 1988; Carneiro et al., 2016). Furthermore, βHB increases expression of orexigenic neuropeptides in cultured hypothalamic cells (Laeger et al., 2012). Evidence for peripheral effects of ketosis can be seen following a LCHF diet in ketotic patients, who have alterations in fasting and post-meal levels of some gut hormones (Sumithran et al., 2013). Exogenous ketones provide a novel tool to study the satiating effect of βHB as ketone ester and salt drinks allow the effect of ketone bodies, per-se, to be separated from the increase in dietary fat and protein accompanying a LCHF diet.

5.2.2 Tolerability of exogenous ketones

LCHF diets can have unpleasant short-term GI and systemic effects (Kossoff et al., 2003, 2006), thought to result from the high proportion of fat in the diet. Some children with epilepsy found following a LCHF diet intolerable due to nausea, vomiting, diarrhoea and lethargy (Hallbook et al., 2015; Bansal et al., 2014). To avoid a LCHF diet, ketosis can be created through consumption of medium chain triglycerides (MCTs), which are readily converted into ketone bodies. However, high levels of MCT consumption also cause severe

GI distress. Symptoms were reported by 100% of participants who consumed 50 - 60 g of MCT prior to exercise (Ivy et al., 1980), and in studies of MCT-containing medicinal products, causing drop-out rates of 25% (Henderson et al., 2009). Therefore, strategies for dietary ketosis need to determine the thresholds of tolerability for different interventions.

Ketone drinks may have fewer effects than LCHF diets or MCTs. A kinetics study of ketone ester drinks (Clarke et al., 2012b) documented the effects on participants who consumed three doses of the ester, in three drinks per day. At the lower doses (0.8 and 2.0 mmoles.kg^{-1} of ketone ester per drink) symptoms were infrequent and mild, however at the highest dose (4.1 mmoles.kg^{-1} of ketone ester per drink) symptoms were more frequent and severe, with two participants discontinued from the study as a result (Clarke et al., 2012b). Whilst oral ketone salts have been studied in a small number of clinical cases in very young and critically ill children, neither GI nor systemic symptoms were recorded (Plecko et al., 2002; Valayannopoulos et al., 2011; Van Hove et al., 2003). Therefore, information regarding the tolerability of both ketone ester and salt compounds is limited compared to the body of literature describing effects of the LCHF diet in different patient groups.

Recently, interest in the use of LCHF diets and exogenous ketones to improve athletic performance has grown. Whilst a performance-enhancing effect has yet to be clearly demonstrated as a result of the LCHF diet, our group has shown that ketone ester drinks can improve the performance of endurance athletes (Cox et al., 2016b). It is unknown if ketone salt drinks would have a similar effect. However, GI effects during exercise may compromise any beneficial impact on performance. Athletes are particularly vulnerable to GI upset as blood flow to the gut is decreased during exercise. Therefore, it is important to investigate the interaction between exercise and ketone drinks to inform their use in a sporting setting.

5.2.3 Aims

Work in this Chapter firstly aimed to investigate the effects of exogenous ketones on appetite and gut hormone secretion, the hypothesis being that exogenous ketones may decrease appetite and modulate the secretion of hormones that change acutely in response to a meal, such as ghrelin and GLP-1. Secondly, the work in this Chapter aimed to describe the type and severity of GI effects following drinks containing different ketone compounds and doses, consumed under a variety of conditions.

5.3 Methods

5.3.1 General data collection

Data were collected in three separate studies of ketone drinks. Participants completed questionnaires that measured 'appetite,' 'symptoms' and 'palatability' at regular intervals after the drink. Data from Studies 1 and 2 were collected as part of the experiments described in Chapters 3 and 4; data from Study 3 were collected in a study of ketone drinks during variable intensity bicycle exercise. All three studies received ethical approval from an external research ethics committee (REC reference numbers: Study 1: 15/SW/0244, Study 2: 14/LO/0288, Study 3: MODREC 298/PPE/11). Participants were recruited and baseline physical measures were collected as described in Chapter 2.1. βHB was measured on whole blood at identical time points to questionnaire completion using a handheld monitor and reagent strips (Precision Xtra, Abbott Diabetes Care, UK).

5.3.2 Study drinks and conditions

Ketone drinks were prepared as described in Chapters 3 and 4 (Studies 1 and 2). In Study 3, the study drink contained 2.2 mmoles.kg^{-1} of ketone ester made up to 500 ml with the citrus water (as used in Study 1) and mixed with 1.4 g/kg dextrose.

Study conditions and visits are summarized in Figure 5.1. During Study 3, participants consumed a ketone drink prior to exercise. The exercise protocol was 65 min: the first 40 min was at a low intensity (40% of the individuals pre-determined maximal wattage), and the final 25 min was a block of intervals at higher intensity (60 / 80% of maximal wattage).

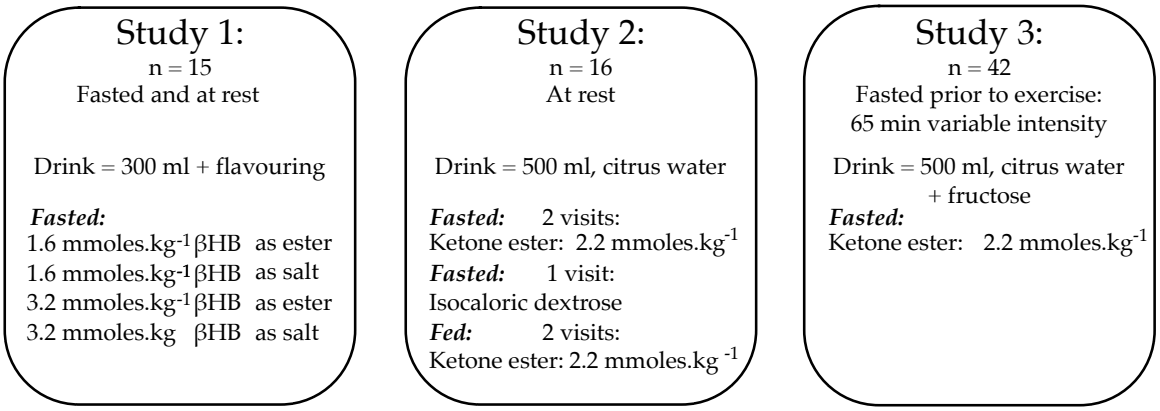

Figure 5.1: *Summary of the study conditions and drinks under which appetite and GI symptoms were measured.*

5.3.3 Effect of ketosis on appetite and gut hormone secretion

5.3.3.1 Appetite and plasma ghrelin

Data were collected from participants (n = 31) as part of Studies 1 and 2. Participants consumed ketone ester, ketone salt or isocaloric dextrose drinks and their hunger/appetite was assessed using a three-point visual analogue scale (VAS) (Figure 5.2). The scale asked participants to consider their 'hunger,' 'fullness' and 'desire to eat.' Similar scales have been routinely used for subjective appetite measurement (Flint et al., 2000).

Blood samples were collected during Study 2, where participants (n = 16) consumed a ketone drink whilst fasted or an isocaloric dextrose drink. Plasma samples from the dextrose visit and the first fasted visit for each participant were analysed for glucose and insulin as previously described (Chapter 2.4), and for active ghrelin using a commercially available ELISA kit (Merck-Millipore, Germany).

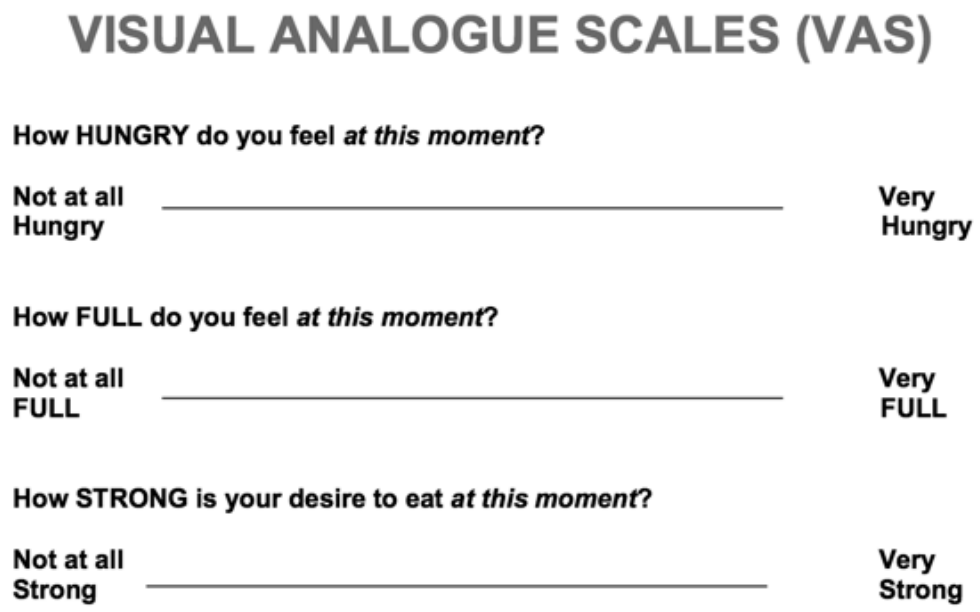

Figure 5.2: *Satiety visual analogue scale used in all studies (not shown to scale)*

5.3.3.2 Effect of βHB on GLP-1 secretion in GLUTag cells

A stable immortalised murine enteroendocrine cell line (GLUTag) was used to investigate whether ketosis affected hormone secretion. GLUTag cells are similar to human L-cells, which are specialised for GLP-1 secretion in response to glucose (Drucker et al., 1994). GLUTag cells were expanded as described in Appendix C.

To investigate the effects of βHB, GLUTag cells were seeded at a density of 50,000 cells per well on to a cell culture treated 24-well plate (Corning, New York, USA). The incubation medium was a Hepes buffer (138 mM NaCl, 4.5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 4.2 mM $NaHCO_3$, 1.2 mM Na_2HPO_4, and 10 mM HEPES), containing no glucose (0 mM), low glucose (5.6 mM) or high glucose (25 mM). Cells were incubated for one hour at 37 °C (n = 6 wells for each concentration) and 3 mM D-βHB was added to 2 wells at each glucose concentration for the hour.

The incubation medium was replaced and GLP-1 secretion was measured after a two

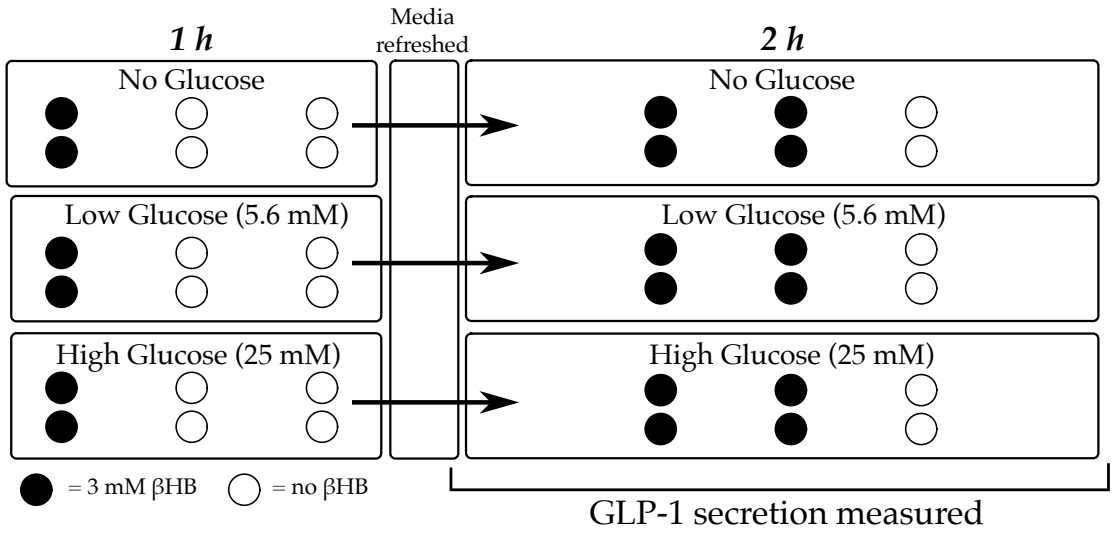

Figure 5.3: *Summary of the conditions under which GLP-1 secretion by GLUTag cells was measured. The 1 h culture period was followed by a change of media; GLP-1 secretion was then measured over the following 2 h. βHB (3 mM) was added as shown. All incubation conditions were studied at: 0 mM glucose (no glucose), 5.6 mM glucose (low) and 25 mM glucose (high).*

hour period, in cells cultured with no, low or high glucose (0 mM, 5.6 mM or 25 mM) at 37 °C. βHB (3 mM) was added to the 2 wells at each glucose concentration that had βHB in the initial hour, and a further 2 wells at each concentration that had not been incubated with βHB. As a control condition, the 2 remaining wells at each glucose concentration did not have βHB at any time. Conditions tested are summarised in Figure 5.3.

At the end of two hour culture, the incubation media from each well was collected into eppendorf tubes kept on ice. The media was centrifuged at 1000 xg for 5 min at 4 °C to remove any floating cells and transferred to fresh, chilled eppendorf tubes. The cells were kept on ice with cold radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris (pH 8.0), 1% (v/v) IGEPAL, 0.5% deoxycholic acid) with added protease inhibitor cocktail solution (Roche, Penzberg, Germany) for 30 min. The resulting cell lysate was centrifuged for 20 min at 4°C at 17,000 xg and protein concentration for each sample was determined using a Bicinchoninic Acid Assay (Pierce BCA; Thermo Scientific, Illinois, USA) according to the manufacturers protocol. GLP-1 was assayed in triplicate in the

supernatant from each well using an HTRF (Homogeneous Time Resolved Fluorescence) assay that is specific for GLP-1 (CISBIO, Codolet, France) according to the manufacturer's protocol.

5.3.3.3 Data processing and statistics

Visual analogue scale data were considered as a distance along the standard line length (mm). Responses for 'hunger' and 'desire to eat' were measured left to right but, as 'fullness' is in opposition to 'hunger,' this score was measured in reverse (right to left). This allowed the three scores to be combined to give an overall measure of hunger, as a % of line length. Paired comparisons were made when the same participant consumed two equivalent drinks. As participants in Study 2 consumed the ketone drink twice and the dextrose drink only once, one mean value for both ketone visits was calculated for the visual analogue scale scores and blood measures to allow paired comparisons. Statistical comparisons between groups were described in the Chapter 2.5, and detailed results of statistical comparisons are reported in Appendix C.

5.3.4 Tolerability of exogenous ketones

5.3.4.1 General study design

Data were analysed from Studies 1, 2 and 3. Participants (n = 73) consumed ketone ester, ketone salt or isocaloric dextrose drinks under different conditions, and the incidence of GI symptoms was recorded using a questionnaire. The effect of ketone dose, meal consumption and exercise on the incidence and overall severity of symptoms reported after the ketone drink was analysed. Additionally, participants who took part in Study 1 (n = 15) consumed ketone ester and ketone salt drinks and completed a visual analogue scale that rated several aspects of drink palatability (Figure 5.4).

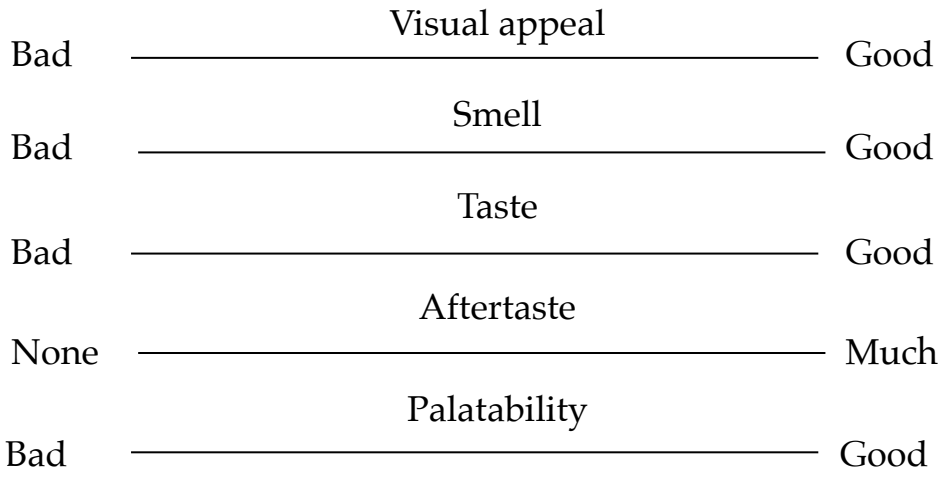

Figure 5.4: *Palatability questionnaire used in Study 1 (not shown to scale)*

5.3.4.2 Data collection

Participants confirmed that they were free of symptoms before study drinks were consumed. At regular intervals following each study drink, participants were asked to complete a questionnaire (See Appendix C, Figure C.1) with 12 individual symptoms grouped into three types: upper abdominal, lower abdominal or systemic symptoms. Symptoms were given a severity value from 0 (none) to 8 (unbearable).

To measure drink palatability, immediately after consuming the study drink participants were asked to complete a five measure visual analogue scale (VAS) on paper that rated the drink for: look, smell, taste, aftertaste and palatability (Figure 5.4). Each line was 150 mm long. Responses were manually measured and the line position was recorded (mm).

5.3.4.3 Data processing and statistical methods

Symptoms were grouped as shown in the questionnaire (upper & lower GI and systemic). Symptom severity was grouped where 1-2 = mild, 3-4 = moderate and >5 = severe. Initial analysis revealed that many of the participants reported no symptoms at any point following the study drinks. Thererfore, a binary incidence outcome was assigned to each of the three

symptom groups for each visit as an initial classification: positive outcome = symptom and negative outcome = no symptom. For the visits where symptoms were reported, the highest severity score for each symptom group was recorded. Furthermore, in order to represent the frequency and intensity of symptoms over the study, the severity values for every symptom report were summed to give an additional 'symptom load' score for each visit, which had no units. Where subjects had completed more than one visit in a given condition (Study 2) a positive outcome was considered as a reported symptom by one subject in either visit. The mean symptom load score for the first and second visit in each condition was calculated and used in the analysis.

Symptoms were also compared in separate cohorts of subjects following drinks of equal ketone ester doses consumed at rest or during exercise. As symptom scores had been collected more frequently during Study 2, some sampling time points were removed from the data to give an equal number of sample time points to Study 3, that were collected at similar intervals. The incidence of symptoms and the symptom load were recalculated for the modified data from Study 2, as above.

When data were collected from the same individual in different conditions (Study 1 and 2), the effect of conditions on incidence was compared using McNemar's test, as this was a binary outcome measure. As 'symptom load' per experiment was numerical, the effect of conditions was compared using Wilcoxon's matched pairs test. As data from Study 2 and 3 were not paired measures from the same individuals, a Fishers exact test was used to compare the incidence of symptoms and a Student's unpaired t-test Mann Whitneys test was used to compare the 'symptom load'.

Symptoms were also compared following drinks of equal ketone doses consumed at rest or during exercise. As symptom scores had been collected more frequently during resting studies, to ensure this did not affect the comparisons, some sampling time points were removed from the resting data to give an equal number of sample time points that were collected at similar intervals. As these observations were not paired measures from

the same individuals, a Student's unpaired t-test was used to compare the 'symptom load'. Detailed results of all statistical comparisons are reported in Appendix A.

Palatability visual analogue scale data were considered as a distance along the 150 mm line length. Statistical comparisons were carried out as described in Chapter 2.5.

5.4 Results

5.4.1 Effect of ketosis on appetite and gut hormone secretion

5.4.1.1 Appetite and plasma ghrelin

Blood ketone levels reached 3 $\pm$ 0.2 mM following the ketone ester drink, but did not rise following the dextrose drink (Figure 5.5 A). Plasma glucose and insulin rose 30 min after the dextrose drink and were higher than following the ketone drink ($p < 0.001$), however after 90 min levels were the same for all drinks (Figure 5.5 B & C). Full statistical effect sizes are shown in Appendix C, Table C.1 and Table C.2. Subjective hunger fell following both ketone and dextrose study drinks, however the ketone ester caused a greater fall in hunger (effect of drink, p = 0.005 vs. dextrose) that persisted for longer than following the isocaloric dextrose drink (Interaction: time x drink, p = 0.04). Hunger was $\approx$ 15% lower between 60-180 min following the ketone ester drink ($p < 0.05$ vs. dextrose) (Figure 5.5 D). The lower hunger seen following ketone ester consumption was associated with significantly lower levels of plasma ghrelin, which were >30% lower after ketones compared to the dextrose drink between 90-240 min (effect of drink, p = 0.02) (Figure 5.5 E).

Both ketone ester and ketone salt drinks had a significant effect on hunger (effect of drink, $p < 0.0001$ in all cases), causing an initial fall from $\approx$ 50 - 60% to $\approx$ 30 - 40%. Hunger rose steadily over the remainder of the study visit, ending at $\approx$ 65 - 75%. There was no consistent effect of dose (low vs. high), or ketone compound (salt vs. ester) depsite large differences in peak βHB (Figure 5.6 A & B, Table 5.1 and Appendix C Table C.1).

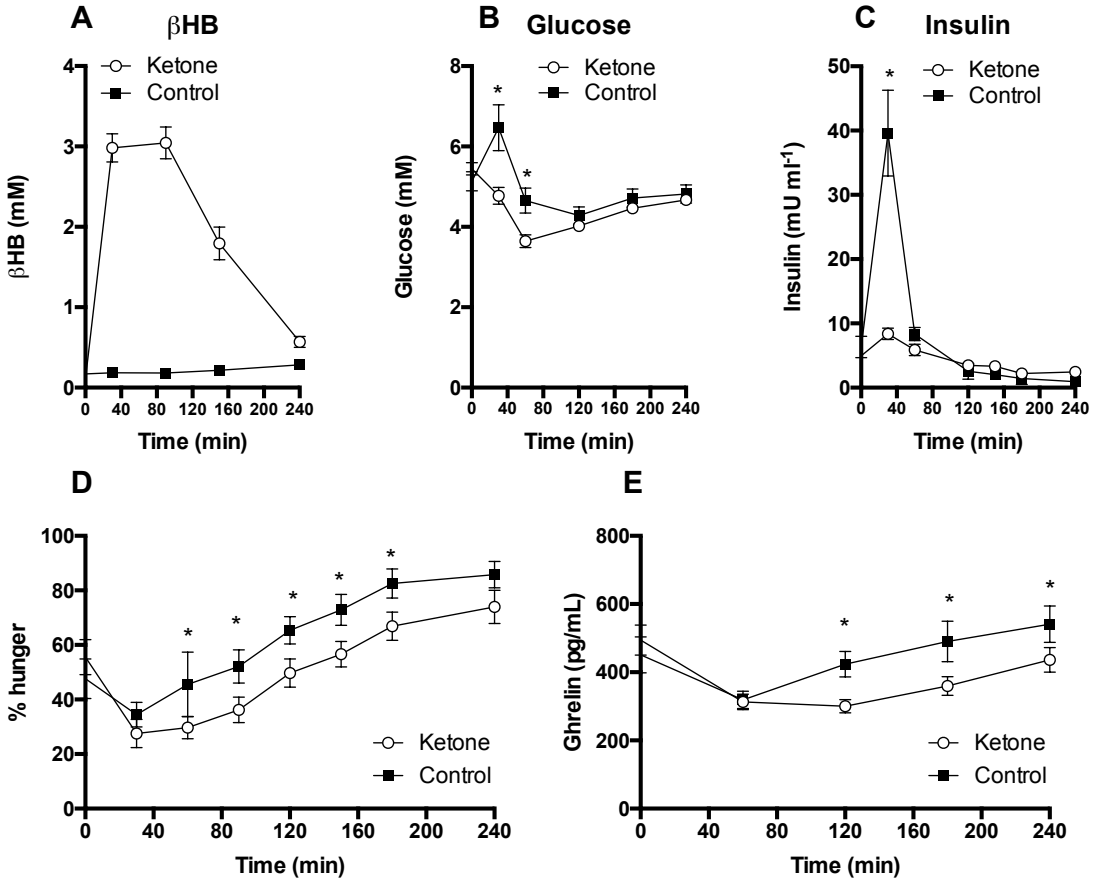

Figure 5.5: *Blood βHB and plasma glucose, insulin, hunger and ghrelin in participants (n = 16) who consumed a ketone ester drink or an isocaloric dextrose control drink at rest. A: Blood D-βHB. B: Plasma glucose. C: Plasma insulin. D: Perceived appetite. E: Plasma ghrelin. Significance was taken as p <0.05 and is indicated as * = ketone ester vs. dextrose control*

5.4.1.2 The effect of βHB on GLP-1 secretion in GLUTag cells

GLP-1 secretion was not significantly increased by with higher glucose concentration in the control condition, without the addition of βHB. However, incubation with 3 mM βHB significantly potentiated secretion of GLP-1 from GLUTag cells by between 150 - 400 pM.μg^{-1}of total protein when stimulated with increasing glucose concentrations for 2 h, compared to stimulation without βHB ($p < 0.05$ in all cases) (Figure 5.7, Appendix C, Table C.3). There was no additional effect of adding 3 mM βHB to the initial 1 h incubation buffer.

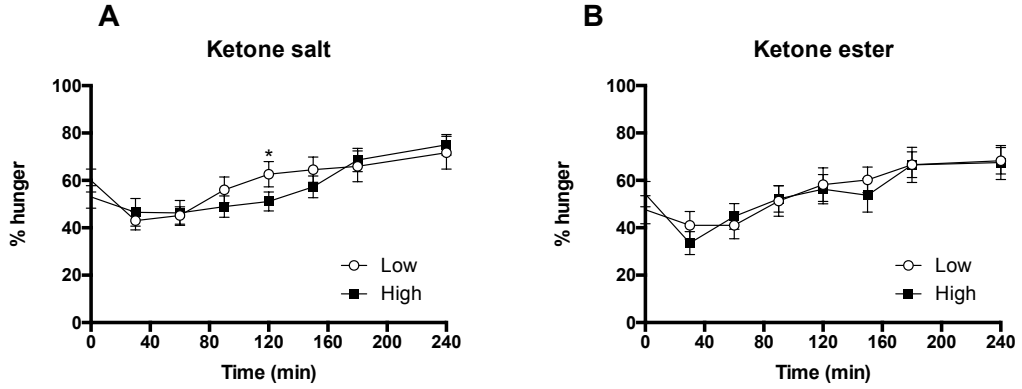

Figure 5.6: *Subjective hunger in participants (n = 15) who consumed either ketone ester or ketone salt drinks at two doses at rest. A: Low and high doses of ketone salt. B: Low and high s of ketone ester. Significance was taken at p <0.05 and is shown as * = between the two experimental groups on a panel.*

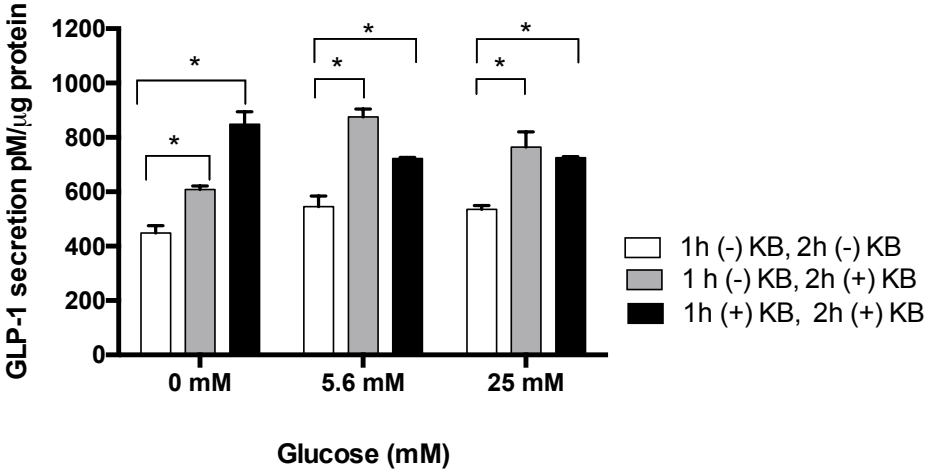

Figure 5.7: *GLUTag cells were incubated for 1 hour in different glucose conditions, with or without 3 mM βHB. The cells were then stimulated with different glucose concentrations, with or without the addition of 3 mM βHB and GLP-1 secretion was measured on the supernatant. Significance was taken as p <0.05 and shown as * <0.05 vs. control*

5.4.2 Tolerability of exogenous ketones

5.4.2.1 Incidence and severity of GI symptoms

Despite differences in levels of blood βHB, there were few significant differences between the incidence and maximal severity of symptoms between ketone compounds and between different conditions (Figures 5.8, 5.9 and 5.10, Table 5.1 and Appendix C, Table C.4 and C.5). The incidence was ≈ 25% for all conditions and all symptom types. The maximal severity was classified as 'mild' for between 69 - 75 % of upper GI, lower GI and systemic symptoms. Severe symptoms only occurred when a ketone ester drink was consumed whilst fed (1 visit), and following high doses of ketone salt (2 visits). When 'symptom load' per experiment was calculated to account for frequency and severity of symptoms, it became apparent that most participants experienced few side-effects. The highest mean 'symptom load' was 13.8 per experiment following the high dose of ketone salt, and the lowest was 0.8 per experiment when a ketone drink was consumed with exercise.

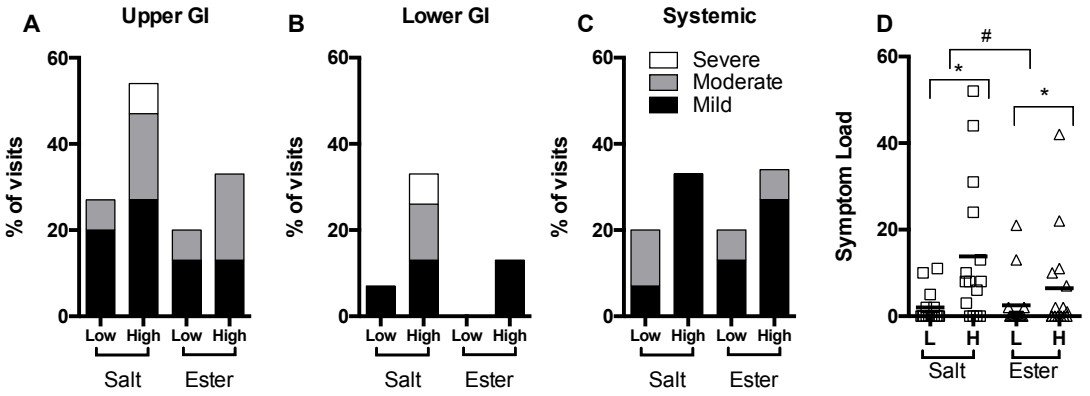

Figure 5.8: *Symptom incidence (the % of total visits where* any *symptom was experienced), maximal severity and 'symptom load' per experiment reported by participants (n = 15) consuming ketone ester and ketone salt drinks at 'low' and 'high' doses. A: Upper GI symptoms B: Lower GI symptoms C: Systemic symptoms D: 'Symptom load' per experiment (sum of all symptom scores) with 'low' (L) and 'high' (H) doses of ketone ester and ketone salt drinks, mean indicated by bar. Significance taken at p <0.05 and shown as * = low vs. high, # = salt vs. ester (same dose).*

Table 5.1: *Peak blood βHB (C_{max} in mM) reached during each of the conditions studied*

Study	Drink	Ketone dose	D- βHB C_{max} (mM)
1	Ester- Low	1.6 mmoles.kg^{-1} βHB	1.7 ± 0.1
	Ester- High	3.2 mmoles.kg^{-1} βHB	3.1 ± 0.2
	Salt- Low	1.6 mmoles.kg^{-1} βHB	0.8 ± 0.1
	Salt- High	3.2 mmoles.kg^{-1} βHB	1.1 ± 0.1
2	Dextrose drink	0	0.1 ± 0.0
	Ester -Fed	2.2 mmoles.kg^{-1} ketone ester	2.4±0.1
	Ester- Fasted/Rest	2.2 mmoles.kg^{-1} ketone ester	3.5±0.1
3	Ester- Exercise	2.2 mmoles.kg^{-1} ketone ester	2.0 ± 0.1

Although incidence of all symptom groups appeared to be higher with higher doses, there were no significant differences in the incidence of upper GI and lower GI symptoms with a high vs. low dose of ketone salt or ester drinks (Figures 5.8 A and B, Appendix C, Table C.4). There were no significant differences in the incidence of any symptom groups between equivalent doses of ketone ester and salts (Figure 5.8 A, B & C).

However, when all symptoms were grouped and the frequency and intensity was accounted for, the 'symptom load' per experiment was significantly increased by increasing the dose of both ketone salt and ester (salt p = 0.008; ester p = 0.03) (Figure 5.8 D and Appendix C, Table C.5). At the low ketone dose, there was no difference in 'symptom load' between compounds, but at the high dose, 'symptom load' was significantly higher with ketone salt vs. ester drinks (p = 0.03) (Figure 5.8 D and Appendix C, Table C.5).

Ketone ester drinks consumed whilst fed or fasted were associated with a significant increase in the incidence of upper GI and systemic symptoms compared to the dextrose control drink (upper: p <0.05 dextrose vs. fed & fasted; systemic: p = <0.001 dextrose vs. fed & fasted, Appendix C, Table C.4), however there were no differences in symptom incidence whether fed or fasted (Figure 5.9 A). Symptom load per experiment was significantly greater following the ketone ester drinks compared to the dextrose drink, but was

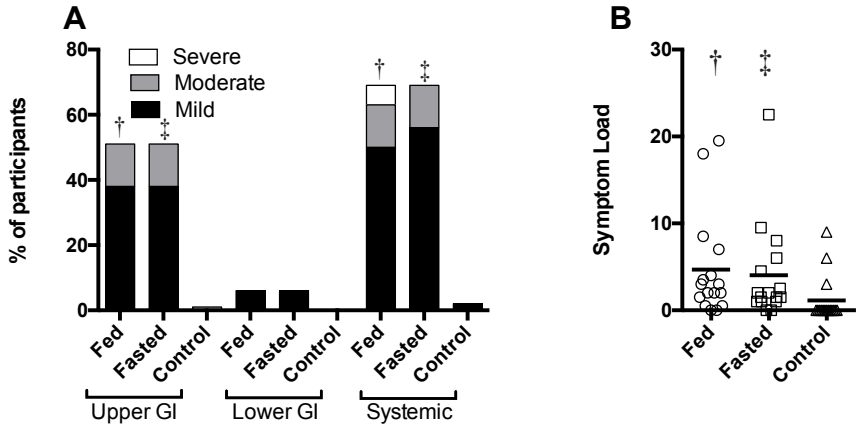

Figure 5.9: *Symptom incidence, maximal severity and 'symptom load' per experiment reported by participants (n = 16) who consumed two ketone ester drinks whilst fed or fasted and one isocaloric dextrose drink. A: The % of total visits where* any *GI symptom was experienced and the max severity of symptom experienced. B: 'symptom load' per experiment (sum of all symptom scores), mean indicated by bar. Significance (p <0.05) shown as † = fed vs. dextrose; ‡ = fasted vs. dextrose*

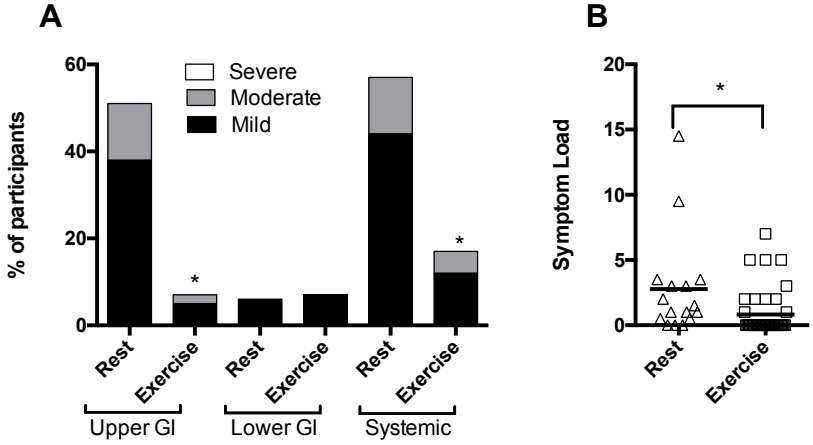

Figure 5.10: *Symptom incidence, maximal severity and 'symptom load' per experiment reported by participants (n = 58) consuming ketone ester drinks at rest or during exercise. A: The % of total visits where* any *GI symptom was experienced and the max severity of symptom experienced. B: 'symptom load' per experiment (sum of all symptom scores), mean indicated by bar. Significance taken at p <0.05 and shown as * = rest vs. exercise*

the same if fed or fasted ($p < 0.05$ in both cases) (Figure 5.9 B and Appendix C, Table C.5).

When ketone ester drinks were consumed before exercise, upper GI and systemic symptoms occurred in significantly fewer visits than when the ketone drinks were consumed at rest (upper GI: $p = 0.02$; systemic: $p = 0.03$) (Figure 5.10 A). Mean 'symptom load' was significantly lower during exercise compared to at rest (rest = 2.8 per experiment vs. exercise = 0.8 per experiment; $p = 0.006$) with peak blood βHB being $\approx$ 1.5 mM lower during exercise (Table 5.1).

Data were collated to inform the development of a generalised linear mixed effects model to predict the importance of different variables (time, βHB level, ketone dose, sex, BMI) on the chance of symptoms. Despite using all available data points, many values were '0' (i.e, no symptoms reported), therefore the model may have been underpowered to accurately describe the data. Further details can be found in Appendix C

5.4.2.2 Palatability of ketone drinks

Neither ketone drink was perceived as palatable, with both low and high doses scoring under 50/150 mm for overall palatability and taste (Figure 5.11 A & B). The ketone ester was significantly worse tasting at a high vs. low dose (39 vs. 19 mm; $p < 0.005$), and worse than the equimolar salt drink at the high dose (19 vs. 35 mm; $p = 0.02$) (Figure 5.11 A). Similarly, the higher dose of the ester had a significantly worse palatability than the high dose salt drink (23 vs. 52 mm; $p = 0.01$), although the high dose salt drink was ranked as having a better palatability than the low (52 vs. 23 mm; $p = 0.01$) (Figure 5.11 B). There were no differences in aftertaste or appearance of the different ketone compounds at either dose (Figure 5.11 C & D). Whilst there were no differences in smell of the low and high dose of the ketone ester drink, the high dose salt smelt significantly worse than the low (91 vs. 51 mm; $p = 0.02$) (Figure 5.11 E).

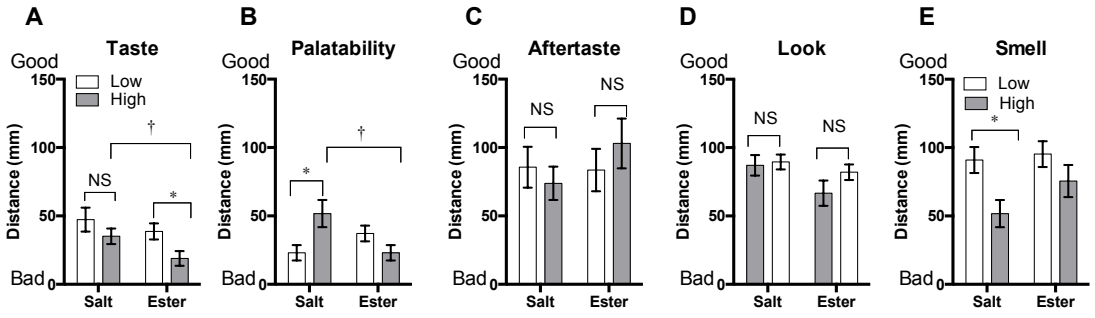

Figure 5.11: *Palatability of ketone ester and ketone salt drinks consumed at two doses was assessed using a questionnaire in n= 15 participants. A: Taste. B: Palatability. C: Aftertaste. D: Look. E: Smell. Significance ($p < 0.05$) shown * low vs. high; † = equivalent dose salt vs. ester*

5.5 Discussion

5.5.1 Main findings

Ketone ester drinks were more satiating than isocaloric dextrose drinks. The mechanism for this may have been due to an effect of βHB on secretion of ghrelin and GLP-1 and was independent of insulin and glucose. Ketone drinks were commonly associated with mild GI side-effects and the type of symptom experienced, incidence and overall severity of symptoms depended on the dose of βHB, the ketone compound itself, and the condition under which it was consumed (i.e rest or exercise).

5.5.2 Effect of ketosis on appetite and gut hormone secretion

βHB from exogenous ketone bodies may have decreased appetite by altering secretion of gut hormones such as ghrelin (orexigenic signal) and GLP-1 (anorexigneic singal) (Figure 5.12). Ghrelin was chosen as an initial target gut hormone as levels change acutely in anticipation of, and in response to, a meal (Nakazato et al., 2001; Bowen et al., 2006). Furthermore, lower levels of fasting ghrelin were observed in patients on a LCHF diet who achieved ketosis during weight loss (Sumithran et al., 2013; Chearskul et al., 2008). In the

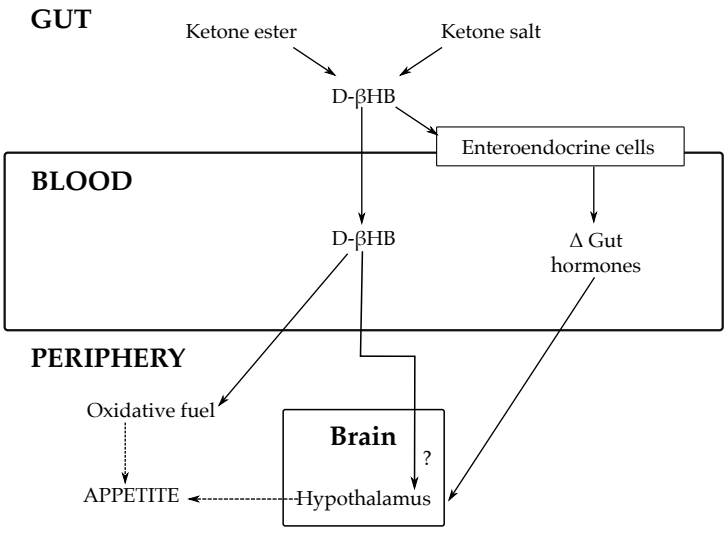

Figure 5.12: *Schematic representation of the potential mechanisms by which ketone drinks may modulate appetite*

LCHF experiments, appetite correlated with the level of ketosis, however this observation could not be solely attributed to βHB as it could not be separated from the other changes accompanying a LCHF diet. The importance of peripheral hormonal action for the anorexigneic action of βHB was suggested by experimental transection of the hepatic branch of the vagus nerve in rodents, which eliminated the hypophagic effect of injected βHB (Langhans et al., 1985). The hepatic vagus receives inputs from the stomach, small bowel and pancreas and is sensitive to the levels of ghrelin (Habara et al., 2014). Therefore, together with the findings described in this Chapter, there is evidence to suggest that the changes in gut hormone secretion resulting from elevated βHB may have increased satiety.

It remains unclear *how* βHB from both endogenous and exogenous sources could directly influence gut hormone secretion, but there may be direct signaling by βHB. Therefore, βHB's effects on GLP-1 secretion in cultured GLUTag cells were measured, which showed that βHB potentiated secretion of GLP-1 by GLUTag cells. This is in contrast to the findings of Sumithran et al. (2013), who saw a fall in GLP-1 secreted by ketotic patients. Future studies of cell in culture could allow pharmacological or knock-down test-

ing of receptors that could explore the link between βHB and gut hormone secretion. For example, ketone bodies have been shown to act antagonistically on GPCR-41, a Gi/Go protein-coupled receptor (Kimura et al., 2011) expressed throughout the small bowel enteroendocrine cells which may have a role in nutrient sensing (Karaki et al., 2008; Symonds et al., 2011) and may offer a target to explore the effect of βHB on gut hormone secretion.

Exogenous ketones may decrease hunger and aid in weight-loss, however such preliminary results should be interpreted with caution. For example, subjective measures of appetite, such as those used in this study, can be influenced by many external cues (social, time-related etc.) (Hill et al., 1995). Although the findings that ketones suppress hunger compared to dextrose are striking, the same effect may not occur should ketones be compared to protein, which is more satiating than dextrose (Poppitt et al., 1998). Finally, an increase in anorexigenic signals resulting from ketone drinks would be irrelevant if these changes do not alter food intake.

As appetite was not the primary endpoint for the studies in this Thesis, future studies could be designed specifically to determine the satiating effect of exogenous ketones compared to other macro-nutrients, looking for changes in *ad libitum* food intake. Many gut hormones are highly unstable and require acid-fixation and rapid analysis to obtain accurate values from plasma (Hosoda et al., 2004), but careful sample protection may reveal the effects of exogenous ketones on peripheral control of appetite. Furthermore, the work in this Chapter did not explore the potential role of central effects of βHB on appetite control that were hinted at by rodent studies using cerebral βHB infusions (Morton et al., 2006; Carneiro et al., 2016). Neuro-imaging studies could be used in future studies of exogenous ketones and appetite to accompany blood analysis, which may clarify the interaction between the central and peripheral changes.

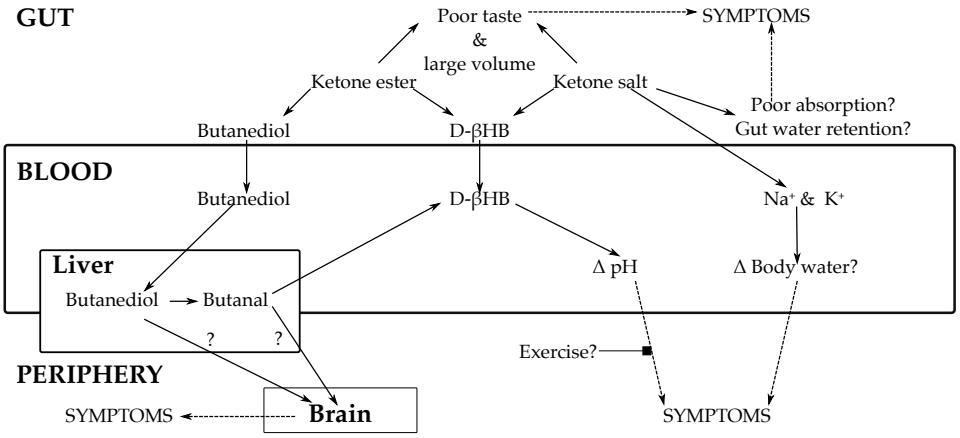

Figure 5.13: *Schematic representation of the potential mechanisms by which ketone drinks may cause GI symptoms.*

5.5.3 Tolerability of exogenous ketones

Several factors may have contributed to the differences in GI symptoms following ketone drinks. These include by-products of ketone compound metabolism, metabolic effects of ketone compounds, the taste of the drinks or their effects in the gut (see summary Figure 5.13). Low levels of symptoms were noted following oral ketone ester drinks (Clarke et al., 2012b), but not in early studies of ketone salts as they were in babies with complicated neurological conditions that obscured symptoms (Plecko et al., 2002; Van Hove et al., 2003).

The results of this study suggest that there is a link between an increase in the dose of both exogenous ketone compounds and the incidence and severity of symptoms. There were striking changes in the incidence of some symptom types (e.g an increase in lower GI symptoms with increasing dose of ketone salt from 7% to 33%) that did not reach statistical significance, perhaps because of a small sample size or due to the stringency of the McNemar's test. However, during these experiments *all* subjects had levels of blood βHB that were elevated up to $\approx$ 4 mM but many experienced no ill-effects, therefore the βHB molecule itself was not consistently linked to symptoms. One must therefore assume that another aspect of these ketone drinks is responsible. The consumption of a large bolus (300

- 500 ml) of an unpalatable drink offers an explanation for some of the symptoms reported in these studies. A further possibility is that the physiological and metabolic effects of the ketone drinks, such as altering acid-base balance or lowering blood glucose, may have caused symptoms. Additionally, as the two drinks have different moieties accompanying βHB delivery, these may also affect the symptoms associated with each drink.

Ketone ester drinks were mainly associated with upper GI and systemic effects. Whilst the unhydrolysed ketone ester could not be detected in the blood after drink consumption (Clarke et al., 2012b), it is possible that butanediol was released into the systemic circulation and may be linked to symptoms. Early studies of this ketone ester did not find levels of blood butanediol above 1 mM, despite a ketone ester delivery of 4 mmoles.kg^{-1} and peak ketosis of 3.3 mM (Clarke et al., 2012b). The central effects of butanediol on humans are unknown, whilst it is less lipid permeable than ethanol it may have a higher potency in the central nervous system (Frye et al., 1981). Furthermore, blood levels of both butanediol or butanal (an intermediate in the conversion of butanediol to βHB) may be higher than expected in patients with compromised hepatic function or in ethnic groups with less active forms of alcohol and aldehyde dehydrogenase enzyme (Mizoi et al., 1983), which could increase symptoms. Although assay development work was undertaken with the Mass Spectrometry Service at Oxford University, it was not possible to assay for butanediol and butanal as part of this project.

High doses of ketone salts caused moderate to severe lower GI symptoms in several subjects. These effects could be caused by the high level of inorganic ions (3.2 g of both sodium and potassium) in the drinks causing water retention in the intestine, which would have increased at the higher dose (Rehrer et al., 1992b; Jeukendrup et al., 2005). The volumes of ketone salt required to deliver equal βHB were higher than the ketone ester (60 ml of salt vs. 20 ml ester for 70 kg person), which made these drinks highly concentrated and unpleasant (Figure 5.11). The salt load also could have caused symptoms, such as headache or nausea, by altering body water levels and blood pressure (Farquhar et al.,

2005). Therefore, the mechanisms underlying some of the GI symptoms seen following ketone ester and salt drinks may differ between the two compounds.

Any acute discomfort resulting from exogenous ketone consumption should be weighed against the difficulties of achieving ketosis through endogenous production. Improperly formulated LCHF diets can lead to headaches, lethargy, dizziness and GI difficulties, such as constipation and diarrhoea, all of which may cause discontinuation of the diet (Hee Seo et al., 2007; Freeman et al., 2007). GI effects are also common with consumption of ketogenic precursors, such as MCTs, which led to a drop out rate of 25% when such a product was studied in a clinical trial (Henderson et al., 2009). By contrast, ketone drinks caused only low levels of symptoms if consumed whilst fed, fasted or before exercise, and some effects may be alleviated by improving drink palatability or reducing drink volume.

Finally, while exogenous ketones have been shown to improve performance of endurance athletes (Cox et al., 2016b), to be a practical intervention they must not result in GI upset that could impair performance. Interestingly, the results of this Chapter demonstrated that symptom incidence and severity was lower when the same dose of ketone ester was consumed prior to exercise compared to rest. Exercise can alter perceptions of painful stimuli and act as a distraction (Janal, 1996) (especially compared to the lack of diversion for participants in the resting studies), or perhaps the group of athletes who took part in the exercise experiments had a higher tolerance for unpleasant stimuli than the volunteers for the resting studies (Janal, 1996). Alternatively, as exercise inherently involves disturbances to whole body homeostasis, such as metabolic acidosis, compensatory changes such as increased respiration (removing acid via CO_2) may have opposed the acid-base perturbations caused by the ketone ester (see Chapter 3). The risk of lower GI distress (such as diarrhoea) is greatly increased during exercise (Rehrer et al., 1992a), therefore ketone salt consumption may further increase the risk of lower GI symptoms in athletes. However, it appeared that as exercise did not increase the risk of unwanted GI side-effects following ketone ester drinks, this exogenous ketone compound may be a tolerable intervention to

potentiate endurance performance.

5.6 Conclusion

In summary, exogenous ketosis suppressed appetite, with this effect potentially mediated by changes in gut hormone secretion. The metabolism of exogenous ketone compounds was associated with mild GI side-effects that appeared to vary according to the ketone compound, dose and conditions of consumption. As symptoms were less frequent and intense during exercise, ketone ester drinks could be well tolerated by endurance athletes to provide a helpful substrate for exercise. Overall, it appears that exogenous ketones offer a route to elevate blood ketones that is possibly more tolerable than a LCHF diet or MCTs and may have further desirable effects, such as appetite suppression.

Chapter 6

Nutritional supplement use in athletes

6.1 Abstract

Nutritional supplements are commonly used in a sporting setting to meet specific dietary demands of training and to optimise performance during competition. Supplement use may depend on factors such as demographics and the perceived vs. proven effects of supplements. Work in this Chapter aimed to study the prevalence of supplement use during training and competition in a cohort of British endurance athletes of different competitive levels. Furthermore, this study sought to establish the perceived difference between the 'average' and 'best' performances of these athletes, in order to compare this to the proven effects of supplements. It was found that supplement use was highly prevalent and increased with athlete performance level, but was unaffected by sex, age or training hours per week. There were differences in the frequency, formulation and function of supplements used during training and competition. The reported difference between 'average' and 'best' performances for each performance level ranged from 2.5 - 8.5%, decreasing as performance level increased. The high level of supplement use in elite athletes reflects this lower variability in performance, and the importance of small improvements, such as may be offered by legal supplements. The high level of variability in lower level athletes suggests that the small effects of supplements may be largely irrelevant, however, as prevalence was still high in this group, this highlights the need for athlete education surrounding supplement use. It is hoped that the results presented in this Chapter will give an insight into the role of supplements in sport, and as exogenous ketones can improve endurance performance (Cox et al., 2016b), these results may provide some context for their use in athletes.

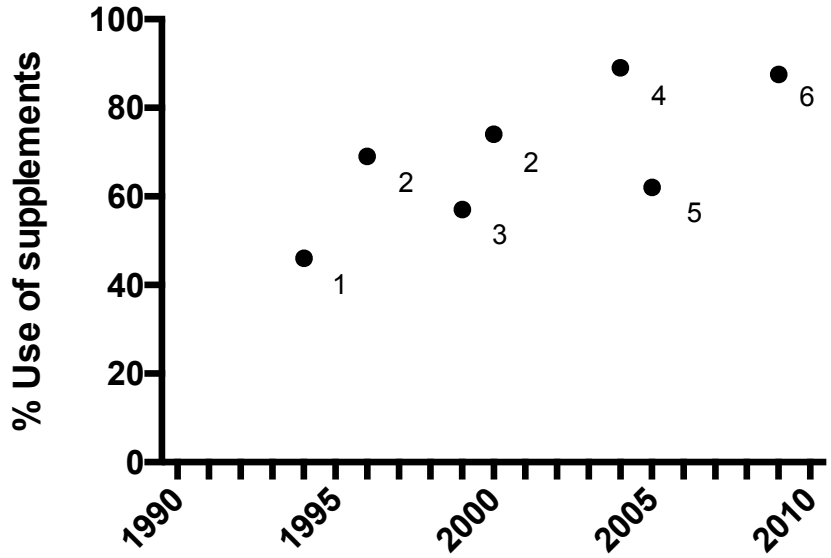

Figure 6.1: *Plot illustrating the reported prevalence rate (%) of nutritional supplement use by athletes over time since 1990. Study 1: Sobal and Marquart (1994); Study 2: Huang et al. (2004) surveyed in 1998 and 2000; Study 3: Krumbach and Ellis D Driskell (1999) ; Study 4: Froiland et al. (2004); Study 5: Nieper (2005), Study 6: Dascombe et al. (2010)*

6.2 Introduction

Sports nutrition supplements were broadly classified by Burke and Read (1993) as either dietary supplements or nutritional ergogenic aids. A dietary supplement "addresses a physiological or nutritional issue (i.e deficiency) arising in sport," and an ergogenic aid "contains a substance that is unessential to the general public, but which may provide a competitive advantage to an athlete." Supplement use in athletes has increased over the last 20 years, from 46% in 1994 to approximately 85% in more recent studies (Figure 6.1). Increased prevalence may be linked to the growth of the sports nutrition market, which grew in value by 40% between 2009 - 14 (Mintel, 2015), despite a lack of scientific evidence supporting the use of many supplements (Braun et al., 2009).

Patterns of supplement use can vary by athlete level (Erdman et al., 2006; Knapik et al., 2016), sex (Erdman et al., 2007) and sport (Dascombe et al., 2010). A further factor that

may influence use is the desired effect, which may vary between training compared to competition due to the different demands of each setting. The primary motivation for dietary supplement use in endurance athletes is often given as either enhanced performance or improved recovery from exercise (Krumbach and Ellis D Driskell, 1999; Froiland et al., 2004). When making informed decisions about supplement use, athletes must account for multiple factors, such as anti-doping rules and potential health hazards. Besides these issues, one consideration is the proven vs. perceived efficacy of each nutritional supplement. Athletes can be influenced by claims of manufacturers or by social pressure from team mates or support staff, resulting in incongruence between their reasons for supplement use and the true action of the supplement used (Petroczi et al., 2007).

There are several issues raised by the growing prevalence of supplement use in sport. Unlike the pharmaceutical industry, production and sale of nutritional supplements is poorly regulated (Petroczi et al., 2011). Manufacturers can make unsubstantiated health claims, or products may contain substances that could lead to a doping violation (Pipe and Ayotte, 2002; UKAD, 2015). Furthermore, consumption of some dietary and herbal preparations is associated with an increased risk of cardiovascular, neurological, metabolic, and haematological adverse effects (Palmer et al., 2003a).

This study aimed to examine the prevalence of supplement use in a cohort of British endurance athletes and to investigate trends in use between training and competition. It further aimed to establish the difference between an 'average' and 'best' performance, as this may help to determine what benefit a supplement must provide in order to be relevant to an athlete. As shown in Chapters 3, 4 and 5, exogenous ketones can reliably and tolerably raise blood βHB levels. Furthermore, ketone esters have been shown to improve endurance performance in rodents (Murray et al., 2016) and in humans (Cox et al., 2016b), and therefore may be a novel nutritional supplement for athletes. The results of this Chapter may give a greater appreciation of the use of supplements by the athletic community and provide context for possible uses of exogenous ketone bodies in athletes.

6.3 Methods

6.3.1 Study population

Ethical approval for the study was obtained through the University of Oxford Central Research Ethics Committee (MSD-IDREC-C1-2014-162). Questions were based on previously published work (Froiland et al., 2004; Nieper, 2005; Dascombe et al., 2010) and confirmed following a pilot study to assess clarity (n = 20) where feedback was sought on language and content. British endurance athletes (n = 181) who took part in cycling, triathlon or rowing, anonymously completed the final version of the on-line survey following an open invitation to participate.

6.3.2 Questionnaire

Demographics assessed included: age, sex and training hours per week. Performance levels were defined as: elite, serious amateur, causal competitor or recreational, and athletes indicated the highest level of competition reached in their sport to confirm their level.

As in similar work undertaken previously (e.g Nieper (2005)), the term "nutritional supplement" was not defined for athletes, as there is no single accepted definition. A distinction was made between pre-mixed and powdered formulation of energy, protein and recovery products. Supplements were grouped by function into three categories: energy nutritional supplements (pre-mixed and powdered energy drinks, energy gels and energy bars), recovery (non-energy) nutritional supplements (vitamins/minerals, electrolytes, protein/amino acid supplements, pre-mixed and powdered recovery drinks and creatine) and ergogenic supplements (caffeine, bicarbonate, beta-alanine and nitrate supplements). Space was provided to record supplements not listed. Athletes selected by recall the regularity with which they used these supplements during training and competition. A supplement was counted if athletes indicated that they used it 'frequently' or 'always'. At the end of the survey,

there was a free response space where athletes were asked to share factors that influenced supplement use.

To determine the perceived improvement between 'average' and 'best' performances, athletes were asked to specify their competition event and to predict a score/time that could be "confidently produced with average physical and mental preparation (typical course, neutral conditions)," and a score/time that they could "realistically hope to be able to achieve on your best day, with ideal physical and mental preparation." The full questionnaire can be found in Appendix D .

6.3.3 Statistical comparisons

Responses were analysed using Prism 6^{TM} and significance was taken as $p < 0.05$. Data from all 181 subjects was analysed throughout. Difference in use by sex was calculated using Fisher's exact test. Difference in use by performance sub-group, age and training hours per week, were compared using a Chi-squared test for trend. Supplement use by function and formulation were reported as the total number of reported uses as, due to the possibility of each athlete reporting multiple products (not a simple yes/no answer), it was not possible to apply a statistical test to asses the overall context-specific differences in use.

The difference between 'average' and 'best' performances were calculated manually for each athlete, expressed as a percentage improvement and reported as mean % ($\pm$ SEM). These calculated performance improvements were checked for outliers using the ROUT method, with alpha set at 0.1 and outlying data points were removed. The resulting data set was tested for normality (D'Agostino-Pearson test) and following this an unpaired, non-parametric test (Kruskal-Wallis) was used to compare the magnitude of performance improvement reported athletes of different levels. Paired post-hoc Dunn's tests were performed to calculate a multiplicity adjusted p value.

6.4 Results

6.4.1 Demographic characteristics

The demographics of respondents (age, sex, training hours/week) are shown in Table 6.1. Eleven respondents had competed at the Olympic Games, and a further fifty two had competed internationally.

Table 6.1: *Demographics of British endurance athletes (n =181) who responded to the on-line survey investigating their use of supplements.*

Characteristic	Group	Number
Sex	Male	119
	Female	62
Age	<18	6
	18-25	105
	26-32	37
	33-30	12
	41-50	14
	50+	7
Training (hours/week)	+36	9
	24-35	59
	18-23	47
	12-17	32
	6-12	26
	<6	8
Level	Elite	44
	Serious Amateur	84
	Casual	41
	Recreational	12
Sport	Rowing	103
	Cycling	58
	Triathlon	18

6.4.2 Prevalence of supplement use

Supplement use was high in this study cohort: 86% of all respondents used supplements. There was no effect of sex (p = 0.17, Figure 6.2 A1 & 2), age (p = 0.77, Figure 6.2 B1 &

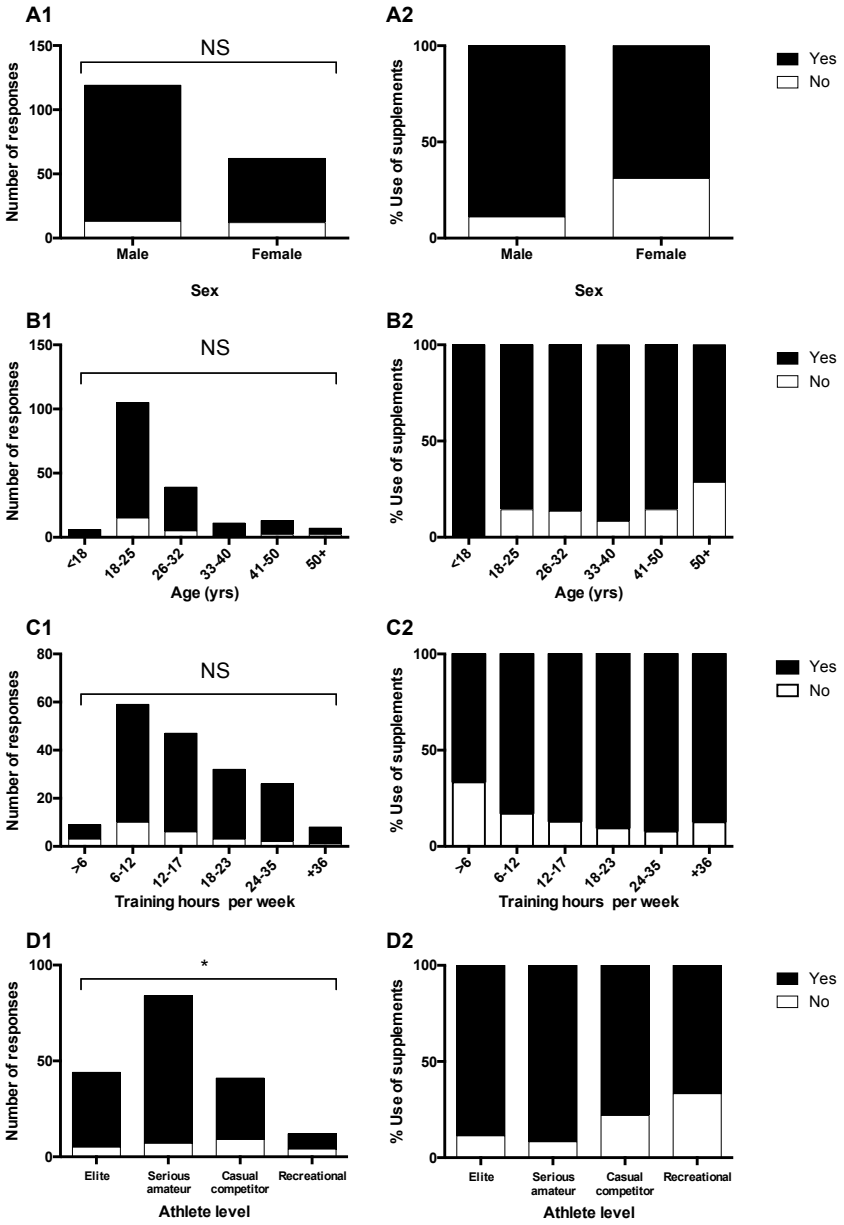

Figure 6.2: *Bar charts illustrating use of supplements by demographic group in British endurance athletes (n = 181). A: Frequency (1) and proportion (2) of supplement use by sex. B: Frequency (1) and proportion (2) of supplement use by age. C: Frequency (1) and proportion (2) of supplement use by training hours per week. D: Frequency (1) and proportion (2) of supplement use by performance level. Significance denoted by (*) and set at $p < 0.05$.*

Table 6.2: *Different types of supplement used in training and competition shown by sex and pooled. Values are frequencies (n) and (%) of total in each group.*

		Male		Female		All	
		n	(%)	n	(%)	n	(%)
TRAINING	Energy	95	37%	39	36%	134	37%
	Recovery	126	49%	50	46%	176	48%
	Ergogenic	37	14%	19	18%	56	15%
	TOTAL	**258**		**108**		**366**	
COMPETITION	Energy	166	58%	73	51%	239	56%
	Recovery	76	27%	40	28%	116	27%
	Ergogenic	42	15%	31	22%	73	17%
	TOTAL	**284**		**144**		**428**	

2) or training hours per week (p = 0.45, Figure 6.2 C1 & 2) on supplement use. There was a significant relationship between increasing performance level and increasing supplement use (χ^2= 8.46, 3. p = 0.04, Figure 6.2 D1 & 2).

6.4.3 Changes in supplement use during training and competition

Many athletes used more than one type and formulation of supplement, resulting in a total number of reported uses greater than total number of survey respondents. The total number of supplements used was higher during competition than training (C = 428; T = 366) (Figure 6.3 A and Table 6.2). This was due to each individual using a greater number of supplements in competition, as 75 athletes used the same number in both settings, however 68 used more products during competition (Figure 6.3 B). A similar number of athletes used no products in either setting (T = 49, C = 50), therefore the mean number of products per athlete was higher during competition (2.4 $\pm$ 0.15) than in training (2.1 $\pm$ 0.15) (Figure 6.3 C and D).

Different supplement formulations were more popular in training compared to competition. In training, powdered energy and recovery supplements were used 1.6 times more often than in competition (T = 128; C = 78), whilst in competition pre-mixed energy and recovery supplements were used more 4.5 times more regularly compared to training (C =

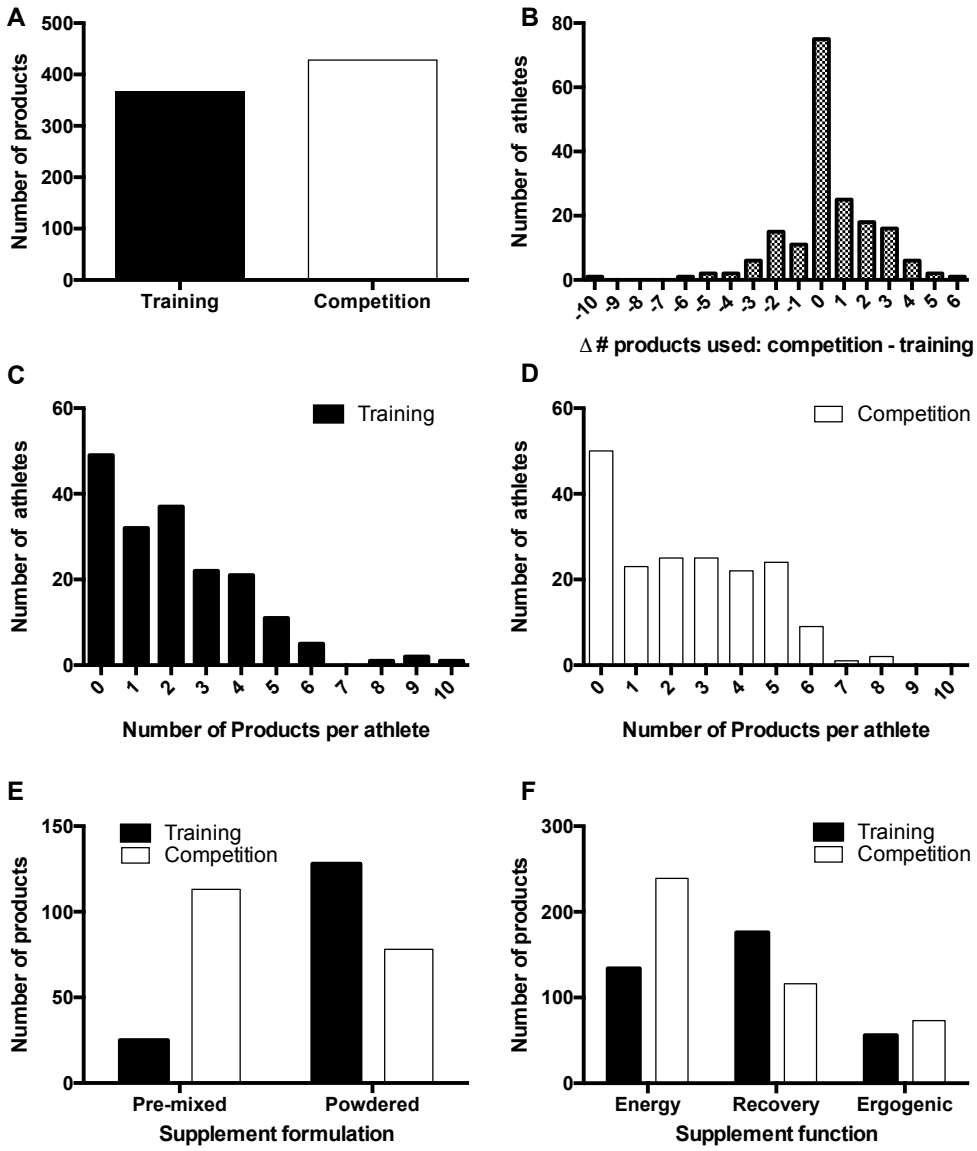

Figure 6.3: *Bar charts illustrating the differences in supplement use by British endurance athletes during training and competition (n = 181). A: Number of supplements used in training (T) or competition (C).B: The difference between the number of supplements that each individual used in competition compared to in training (where '0' denotes same number of products used in each context). C: The number of athletes using each amount of products during training. D: The number of athletes using each amount of products during training. E: The number of uses of pre-mixed and powdered supplement formulations in training and competition. F: The number of uses of supplement by function in training and competition. Abbreviations: C, competition; En, energy; Erg, ergogenic; PM, pre-mixed; P, powdered; Rec, recovery; T, training.*

113; T = 25) (Figure 6.3 E).

There were differences between the function of products used in each setting which were consistent between male and female athletes (Table 6.2). Total use of energy supplements was over 1.8 times greater during competition than training; similarly ergogenic supplements were used 1.3 times more in competition but recovery products were used 1.5 more frequently during training (Figure 6.3 F).

6.4.4 Improvement between an athletes 'average' and 'best' performances

When data for all athletes was pooled, performance level significantly affected the improvement between 'average' and 'best' performances ($H^2 = 64.9$; $p < 0.0001$). Elite athletes felt a 2.1$\pm$ 0.2% improvement was possible, for serious amateur athletes this was 3.4 $\pm$ 0.3%, increasing to 7.3$\pm$ 0.6% for casual competitors and was highest in recreational athletes (8.7 $\pm$ 1.3%)(Figure 6.4A). Supplement use by sub-group increased as the difference between 'average' and 'best' performances decreased (R^2 = - 0.87, Figure 6.4B), however this cannot be considered independently of the correlation between performance level and use.

There was significant effect of sport (p = 0.01) on the improvement between 'average' and 'best' performances (Figure 6.4C), with rowers reporting a significantly smaller improvement than both cyclists (-4.1 $\pm$ 0.7 %, p = $<$0.0001) and triathletes (-3.6 $\pm$ 1.1 %, p = 0.002). The effect of level remained significant when all sports were considered separately (p = 0.007), although this was only significant in rowing following corrections for multiple comparisons. This is likely due to the comparatively smaller number of cyclists and triathletes who completed the survey (Table 6.1). There were no significant differences between male and female athletes (p = 0.46, Figure 6.4C).

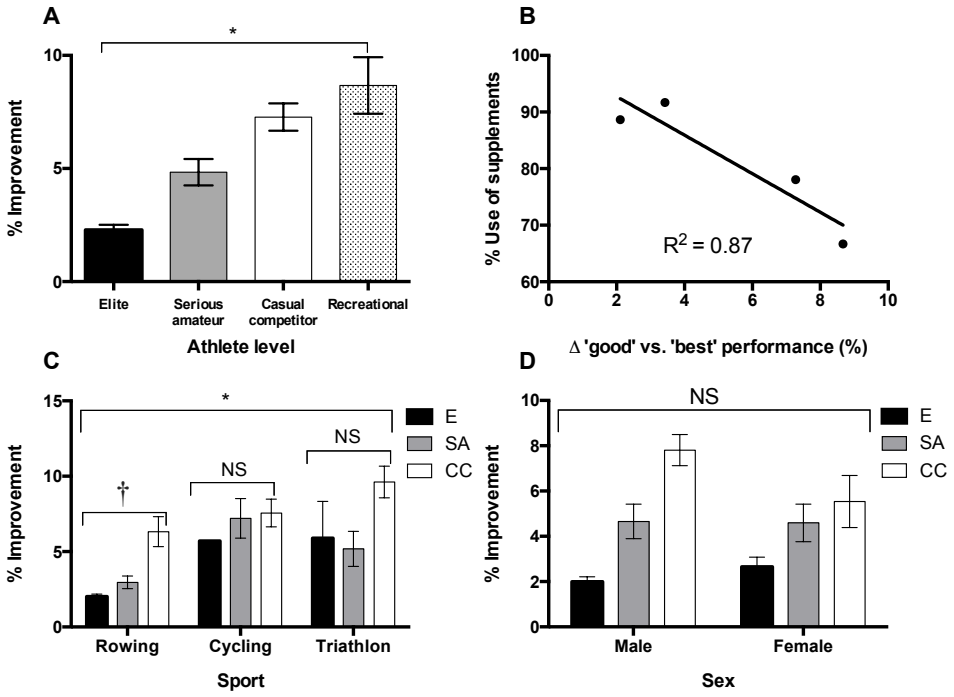

Figure 6.4: *The relationship between athlete level, sport, improvement between 'average' and 'best' performances and supplement use in British endurance athletes (n = 181). Values are mean ± SEM, significance set at p <0.05 and denoted by * = overall effect of level, † = effect of level within sport. A: Mean improvement by performance level. B: Mean improvement by performance level plotted against frequency of supplement use in that performance level. C: Mean improvement by sport, rowers (n = 96); cyclists (n = 67), triathletes (n = 18). D: Mean improvement by sex. Abbreviations: CC, casual competitor; E, elite; NS, not significant; SA, serious amateur.*

6.4.5 Other factors affecting use

In the free response section athletes commented about factors that affected their use of supplements (n = 63). The factors most frequently mentioned were that many preferred home-made or food alternatives (n = 22), that the cost of supplements was high (n = 12), that taste was important in their choice of supplement (n = 9) and that they had doubts as to the additional benefits of supplements (n = 6).

6.5 Discussion

6.5.1 Main findings

Use of supplements by British endurance athletes was similar in athletes of all ages and both sexes, but varied between training and competition. Supplement use was higher in elite vs. lower level athletes, and the difference between performances perceived as 'average' and 'best,' was smaller in elite vs. lower level athletes. The smaller perceived differences in performance of elite athletes may be linked to the increased supplement use in this population.

6.5.2 Prevalence of supplement use

Supplement use was highly prevalent in this study cohort, despite many leading sports organisations advising against supplementation (Maughan et al., 2007; Thomas et al., 2016). The rationale for the official position is that the effect of supplements on performance is small compared to other factors such as talent, training, psychology and robustness. Overall prevalence of supplement use was not higher than in earlier studies (Figure 6.1), suggesting that even with increased availability, ubiquitous supplementation is unlikely. The growth in the supplement market may be attributed to high prevalence in lower performance athletes ($>$60%), as these athletes represent a large proportion of the sporting community. The relationship between increasing performance level and higher rates of supplement use is in agreement with the existing literature (Erdman et al., 2006; Knapik et al., 2016). With respect to other demographic factors, in this cohort there were no differences in use between male and female athletes or in athletes of different ages, however a recent meta-analysis concluded that a lack of homogeneity between study populations precludes generalised statements about supplement use (Knapik et al., 2016).

6.5.3 Supplement use in training and competition

Use of nutritional supplements varied between training and competition to meet the context-specific demands, with overall use of supplements and the number of products used by each athlete being higher in competition than training. The differences in formulations of supplement used in training and competition are likely to be largely practical. For example, these results showed an increased use of pre-mixed supplements during competition, which are easier to transport, prepare and consume. The changes in the function of products used may be motivated by the belief that supplementation has a relevant impact on performance: as illustrated by the increased use of supplements with an 'energy' or 'ergogenic' function during competition. This is not without grounds, as providing adequate metabolic substrate (usually as carbohydrate) to sustain exercise is a crucial determinant of exercise capacity, and ingestion of supplemental carbohydrates during exercise can significantly improve endurance performance (Carter et al., 2003). Similarly, evidence supports the use of *some* ergogenic supplements during competition, for example supplements such as caffeine and bicarbonate can improve performance by 5 - 20% (Astorino and Roberson, 2010; Matson and Tran, 1993). Supplement consumption may also be ergogenic due to a strong placebo effect (Clark et al., 2000; Hopkins et al., 1998). All considered, the widespread use of supplements seen in this cohort during competition is unsurprising.

Supplements that functioned to enhance recovery were most popular during training. The impact of using these supplements during training on eventual competitive performance is unclear, and difficult to quantify. On the one hand, supplements that reduce the physiological stress of training and promote recovery may increase the quality and/or quantity of training completed, therefore improving competitive performance. An opposing approach to athletic training emphasises the need to *increase* physiological stressors to accelerate adaptations that improve exercise capacity and thereby performance (Hawley et al., 2006). One example is the use of antioxidants, which are commonly used in the

belief they reduce muscle damage, immune dysfunction and fatigue, thereby improving performance, whilst some evidence suggests that they in fact impair training adaptations (Braakhuis and Hopkins, 2015). The emerging paradigm is that both diet and supplement use should be periodised to achieve a balance between supporting and stressing an athlete's body to maximise the gains of training (Thomas et al., 2016). The high use of supplements in training seen in this cohort (a mean of 2 supplements per athlete) suggests a need for education to ensure that products used during training do not compromise adaptation to exercise and are only used in appropriate settings to augment training strategies.

6.5.4 Athlete level, improvement and supplement use

At an elite level, tiny margins can make the difference between winning and losing. Although there is a role for natural talent, performance is built on a foundation of training (e.g quantity, quality, type), lifestyle choices (e.g diet, recovery), and resilience (physical and mental). As a result of higher quality training, elite athletes can perform more consistently than those of a lower standard (Paton and Hopkins, 2001; Malcata and Hopkins, 2014). The lower level of variation between an 'average' and 'best' performance reported by the elite vs. lower-level athletes could be reflective of their consistency, however it also highlights the importance of smaller improvements to these athletes. Elite athletes often have a high level of investment in the outcome of a competition (both personal and/or financial), therefore the small improvements offered by supplements are likely to be relevant for these individuals.

In contrast, the performance of lower-level athletes is more variable (Paton and Hopkins, 2001; Malcata and Hopkins, 2014), emphasised by the greater difference between their 'average' and 'best' performances. As a result, one might conclude that the small benefits offered by supplements are unlikely to compensate for the variability in performance of these athletes. However, whilst supplement use in lower level athletes was less than in high-performance athletes, it was still highly prevalent (>60%). Lower-level athletes have

reduced access to information about supplements and may make decisions independently and based on the claims of product manufacturers, which can be (deliberately) misleading. This may result in inappropriate and potentially dangerous use of supplements, which do not have a relevant performance effect in these athletes (Petroczi et al., 2007; Petroczi and Naughton, 2007; Palmer et al., 2003b).

6.5.5 Ketone supplements in sport

Exogenous ketone bodies could be considered as either a dietary supplement or an ergogenic aid due to their dual roles as a fuel and a metabolic signal. Work by Murray et al. (2016) revealed that ketone ester supplementation improved the endurance capacity of rats, and similar improvements were described by Cox et al. (2016b) in humans, where ketone ester drinks improved cycling performance (+ 2% vs. carbohydrate alone). Furthermore, exogenous ketosis may trigger metabolic changes such as glycogen sparing (Cox et al., 2016c) or decreased protein breakdown (Cox et al., 2016a) that augment recovery during heavy periods of training.

However, the potential use of exogenous ketones in a sporting setting is not without issues. Similarly to other ergogenic supplements, the synthetic nature of exogenous ketones means that they could be seen as ethically questionable compared to supplements providing a source of dietary macro-nutrients such as carbohydrate. Furthermore, if exogenous ketones were to be used in training, studies should characterise the longer term metabolic effects to ensure that the beneficial effects of ketones on protein handling, glycogen use and re-synthesis do not blunt the desired physiological adaptations to training (Hawley et al., 2006). Current understanding of how ketones improve performance is limited, and it is assumed that their combustion as an oxidative fuel source and ability to alter metabolism of fat and carbohydrate underlie the acute ergogenic effect (Cox et al., 2016b). Should it be discovered that ketone bodies act as a longer-acting adaptive signal their use may be prohibited, although distinguishing between exogenous and endogenous ketones may prove a

challenge for doping authorities. Finally, as demonstrated in Chapters 3 and 5, excessive consumption of exogenous ketones may increase the risk of metabolic or GI side-effects, therefore athlete education will be important if exogenous ketone supplements become readily available.

6.6 Conclusion

The use of supplements by British endurance athletes was high, and exhibited context-specific differences between training and competition. Higher level athletes reported a greater prevalence of use, and a smaller difference between their 'average' and 'best' performances, therefore a supplement may only need to offer a small ergongenic effect to be relevant to these athletes. Despite greater variability in performance of lower level athletes, supplement use was still common, indicating the importance of athlete education to ensure appropriate use of supplements. These findings broaden knowledge of current supplementation practices and provide context for the use of exogenous ketones in sport.

Chapter 7

Effect of glycogen on cardiac D-βhydroxybutyrate oxidation

7.1 Abstract

Endogenous ketone bodies are normally elevated only when glucose and insulin are low, whereas exogenous ketone bodies can elevate blood βHB in the presence of replete liver and muscle glycogen (carbohydrate) stores. These exogenous ketones can act as an oxidative fuel, but the optimal metabolic conditions for their oxidation are unknown. The experiments in this Chapter used an isolated perfused rat heart to determine whether the amount of glycogen stored in the heart could alter βHB oxidation rates. Glycogen can be metabolised (via glycolysis) to be oxidised in the Krebs cycle or used in a process called anaplerosis to regenerate Krebs cycle intermediates that have been depleted when used in other biosynthetic reactions. The availability of glycogen for use in anaplerosis may alter the ability of the muscle to oxidise βHB. Here, cardiac glycogen content was altered using different buffer substrates, achieving an 8-fold difference between the 'Low' and 'High' conditions of 5.3 $\pm$ 1.6 and 43.5 $\pm$ 5.1 μmoles glycosyl units.g wet weight $^{-1}$ respectively. βHB oxidation rate, measured using sodium [^{14}C] hydroxybutyrate, was related to glycogen content (R^2= 0.32, p = 0.01), and doubled from 0.7 $\pm$ 0.1 to 1.4 $\pm$ 0.3 μmoles.min^{-1}g.wet weight between 'Low' and 'High' glycogen levels. Metabolomic heart tissue analysis showed 40% higher levels of glycolytic intermediates and 70% higher levels of Krebs cycle intermediates in hearts with 'High' glycogen, and glycogen content was directly related to the Krebs cycle total pool size (R^2= 0.49, p = 0.01). Conversely amino acid levels were 3-fold higher in 'Low' glycogen hearts, suggesting that they were being used as an alternative to glycogen for Krebs cycle anaplerosis. These findings suggest that muscle glycogen facilitates βHB oxidation by providing a substrate for anaplerosis, thereby decreasing proteolysis. In short, the capacity to oxidise βHB was increased by high levels of glycogen in cardiac muscle.

7.2 Introduction

In 1995 Taegtmeyer famously stated that "not all metabolic substrates are equal,"(Taegtmeyer and de Villalobos, 1995) and whilst substrate oxidation is largely dependant on availability, it can be modulated by competing substrates (e.g fat oxidation decreases carbohydrate oxidation Hue and Taegtmeyer (2009)). During endogenous ketosis, ketones offer a replacement fuel for carbohydrate that is intended for the brain. However, during exogenous ketosis, ketones compete with carbohydrate for oxidation throughout the body. Work in earlier Chapters has demonstrated the efficacy of exogenous sources of ketones to elevate blood βHB, however it is important to understand how to optimise the use of the resulting βHB as a metabolic substrate. It is unknown whether oxidation of ketone bodies in muscle is affected by changes in levels of carbohydrate stored in the form of intracellular glycogen.

In the fed state, the heart obtains ≈ 60-90% of the acetyl-CoA required for Kerbs cycle function from the β-oxidation of fatty acids, and the remaining 10-40% from the oxidation of pyruvate from plasma glucose and glycogen (Gertz et al., 1988; Lopaschuk et al., 2010). Ketone bodies are taken up and oxidised by the heart in a concentration-dependent manner (Sultan, 1988). However, they cannot be the sole substrate, as a gradual functional decline has been observed in the isolated heart perfused with acetoacetate or βHB alone (Taegtmeyer et al., 1980; Taegtmeyer, 1983), which could be prevented by the addition of substrates such as glucose and lactate (Sultan, 1997; Russell and Taegtmeyer, 1991).

Ketone bodies can only enter the Krebs cycle as 2-carbon intermediates, via condensation of acetyl-CoA with oxaloacetate; these carbons replace the two that are lost as CO_2 molecules produced with each full turn of the cycle (Owen et al., 2002; Koeslag et al., 1980) (Figure 7.1). Therefore, entry of 4-carbon substrates (anaplerosis) is vital to maintain ketone oxidation in the Krebs cycle, as intermediates are constantly removed for use in bio-synthetic reactions (cataplerosis) (Owen et al., 2002). Sources of anaplerotic substrates include carbohydrate from circulating glucose or glycogen, and amino acids via transam-

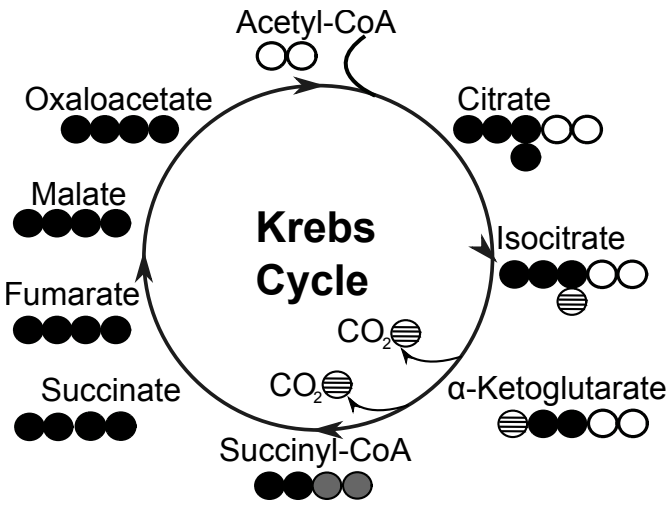

Figure 7.1: *Entry and exit of 2 carbon units from the Krebs cycle pool. Acetyl-CoA adds 2C units to the cycle, however these are effectively 'lost' with each full turn, as two CO_2. White C-units are not lost in their first 'turn' as part of the circle, indicated as they turn from white to grey.*

ination (Figure 7.2). Pyruvate is one of the key anaplerotic substrates in the heart, being used almost equally for decarboxylation to acetyl-CoA or carboxylation to oxaloacetate (Comte et al., 1997).

Exogenous ketosis, when muscle and liver carbohydrate (glycogen) stores are replete, represents a novel physiological state, with an unknown hierarchy of substrate utilisation. This is of particular relevance to endurance athletes, in whom carbohydrate availability is a key determinant of performance (Bergstrom et al., 1967). Ketone drinks improved endurance performance despite strikingly different carbohydrate metabolism to that usually seen during exercise (Cox et al., 2016b). In this study, exogenous ketosis was associated with lower muscle glycolytic intermediates, glycogen utilisation and blood lactate levels. Ketone oxidation was estimated to account for 10-18% of oxygen consumption, in contrast to the observations that ketone oxidation is limited in 'starved' skeletal muscle (Fery and Balasse, 1983). Therefore, it was suggested that carbohydrate was required for maximal oxidation of ketone bodies (Cox et al., 2016b). Usually, athletes commence exercise with replete glycogen stores, which are dramatically depleted if the effort is prolonged

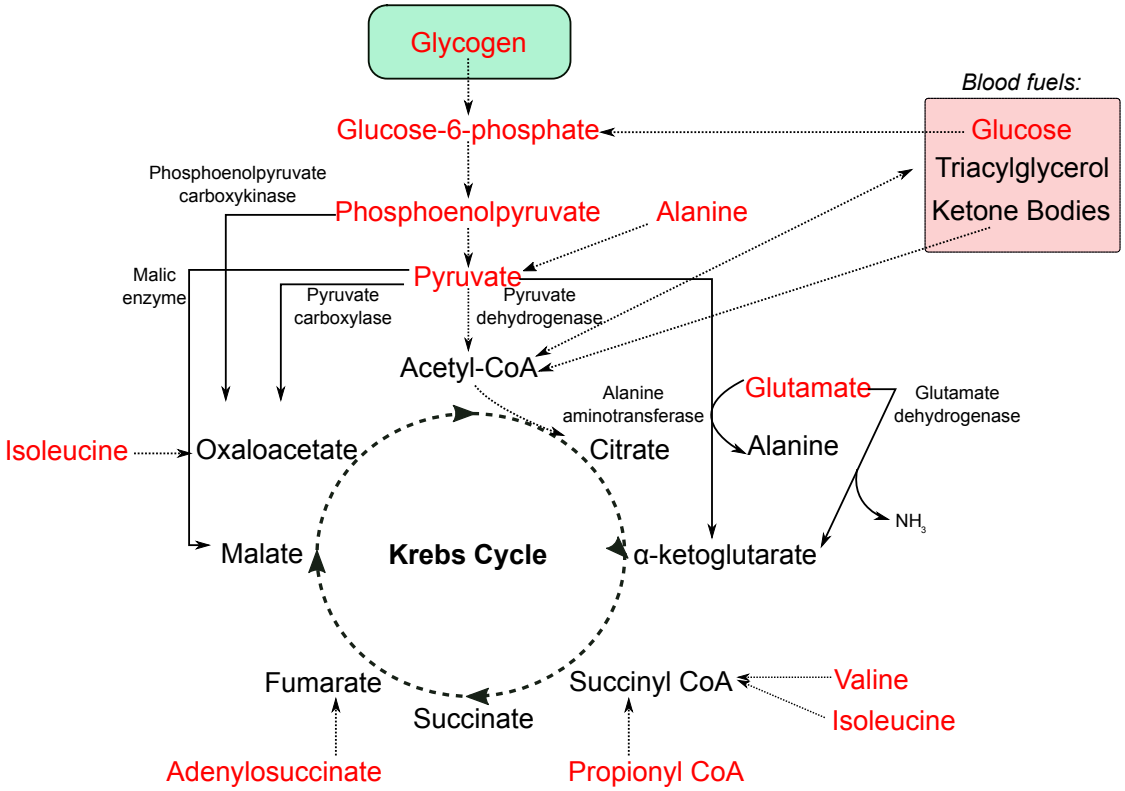

Figure 7.2: *Summary of the major anaplerotic pathways. Substrates shown in red are able to act anaplerotically. Glycogen can be used for anaplerosis through conversion of pyruvate to oxaloacetate by pyruvate carboxylase, to malate by malic enzyme, to α-ketoglutarate by alanine aminotransferase or phosphoenolpyruvate to oxaloacetate by phosphoenolpyruvate carboxykinase. Adapted from Gibala et al. (2000)*

(Bergstrom et al., 1967). As a result, it is important to investigate how ketone oxidation varies with changes in glycogen levels.

Here, isolated perfused rat hearts were used to determine the effect of glycogen levels on βHB oxidation because muscle glycogen levels can be altered and functional and metabolic measurements made. Cardiac glycogen may act as a source of anaplerotic intermediates to increase ketone oxidation, whereas low glycogen may cause depletion of intermediates and limit the condensation of acetyl-CoA from ketone bodies in the Krebs cycle.

7.3 Methods

Glycogen levels in the perfused rat heart were altered by using different metabolic substrates in the perfusion buffer in the absence of ketone bodies (-K perfusion). Hearts were subsequently perfused with ^{14}C-labelled D-βHB to directly measure βHB oxidation (+K perfusion). Cardiac function was recorded throughout perfusions, and analysis of heart tissue metabolites was undertaken following the experiments (Figure 7.3).

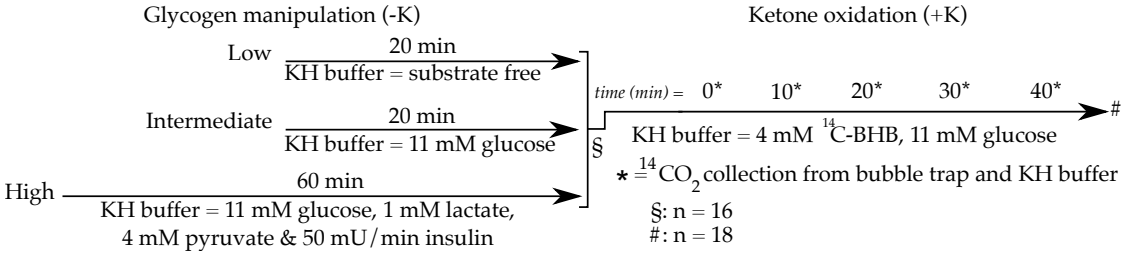

Figure 7.3: *Schematic illustrating the overall perfusion study design and the sampling schedule for each of the perfusion experiments. Abbreviations: $ and # = hearts freeze-clamped for analysis, KH = Krebs-Henseleit, BHB = D-βHB.*

7.3.0.1 Heart Perfusion

Male Wistar rats (body weight 150 - 200 g) were purchased from Harlan (UK) and kept in controlled conditions (19-23 °C, humidity 45-65%, 12:12-h light:dark cycle) in the animal facilities at the University of Oxford. Animals consumed standard rodent chow (Teklad Global Diets 2186) *ad-libitum* and were not fasted prior to experiments. Animals were anaesthetized with an intraperitoneal injection of sodium pentobarbitone (0.3 $ml.kg^{-1}$). Hearts were rapidly excised and arrested in ice-cold Krebs-Henseleit (KH) buffer. Excess tissue was removed, and the hearts were cannulated, via the ascending aorta, for retrograde perfusion using the Langendorff method with KH buffer containing 118 mM NaCl; 4.7 mM KCl; 1.2 mM $MgSO_2$; 1.75 mM $CaCl_2$; 0.5 mM Na_2EDTA; 25 mM $NaHCO_3$; pH 7.4. The buffer, aerated with 95% O_2 and 5% CO_2 at 37 °C, was perfused through the hearts at a constant pressure of 100 mmHg. The pulmonary artery was incised to prevent venous pressure accumulation and the left ventricle was vented by insertion of a drain through the apex of the left ventricle. A water-filled latex balloon, attached via polyethylene tubing to a pressure transducer, was inserted into the left ventricular cavity via the mitral valve and inflated sufficiently to result in an end-diastolic pressure of $\approx$ 4 mmHg. Left ventricular pressures and heart rates were recorded using a PowerLab data acquisition system. Cardiac contractile function was expressed as a rate-pressure product, this being the product of left ventricular developed pressure (mmHg) and heart rate (beats per min), where developed pressure was the systolic pressure minus end-diastolic pressure.

7.3.0.2 Glycogen manipulation

In order to alter cardiac glycogen levels, hearts underwent one of three different -K perfusion protocols, based on the work of Laughlin et al. (1994), Cross et al. (1996) and Dhalla et al. (1972) (Figure 7.3):

1. 'Low': To deplete glycogen, hearts were perfused for 20 min with non-recirculating

KH buffer that contained no metabolic substrate (95% O_2 and 5% CO_2 at 37 °C).

2. 'Intermediate': For slightly higher glycogen, hearts were perfused for 20 min with non-recirculating KH buffer that contained 11 mM glucose (95% O_2 and 5% CO_2 at 37°C).

3. 'High': To elevate glycogen, hearts were perfused for 60 min with non-recirculating KH buffer with 11 mM glucose, 1 mM lactate, 4 mM pyruvate and insulin at 50 mU/min (95% O_2 and 5% CO_2 at 37 °C).

In order to validate these perfusion conditions, a subset of hearts (n =16) were freeze-clamped in Wollenberger tongs cooled in liquid nitrogen for analysis of glycogen and triacylglycerol content.

7.3.0.3 Ketone body oxidation

After altering the glycogen content, hearts (n = 18) were perfused with 200 ml of recirculating KH buffer aerated with 95% O_2 and 5% CO_2 at 37 °C with 11 mM glucose and 4 mM D-βHB for 40 min (Figure 7.3). When heart function had stabilised, 0.2 MBq (100 μl) ^{14}C labelled D-βHB was added to the buffer reservoir. Evolved CO_2 and HCO_3^- were collected every 10 min for 40 min. The perfusion apparatus was made air-tight and interconnected with tubing; air was drawn through the tubing using a vacuum pump and passed through a bubble trap containing 15 ml of a chemical CO_2 absorber (Carbosorb, Perkin Elmer, Massachusetts, USA), which was replaced every 10 min. Care was taken not to expose the heart itself to variations in its surrounding atmospheric pressure, which may cause tampenade. A 1 ml aliquot of perfusate was collected using a syringe and placed in the central well of a sealed Ehrlenmeyer flask containing 1 M H_2SO_4, surrounded by 5 ml Carbosorb and left overnight. Carbosorb was removed from the Ehrlenmeyer flask (5 ml) and from the bubble trap (5 ml from 15 ml) and mixed with scintillation cocktail (Permafluor E+, Perkin Elmer, Massachusetts, USA) and radioactivity was counted.

7.3.0.4 Tissue analysis

Hearts were freeze-clamped in Wollenberger tongs cooled in liquid nitrogen at the end of the perfusion. Tissue was crushed and stored at -80°C prior to analysis. Cardiac glycogen and TG were measured in all hearts. Glycogen was measured in 0.3 g (wet weight) of tissue using an enzymatic method (Bergmeyer, 1974). Following an acid extraction, glycogen was hydrolysed to glucose using amyloglucosidase (Sigma Aldrich, Poole, UK) and glucose was measured using a spectrophotometric glucose (hexokinase) assay (Sigma Aldrich, Poole, UK). TG was measured using 0.3 g (wet weight) of tissue using an enzymatic method: following a Folch total lipid extraction, TG was assayed using the GPO-PAP method (Randox Laboratories, Crumlin, UK). Metabolomic analysis for metabolites and amino acids was carried out with Oxford University Mass Spectrometry Services on tissue samples from all hearts that were perfused with ^{14}C-βHB (n = 18). Tissue (50 mg) was extracted in methanol and homogenised using a Precellys homogeniser. The sample was centrifuged (1500 G, 20 min) and transferred to a Waters Total Recovery Vial before analysis by lipid chromatography coupled to mass spectrometry (Details in Appendix E).

7.3.1 Statistical analysis

Total ^{14}C-βHB oxidation was calculated from the sum of the $^{14}CO_2$ absorbed in the flask and bubble traps and normalised to heart wet weight. As there were large differences in the peak area of each metabolite, in order to represent the relative changes in concentration between conditions and to approximate the relative amount of metabolites in a given pathway (i.e. the Krebs cycle), the peak area for each sample were normalised to the mean peak area of the 'intermediate' glycogen hearts. Results throughout are given as mean ± SEM. Statistical analysis was as described in Chapter 2.5 and significance was taken at $p < 0.05$. Detailed results of statistical comparisons are reported in Appendix E.

7.4 Results

7.4.1 Cardiac function, glycogen and triacylglycerol

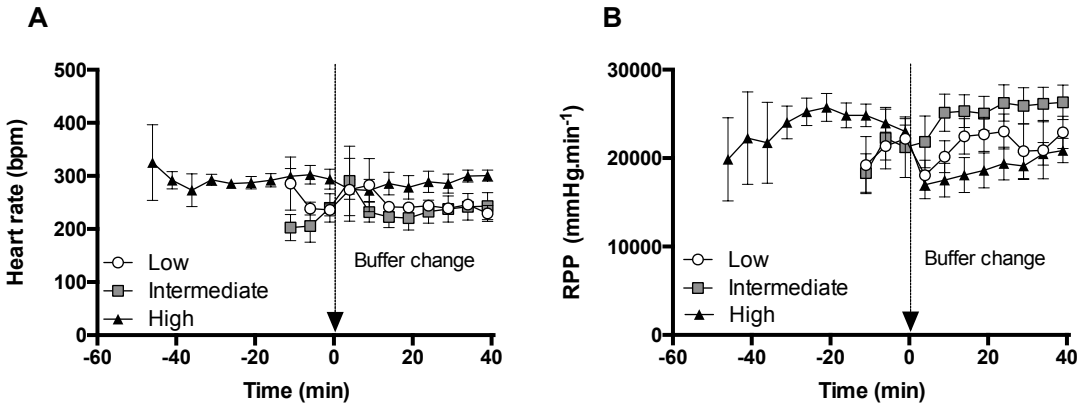

Figure 7.4: *Cardiac function over time in isolated perfused rat hearts during glycogen manipulation protocol and following a change in perfusion buffer to one containing 4 mM β-HB and 11 mM glucose (40 min) (n = 18). Heart rate (beats per min- bpm) (A) and the calculated rate pressure product (RPP- mmHg.min^{-1}) (B) are shown. Values were omitted for the period where the hearts were stabilising as variability was very high (initial 5 - 10 min of manipulation). Values were pooled for all hearts and for every 5 min and are shown as means ± SEM*

Table 7.1: *Mean heart rate (HR) (beats per min- bpm) and rate pressure product (RPP) values for isolated perfused rat hearts that underwent both the -K and the +K perfusions (n = 18). Values are mean ± SEM, no singificant differences were seen.*

Group	-K HR (bpm)	+K HR (bpm)	-K RPP (mmHg.min^{-1})	+K RPP (mmHg.min^{-1})
'Low'	258 ± 18	245 ± 6	20,706 ± 1680	22,701 ± 1950
'Intermediate'	214 ± 29	217 ± 12	21,352 ± 2610	23,908 ± 720
'High'	325 ± 30	287 ± 18	23,567 ± 2150	19,271 ± 1680

There were no significant differences in functional parameters for hearts in the three groups during the perfusions (Table 7.1). Heart rate and RPP were stable throughout perfusion under all conditions (Figure 7.4).

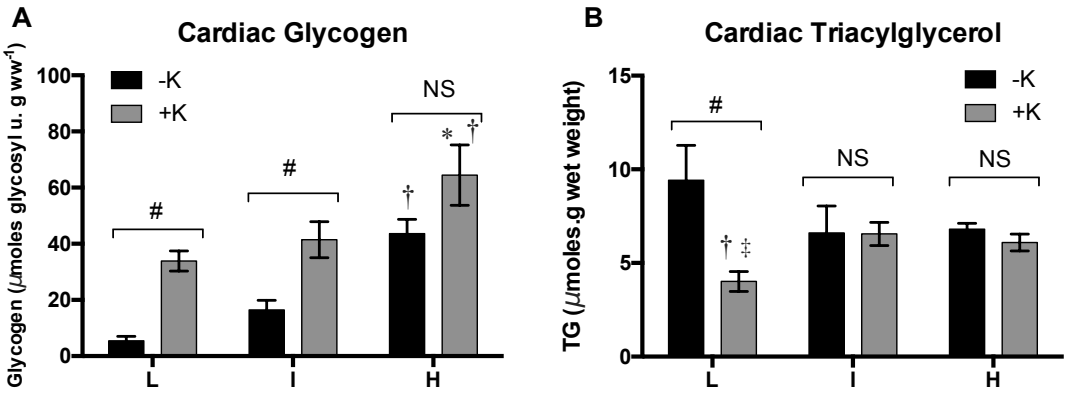

Figure 7.5: *Levels of glycogen (A) and triacylglycerol (B) in hearts following glycogen manipulation (-K, n = 16) or following the 40 min perfusion with D-βHB and glucose (+K, n = 18). Significance was taken at p <0.05 and shown as # = -K vs. +K; † = 'High' vs. 'Low'; * = 'High' vs. 'Intermediate;' ‡ = 'Intermediate' vs. 'Low.' Values are means ± SEM*

Levels of cardiac glycogen and TG were altered by the -K and +K perfusion protocols, demonstrating the effect of substrate provision on endogenous fuel stores (Figure 7.5). Following the -K perfusion, glycogen levels in the 'High' group were 8- and 3-fold higher than in the 'Low' and 'Intermediate' conditions, respectively (L = 5.3 ± 1.6 μmoles glycosyl units.g wet weight $^{-1}$; I = 16.3 ± 3.4 μmoles glycosyl units.g wet weight $^{-1}$; H = 43.5 ± 5.1 μmoles glycosyl units.g wet weight $^{-1}$) ($p<0.001$) (Appendix E, Table E.1). After the +K perfusion, differences in cardiac glycogen between the groups persisted. Glycogen levels were 2- and 1.5-fold higher in the 'High' condition than in the 'Low' and 'Intermediate' conditions respectively (L = 33.8 ± 3.5 μmoles glycosyl units.g wet weight $^{-1}$; I = 44.1 ± 6.4 μmoles glycosyl units.g wet weight $^{-1}$; H = 64.4 ± 10.7 μmoles glycosyl units.g wet weight $^{-1}$) ($p = 0.014$). The mean glycogen level was higher in all hearts that had been perfused with 4 mM βHB and 11 mM glucose compared to hearts that were freeze-clamped directly following the -K protocol, suggesting that glycogen synthesis had occurred in all hearts when perfused with βHB and glucose regardless of pre-existing glycogen levels (Figure 7.5 A).

In hearts freeze clamped directly after the -K protocol, there were no differences in

cardiac TG (Figure 7.5B, Appendix E, Table E.1). By contrast, hearts following the +K perfusion had significantly decreased TG when glycogen was 'Low' compared to the 'Intermediate' and 'High' conditions (p =0.009), in which TG levels were the same. This represented a significant fall in mean myocardial TG compared to levels after the -K perfusion (p=0.006)(Figure 7.5B).

7.4.2 Intermediates of glycolysis and the Krebs cycle

Exploratory statistical analysis showed highly significant differences between the 'Low' and 'High' glycogen groups in intermediates of glycolysis and the Krebs cycle (See Appendix E, Figure E.1). Levels of glycolytic intermediates were similar for the 'Low' and 'Intermediate' glycogen levels, however there were 1.5-2-fold higher levels of glycolytic intermediates when glycogen was 'High' (Figure 7.6). All glycolytic intermediates were at their highest concentrations with 'High' glycogen, except for phosphoenolpyruvate. Similarly, Krebs cycle intermediates in 'High' glycogen hearts were 1.5-2-fold higher than in the hearts with lower glycogen (Figure 7.6). See Appendix E, Table E.2 for all p-values.

7.4.3 Amino Acids

Muscle amino acid levels differed between 'Low' and 'High' glycogen hearts (Figure 7.7). Exploratory statistical analysis of 'variable importance' was able to predict which amino acids predicted the difference between the 'Low' and 'High' groups (See Appendix E, Figure E.2). These were: alanine, glutamic acid, isoleucine, leucine, lysine, phenylalanine, serine, threonine, valine. Further analysis focused on the amino acids known to be metabolically linked to the Krebs cycle. In 'Low' glycogen hearts, levels of branched chain amino acids (leucine, isoleucine and valine) were 4.2-fold higher ($p < 0.05$ in all cases). Glutamic acid was also significantly higher in 'Low' glycogen hearts, however there were no significant differences in the levels of alanine and lysine, despite lysine being 4.4-fold higher in hearts with 'Low' glycogen. See Appendix E, Table E.3 for all p-values.

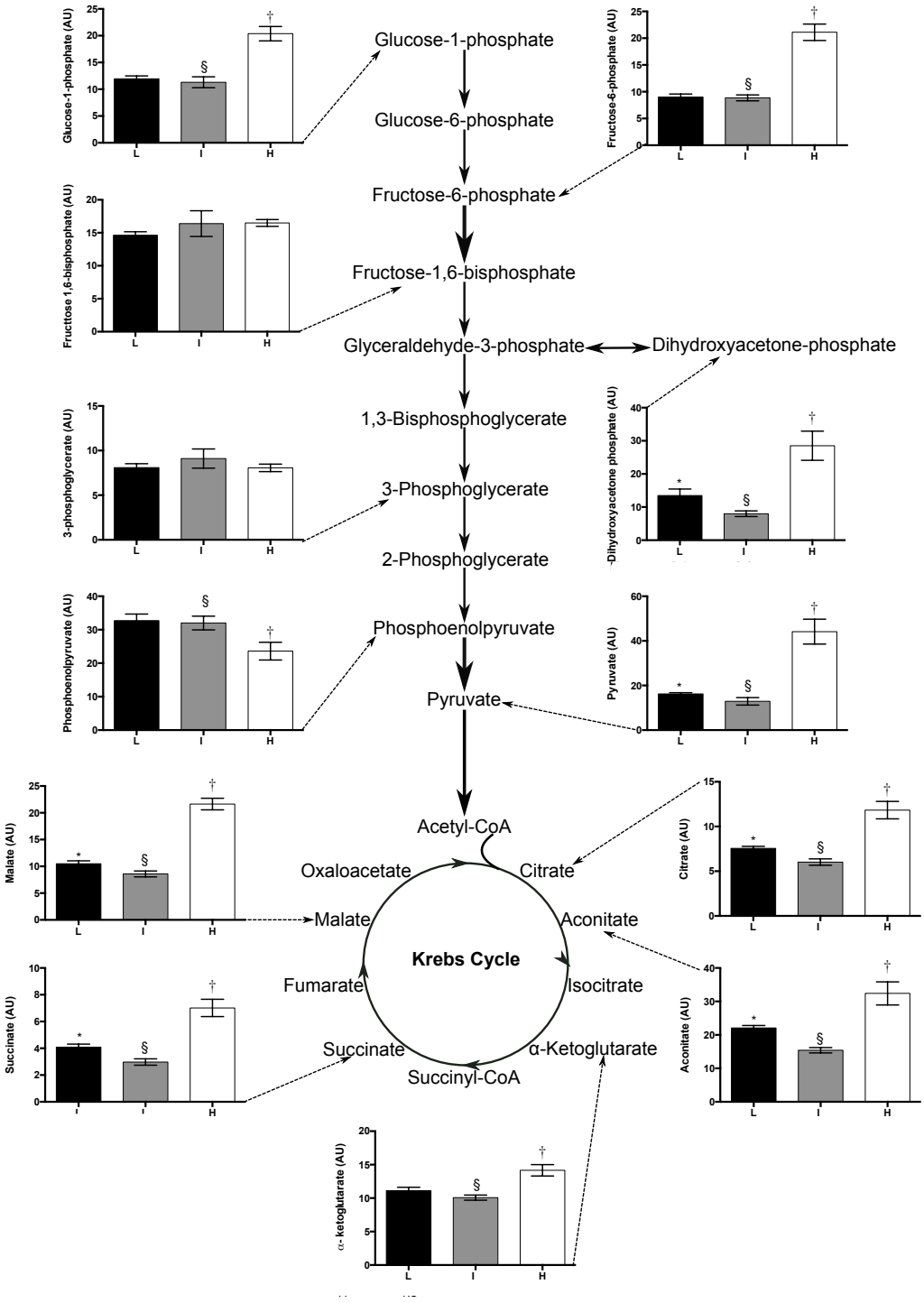

Figure 7.6: *Glycolytic and Krebs cycle intermediates measured using metabolomic analysis of tissue from hearts that had been perfused with β-HB and glucose for 40 min following glycogen manipulation to give 'Low' vs. 'Intermediate' and 'High' glycogen levels (n = 18). Significance was taken at p <0.05 and shown as † = 'High' vs. 'Low'; § = 'High' vs. 'Intermediate'; * = 'Intermediate' vs. 'Low'. Values are given in arbitrary units (AU) and shown as means ± SEM. Abbreviations; H = High; I = Intermediate; L = Low.*

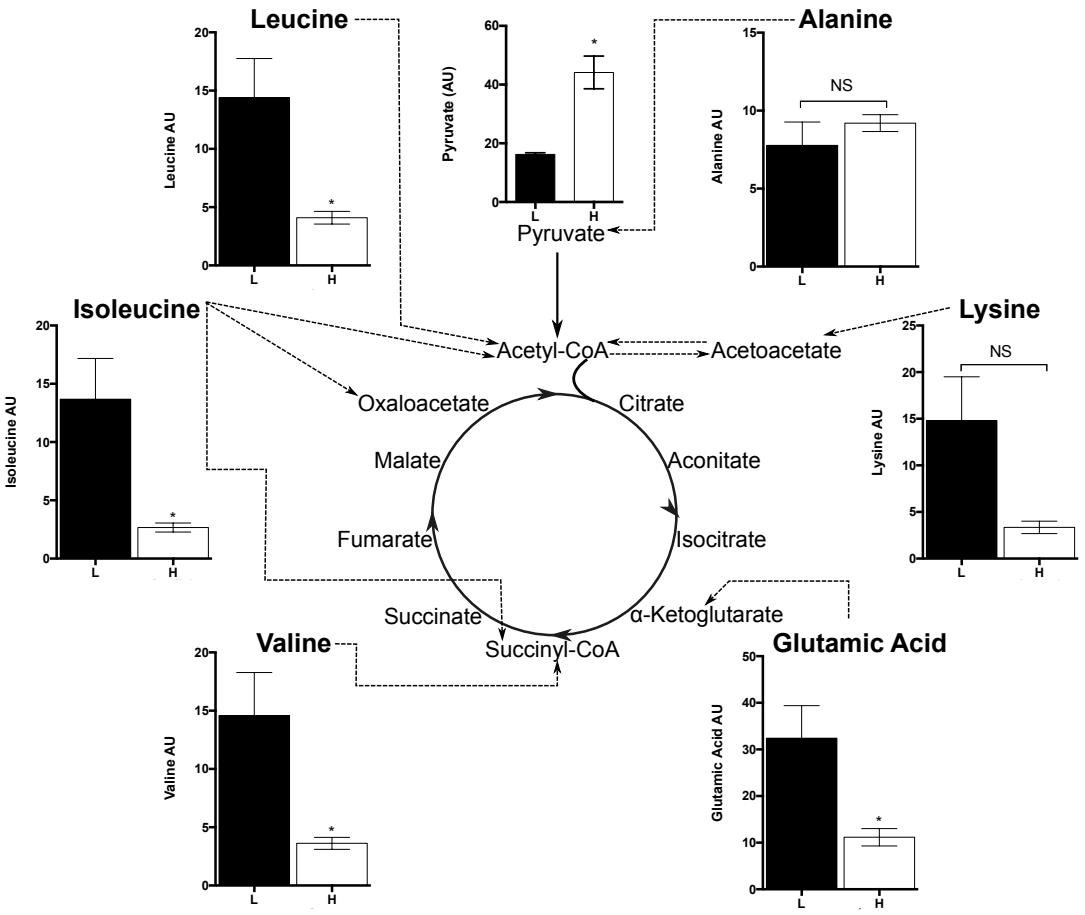

Figure 7.7: *Amino acid levels in hearts following glycogen manipulation and perfusion with D-βHB, shown with the potential point of entry into the Krebs cycle. Significance was taken at $p < 0.05$ and shown as * = 'Low' vs. 'High'. Values are given in arbitary units (AU) and shown as the mean ± SEM. Abbreviations; H = High; L = Low.*

7.4.4 βHB oxidation

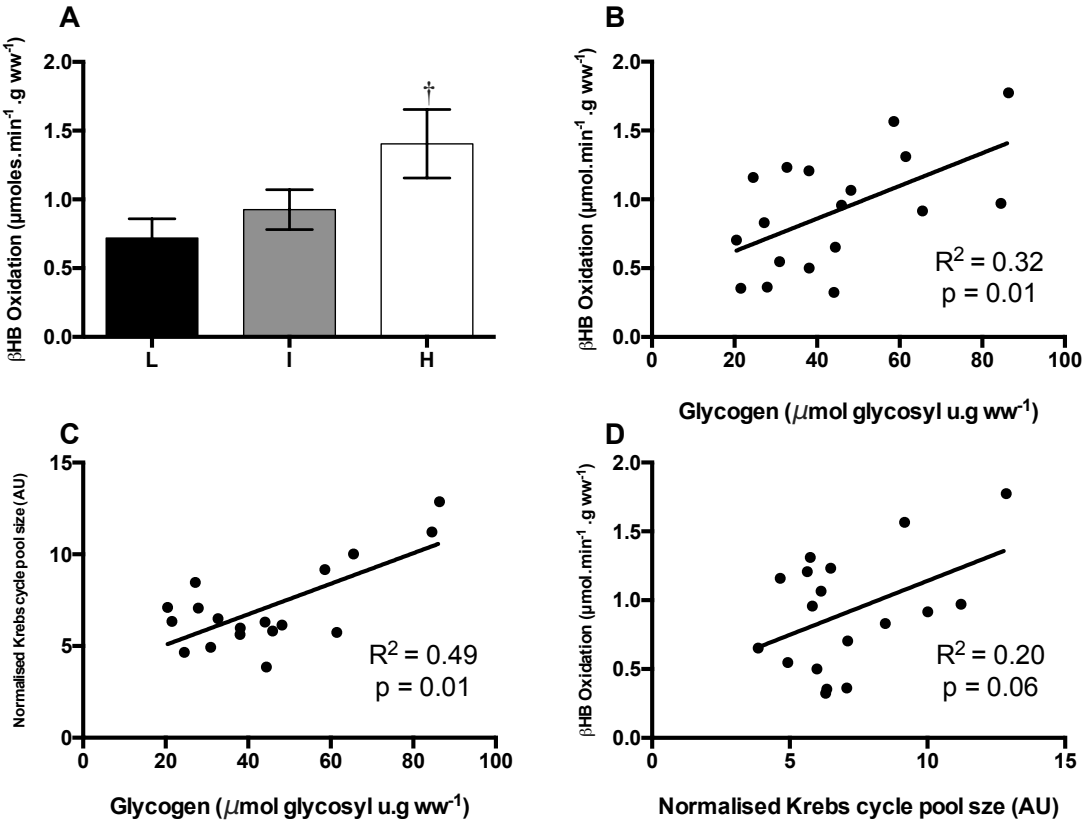

Figure 7.8: *D-βHB oxidation was measured in hearts following glycogen manipulation (n = 18). A: Mean βHB oxidation for different glycogen levels. B: Individual βHB oxidation rates plotted against the glycogen concentration at the end of each perfusion. C: Individual Krebs cycle pool size calculated from the results of the metabolomic analysis plotted against the glycogen concentration at the end of each perfusion. D: Individual βHB oxidation rates plotted against the total Krebs cycle pool size. Significance was taken at p <0.05 and shown as † = 'High' vs. 'Low.' Values are mean ± SEM*

There were significant differences in βHB oxidation with different glycogen levels (p <0.05) (Appendix E, Table E.1). The rate of βHB oxidation was lowest with 'low' glycogen (0.72 ± 0.14 μmoles min^{-1}.g wet weight^{-1}), and 2-fold higher with 'high' glycogen (1.41 ± 0.25 μmoles min^{-1}.g wet weight^{-1}) (p <0.05) (Figure 7.8 A). Plotting the individual oxidation rates against the glycogen levels for each heart gave a significant relationship

between post +K perfusion cardiac glycogen concentration and βHB oxidation (R^2 = 0.32, p = 0.01) (Figure 7.8 B). The total Krebs cycle pool size calculated for each heart was significantly related to the post +K perfusion cardiac glycogen concentration (R^2 = 0.49, p= 0.01) (Figure 7.8 C). The relationship between the total Krebs cycle pool size and βHB oxidation was approaching significance (R^2 = 0.20, p = 0.06) (Figure 7.8 D).

7.5 Discussion

7.5.1 Main findings

The pre-existing glycogen content altered βHB oxidation in the isolated perfused rat heart, with higher oxidation rates occurring with higher glycogen levels. Metabolomic analysis showed that the higher βHB oxidation may have been due to an anaplerotic effect of glycolysis from glycogen, permitting the oxidation of βHB-derived acetyl-CoA in the Krebs cycle and reducing proteolysis used to provide branched chain amino acids as an alternative oxidatitve and anaplerotic substrate for the muscle.

7.5.2 Changes in cardiac function, glycogen and triacylglycerol levels

As function was the same for all hearts, irrespective of perfusion substrate, it seemed that energy requirements in the non-working, retrograde-perfused heart could be met by the available fuel stores, even in the absence of other substrates. The levels achieved through the differing perfusion conditions were in agreement with previous studies (Table 7.2) and, as seen previously (Dhalla et al., 1972; Cross et al., 1996), it appeared that cardiac glycogen was the major contributor to energy provision during substrate-free perfusion. Glycogen levels fell in the absence of circulating substrate, but myocardial TG remained high, suggesting that a lack of anaplerosis may have prevented TG oxidation.

Perfusion with D-βHB plus glucose increased cardiac glycogen in all rat hearts, as

Table 7.2: *Reported values for cardiac glycogen levels measured on tissue obtained from freshly excised rat hearts or after perfusion with different substrates*

Condition Studied:	Glycogen Concentration (μmoles.g.wet weight^{-1})	Reference	*Current Results* (μmoles.g.wet weight^{-1})
Freshly excised heart:			
Fed	13-18	(Opie et al., 1963; Fisher and Williamson, 1961)	
Fasted	32	(Opie et al., 1963; Evans, 1934)	
Perfused heart:			
Substrate free	4-8	(Cross et al., 1996; Opie et al., 1963)	*5*
Glucose	13-19	(Cross et al., 1996)	*16*
Glucose + Insulin	30	(Cross et al., 1996)	*45*

reported for dog heart (Laughlin et al., 1994), rat diaphragm (Garland et al., 1964) and soleus muscle (Maizels et al., 1977). However, there was no further increase when glycogen was already 'high' (Figure 7.5 A), suggesting that levels may have been approaching an upper limit that prevented further glycogen storage (Table 7.2). Myocardial TG may have been an important fuel for hearts with 'Low' glycogen, as levels had fallen significantly following the +K perfusion, where glucose in the buffer may have allowed some anaplerosis to continue.

7.5.3 βHB oxidation and intermediary metabolism

The heart is able to use of a range of substrates during fasting, when glycogen increases (Opie et al., 1963; Evans, 1934). Here, it has been shown that glycogen increases the oxidation of ketone bodies, probably via anaplerosis, which may explain the increased ketone oxidation in hearts from fasting animals (Sultan, 1990).

Hearts with 'High' glycogen levels had higher levels of many glycolytic and Krebs cycle intermediates compared to 'Low' glycogen levels, and the total Krebs cycle pool size was significantly related to cardiac glycogen levels. This suggested that glycogen breakdown via glycolysis provided a substrate for anaplerotic reactions to allow increased βHB oxidation. Anaplerotic fates of glycogen include conversion of pyruvate to oxaloacetate by pyruvate carboxylase, to malate by malic enzyme, to α-ketoglutarate by alanine aminotransferase or phosphoenolpyruvate to oxaloacetate by phosphoenolpyruvate carboxykinase (Gibala et al., 2000) (Figure 7.2). Interestingly, whilst all glycolytic intermediates

were increased in the 'High' vs. 'Low' glycogen, phosphoenolpyruvate (PEP) was lower, indicating that PEP conversion to oxaloacetate may have been greater in the 'High' vs. 'Low' glycogen condition or, alternatively, that conversion of PEP to pyruvate was lower in the 'Low' vs. 'High' condition due to lower pyruvate dehydrogenase flux, which may have resulted in PEP accumulation. Unfortunately, oxaloacetate could not be accurately measured in the metabolomic analysis, thus omitting an important part of the puzzle that could be addressed in future research.

The higher levels of Krebs cycle intermediates in the 'High' glycogen condition and significant relationship between total Krebs cycle pool size and cardiac glycogen supports the hypothesis that glycogen allowed this expansion through anaplerosis. Increased levels of ketone bodies are proposed to inhibit some key steps in the Krebs cycle (Taegtmeyer, 1983), primarily α-ketoglutarate dehydrogenase (Taegtmeyer, 1983; Russell and Taegtmeyer, 1991), and thus impair function as the sole substrate. However, this can be overcome by anaplerosis, which provides Krebs cycle intermediates, malate and oxaloacetate, that maintain flux through the cycle. Here, levels of malate were significantly higher with higher glycogen. Although oxaloacetate could not be measured, it is assumed to be in near-equilibrium with malate (Taegtmeyer, 1983), therefore in the 'High' glycogen hearts both malate and oxaolacetate could have provided a substrate for condensation of acetyl-CoA derived from ketone bodies and TG, to form citrate. This is supported by the finding that both citrate and a downstream metabolite, aconitate, were significantly higher in the 'High' glycogen arm. Therefore, it appears that glycogen was associated with an increase in the ability of the Krebs cycle to accept acetyl-CoA derived from βHB and TG.

Endogenous TG is an alternative fuel which may have sustained function in the hearts with 'Low' glycogen content, as levels fell during perfusion with βHB and glucose. Although ketone bodies have been shown to inhibit oxidation of exogenous fat by the heart (Forsey et al., 1987), our understanding of fuel selection during ketosis has been challenged by recent observations of Cox et al. (2016b), who showed that exogenous ketone bodies *in-*

creased endogenous fat utilisation during exercise. Therefore, a previously undocumented effect of exogenous βHB on endogenous lipid handling is possible and the energy needs of the 'Low' glycogen hearts may have been met by an increase in TG utilisation as well as increased proteolysis. One caveat is that the rate of uptake and oxidation of glucose from the perfusion buffer was not measured and may have increased to compensate for low glycogen availability and low βHB oxidation. However, this does not seem likely, given that it has been repeatedly shown that ketone bodies decrease exogenous glucose oxidation in the perfused heart (Williamson and Krebs, 1961; Sultan, 1992).

A further effect of higher cardiac glycogen was that it decreased the need for protein breakdown for anaplerosis and terminal oxidation, as shown by the lower levels of muscle amino acids in the 'High' glycogen hearts. In conditions of high energy demand, such as during exercise, muscle proteolysis is increased to provide amino acids, which can act as a substrate for the Krebs cycle via succinyl CoA and oxaloacetate (van Hall et al., 1995). However, protein breakdown cannot go on indefinitely without endangering tissue function. Hearts with 'Low' glycogen were characterised by low carbohydrate availability as well as low rates of ketone body oxidation, and therefore may have been particularly reliant on proteolysis to provide an oxidative substrate and an alternative source of 4-carbon intermediates for Krebs cycle anaplerosis.

Ultimately, metabolomic analysis can only offer a snapshot of tissue metabolism at a single time point and does not shed insight on the flux through each pathway (Taegtmeyer, 1983). For example, the presence of a metabolite at high concentrations could be due increased flux or to due accumulation resulting from inhibition of a downstream step. This analysis did not distinguish between metabolite concentrations in the mitochondrial and cytosolic compartments, which may be different. Therefore, other experimental and analytical methods (eg. ^{13}C labelling) could be employed to directly measure anaplerotic flux during βHB metabolism in the presence of replete carbohydrate stores (Malloy et al., 1990). Attempts were made to address this question through development of a model to measure

pyruvate dehydrogenase (PDH) flux in the skeletal muscle of an intact animal (See Appendix E). Although the technique detected a significant difference between rest and and exercise, there was no detectable difference in PDH flux with exogenous ketones due to insensitivity in the technique. However, PDH flux has been shown to be inhibited during the *in vivo* rat heart during exogenous ketosis (Murray et al., 2016).

Metabolic differences between cardiac and skeletal muscle may have arisen out of evolutionary necessity. If skeletal muscle readily used ketone bodies during starvation, the rate of ketogenesis by the liver may not be sufficient to meet cerebral requirements. Skeletal muscle represents a large potential sink for ketone bodies in the periphery despite its relatively low ketolytic capacity compared to cardiac muscle (Beis et al., 1980), as it accounts for a large proportion of the body. Furthermore, skeletal muscle operates over a broad range of ATP turnover rates, which can increase between 60- and 100-fold during exercise (Gibala et al., 1998). If ketone bodies were readily oxidised by skeletal muscle to meet an increased ATP demand, the fuel supply to the brain and the heart may be at risk (Cox et al., 2016b). During starvation, skeletal muscle glycogen stores become depleted, therefore use of βHB in skeletal muscle would fall, thereby 'sparing' blood ketone bodies for oxidation in organs with stored glycogen, such as the heart and brain.

These findings have interesting implications for fuelling strategies in athletes using exogenous ketones, whereby athletes with replete carbohydrate stores may be better able to oxidise βHB and as a result, slow the use of glycogen. Conversely, athletes with low glycogen stores may be less able to oxidise exogenous ketone bodies, as seen in fasting (Fery and Balasse, 1983), and may rely on muscle protein breakdown to fuel muscle contraction. Cox et al. (2016b) demonstrated that ketone ester mixed with carbohydrate in drinks consumed prior to exercise, decreased overall glycogen use and blunted the exercise induced rise in muscle branched chain amino acid levels during exercise, suggesting that ketones worked in synergy with exogenous and endogenous sources of carbohydrate to provide a fuel for the muscle. This hypothesis is supported by the results of this study, which demonstrated

that higher levels of glycogen increased βHB oxidation.

7.6 Conclusion

In conclusion, the work in this Chapter has demonstrated that glycogen increased oxidiation of βHB in the heart. The proposed mechanism is the anaplerotic use of the 4-carbon units derived from pyruvate carboxylation, which increased the ability of the Krebs cycle to accept 2-carbon units (acetyl-CoA) from βHB metabolism. This appeared to reduce the requirement for proteolysis and the use of branched chain amino acids in the Krebs cycle. Therefore, the availability of intra-muscular carbohydrate may alter the use of exogenous ketones as oxidative substrate in muscle.

Chapter 8

General Discussion

8.1 Main Findings

The primary aim of the work in this Thesis was to characterise some of the metabolic, signalling, hormonal and GI effects of exogenous ketone bodies. The main findings were:

1. Lower blood levels of D-βHB were seen when equivalent amounts of total βHB were given as a salt compared to an ester, due to delivery of the L-isoform of βHB in the salt, which was not readily removed from the blood.

2. Both exogenous ketone compounds lowered blood glucose, FFA and TG levels to the same extent but had disparate effects on acid-base balance, as ketone salt drinks raised blood pH and ketone esters lowered blood pH.

3. Uptake and elimination of βHB from ketone ester drinks was repeatable between and within individuals, whether fed or fasted. Ketone ester metabolism was unaffected by pre-existing ketosis, or by the method of administration (bolus feeding vs. continuous infusion) but was altered by a meal.

4. Ketone drinks suppressed appetite more than isocaloric dextrose, which may be linked to altered secretion of gut hormones including ghrelin and GLP-1.

5. Ketone drinks caused low-level, mild GI effects. 'Symptom load' increased at higher ketone doses and was greatest following the high dose of ketone salt. Symptoms were both less frequent and less intense when ketone ester drinks were consumed before exercise compared to remaining at rest.

6. Exogenous ketones could be used as a supplement for endurance athletes. Use of nutritional supplements was found to be widespread in British endurance athletes and consumption habits varied between training and competition. Expected performance improvement decreased with increasing athlete level.

7. In an isolated perfused rodent heart, high levels of glycogen increased βHB oxidation, possibly through an anaplerotic effect of pyruvate derived from glycogen, which replenished Krebs cycle intermediates.

8.2 Metabolism of exogenous ketones

Current understanding of ketone metabolism is largely based on studies of endogenous ketosis resulting from starvation or a ketogenic diet. Although some previous work used exogenous ketone salt infusions as a tool for metabolic investigations, prior to this Thesis the knowledge of the metabolic 'fingerprint' of exogenous ketosis following oral ketone supplementation was limited. There is a growing appreciation of the potential benefits of ketosis for health and performance (Westman et al., 2003; Volek et al., 2015) which has led to a search for strategies that achieve ketosis without the dietary restriction of a LCHF diet, such as the consumption of exogenous ketones. Therefore, the work in this Thesis sought to understand the metabolic effects of two exogenous sources of ketones developed for oral consumption.

8.2.1 Exogenous ketosis

The findings of this Thesis illustrated that individual ketone compounds had some disparate physiological effects. Firstly, ketone drinks that were equimolar with respect to total βHB gave lower levels of blood D-βHB when containing a ketone salt compared to a ketone ester, as the salt was a racemic mixture of D and L-βHB isoforms. This meant that the characteristics of each ketone compound for D-βHB delivery could not be compared per-se, as the amount of D-βHB was not equivalent. However, ketone salt compounds are naturally formed as racemic mixtures, and this phenomenon offered a novel insight into stereo-specific differences in the metabolism of βHB. Current understanding of L-βHB metabolism is predominantly based on animal models, and suggests that whilst L-βHB is

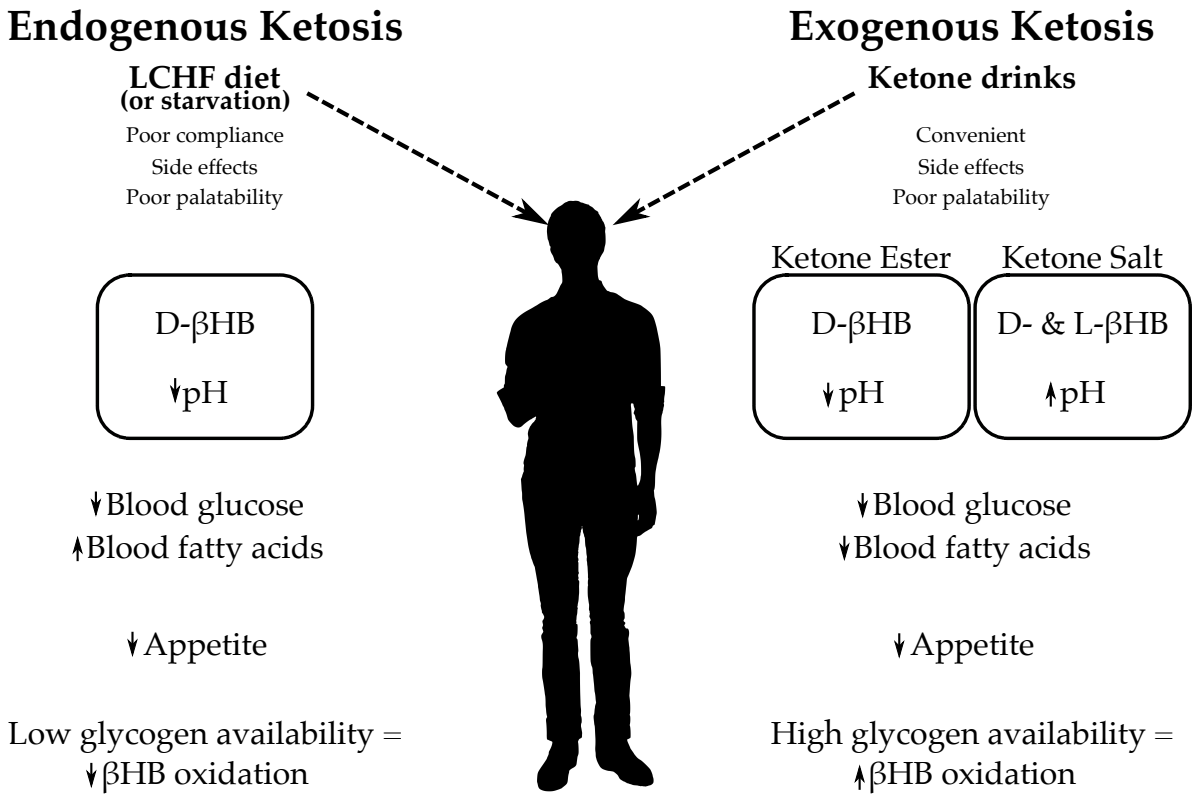

Figure 8.1: *Summary of the main findings of this Thesis, highlighting the differences between endogenous and exogenous ketosis and the differences between ketone compounds.*

not naturally produced at high levels within the body (Scofield et al., 1982), some may be oxidised or used for lipid biosynthesis (Webber and Edmond, 1977; Scofield et al., 1982; Lincoln et al., 1987; Desrochers et al., 1992). The metabolic fate of L-βHB in humans is unknown, but here it was seen that removal from the blood was slower than that of D-βHB, so it may not contribute to any measurable extent to oxidative metabolism. One putative role for L-βHB is as an intracellular 'signalling' metabolite that modulates the effects of D-βHB on substrate metabolism (Tsai et al., 2006), and whilst the effect of ketone salts and esters on blood glucose and lipids appeared to be similar, it is unclear if L-βHB from ketone salts interacted with the metabolism of D-βHB. Until its metabolic fate is understood, it cannot be assumed that the D- and L- isoforms of βHB are metabolically equivalent, and if the aim of supplementing ketone bodies is to provide an oxidative fuel, the D- isoform alone may be preferable.

A further physiological difference between the two ketone compounds was their effect on acid-base homeostasis. Ketone salt drinks delivered $\approx$ 6.4 g of inorganic ions in each drink, which transiently alkalinised the blood, as seen with previous salt infusions (Fery and Balasse, 1988; Muller et al., 1984), this ion load may preclude long term consumption (He and MacGregor, 2008). Ketone ester drinks had the opposite effect on blood pH, causing a mild metabolic acidosis that increased with levels of ketonaemia. Although the acid load delivered in the ketone ester at the doses studied here is readily buffered in healthy individuals, chronic metabolic acidosis has clinical consequences that depend on the degree of acidaemia. Therefore, increasing the physiological stress through higher doses or long-term consumption of either ketone compound should be undertaken with caution. These potential drawbacks of ketone drinks should be compared to the potential health implications of using a LCHF diet to achieve ketosis, which also include acidosis, hyperlipidaemia, increases in serum uric acid and renal stones (Keene, 2006; Kwiterovich et al., 2003). If long-term ketosis is sufficiently beneficial, the metabolic implications of each strategy for ketosis should be considered and an appropriate course of action taken to monitor these

effects.

Both ketone compounds were relatively well tolerated. Comparatively, LCHF diets and ketone precursors, such as MCTs, are associated with poor palatability and GI effects, which have impeded their widespread use (Amari et al., 1995). Prior to this Thesis, limited information was available on the side-effects of ketone ester drinks (Clarke et al., 2012b) and none was available for ketone salt drinks. While both compounds were unpalatable, the associated GI and systemic symptoms were predominantly 'mild,' although they increased in intensity and frequency at higher doses of both compounds. A limitation of this work was that it was unable to identify the underlying causes of the symptoms, however the differences in 'type' of symptom between each compound suggest that separate mechanisms were responsible in each case. These findings suggest that consuming the lowest amount of ketone compound required for a useful level of ketosis would minimise symptoms.

8.2.2 Factors altering metabolism of exogenous ketones

Novel differences between ketone ester metabolism in the fed and fasted state were seen: ketosis could still be attained when ketone ester drinks were consumed alongside food, however levels were lower than when fasted. A meal may decrease gut ketone uptake, as has been seen with similar small hydrophilic molecules (e.g. ethanol (Fraser et al., 1995)), alternatively insulin released following carbohydrate consumption may have induced an increase in peripheral uptake of βHB (Balasse and Havel, 1971; Keller et al., 1988). It is unclear from these results the contribution of each of these processes to the observed differences, therefore future work could use an isotopically labelled ketone ester and measure label reappearance in expired CO_2, to determine changes in the metabolic fate of βHB following a meal. These observations have practical implications for ketone ester consumption, namely that should maximal ketone levels be desired, co-ingestion of a meal should be considered.

Ketone ester metabolism and resulting βHB appearance and removal appeared largely

unchanged by existing hyperketonemia from exogenous ketones, although it is unknown if this would continue at higher doses. Accumulation of endogenous ketones, such as seen in diabetic ketoacidosis, has been attributed to a decrease in peripheral ketone utilisation over blood levels of $\approx$ 8-12 mM (Fery and Balasse, 1985), but at the lower levels studied here, no such effect was evident.

Finally, differences in ketone kinetics between and within individuals was examined to determine the repeatability of ketone metabolism following ketone ester drinks. Variations in peak blood ketone levels within each individual were small ($<$1%), however there was a difference of $\approx$ 2 mM between the lowest and highest peak βHB levels following body weight-adjusted drinks. Therefore, to minimise the amount of ketone consumed to achieve ketosis, ketone kinetics should be determined separately for each individual to allow fine control of ketosis. These results should be interpreted cautiously as the study populations were fairly homogeneous and factors such as body composition, genotypic differences in enzyme expression or disease may impact on ketone kinetics, or increase the variability in blood ketone levels, and should be investigated in future work.

Given the variability in ketone kinetics, accurate estimation of blood βHB levels is desirable to achieve precise control of ketosis. Non-invasive measurements, such as breath acetone meters or urine ketone sticks are routinely used to estimate blood ketone levels in diabetic patients or individuals following a LCHF diet (Tsuguyoshi et al., 2013; Taboulet et al., 2007). These methods rely on the proportional relationship between blood, urine and breath acetone levels, but can be inaccurate at higher ketone levels (Goschke and Lauffenburger, 1975; Taboulet et al., 2007). Here, it was found that the relationships between blood βHB and breath acetone were not proportional following ketone ester drinks, also urinary ketone levels did not accurately reflect blood βHB levels. Therefore, direct blood βHB measurement is preferable to accurately monitor exogenous ketosis.

8.2.3 Exogenous ketones as a metabolic signal

A key benefit of a LCHF diet is decreased levels of blood glucose, which could, for example, improve clinical outcomes in diabetes (Feinman et al., 2015). Even without dietary carbohydrate restriction, oral exogenous ketones lowered blood glucose, demonstrating a direct effect of βHB itself on whole-body glucose metabolism, as seen during ketone salt infusions (Balasse and Ooms, 1968; Mikkelsen et al., 2015). Putative mechanisms for this effect include decreased hepatic glucose release, increased insulin release or increased tissue glucose uptake (Biden and Taylor, 1983; Miles et al., 1981; Kashiwaya et al., 1997), however exploring the role of these two mechanisms was not possible from the results of this Thesis.

During starvation and LCHF diets blood lipids are elevated due to low levels of insulin, and endogenous ketone bodies regulate their own production through inhibition of peripheral lipolysis (Robinson and Williamson, 1980; Taggart et al., 2005). During exogenous ketosis this signalling effect resulted in low levels of blood free fatty acids, far below levels seen during starvation or a LCHF diet (Cahill et al., 1966; Kwiterovich et al., 2003). The ability of exogenous ketones to simultaneously lower blood glucose and blood lipids could be beneficial in the management of the metabolic syndrome.

Finally, *in vitro* and human studies have hinted that signalling effects of ketones may modulate complex behaviours, such as food intake (Laeger et al., 2012; Sumithran et al., 2013). Whilst reduced appetite had been documented during endogenous ketosis (Gibson et al., 2015), the role of βHB was unclear. The results presented in this Thesis suggest that ketone bodies themselves may have a central and/or peripheral effect that reduces appetite. However, as the human experiments here were not specifically designed to investigate appetite, the findings should be interpreted with caution. As ketogenic (high fat) diets can decrease metabolic efficiency by increasing uncoupling protein expression (Murray et al., 2009; Cole et al., 2011), it is not a given that a decrease in appetite with exogenous ketone

consumption would lead to weight loss. Furthermore, ketone drinks do represent a source of dietary calories and their addition to the diet should be compensated for by reducing calories from other macro-nutrients. Ultimately, the convenience of ketone drinks means that the role of βHB in appetite control is an interesting avenue for future investigation

The minimum level of ketosis required to achieve metabolic effects has yet to be defined. This is a particularly pertinent question if looking to minimise ketone consumption to reduce side-effects. Here, there was no significant dose-response relationship between the levels of D-βHB and the fall in blood glucose and FFA when two doses of two ketone compounds were studied. This is in agreement with Mikkelsen et al. (2015), who demonstrated a fall in arterial glucose appearance and lipolysis with low rate βHB infusion, but little further effect when the infusion rate was increased. Similarly, there did not appear to be increased appetite suppression with rising ketosis, in fact a meta-analysis by Gibson et al. (2015) indicated that βHB may suppress hunger even at levels $<$0.5 mM. Therefore, the beneficial signalling effects of βHB may be achieved at relatively low levels of ketosis.

8.2.4 Ketosis in athletes

The implications of a 'ketogenic' LCHF diet for endurance performance are currently under debate (Phinney, 2004; Burke and Kiens, 2006; Burke, 2015; Volek et al., 2015; Burke et al., 2016). On the one hand, carbohydrate is widely believed to be vital to sustain performance at exercise intensities over 60% $VO_{2\,max}$ (maximal oxygen uptake). On the other, 'keto-adapted' athletes have an increased ability to oxidise fat at high intensities (Volek et al., 2016, 2015), which may improve performance in ultra-distance events, as there is much higher potential energy stored in adipose tissue triacylglycerol compared to hepatic and muscle glycogen. However, no improvements in performance have yet been seen as a result of a LCHF diet (Burke, 2015), and some investigators have suggested that the diet could impair performance by decreasing high-intensity (glycolytic) capacity (Stellingwerff et al., 2006).

Acute consumption of exogenous ketone supplements may offer some of the benefits of the LCHF diet without adaptations that impair glycolytic capacity. Ketone drinks may improve performance by providing an alternative oxidative fuel source, sparing glycogen and increasing intramuscular fat oxidation (Cox et al., 2016b). In this Thesis, it was shown that athletes commonly use energy or ergogenic supplements during competition, with an expected variability in performance of ≈2% at the elite level. As ketone ester drinks resulted in a ≈2% improvement in endurance performance (Cox et al., 2016b), this effect may be relevant to endurance athletes looking to exploit the possible benefits of ketosis, without the need for dietary manipulation. In fact, as the ability to oxidise βHB increased with high muscle glycogen in the isolated perfused heart, these drinks may be of most benefit in athletes with a normal mixed diet as there may be an obligate role for carbohydrate to maximise the oxidation of exogenous ketones.

To enhance performance, ketone drinks should deliver sufficient βHB for use as an oxidative substrate and be well tolerated at the required doses. The optimal level of ketosis for athletes has yet to be determined, but there may be a threshold above which ketones offer no additional benefit to performance. Due to the supposed limits to muscle ketone uptake (Fery and Balasse, 1983), an advantage above a ketosis of 8-12 mM is unlikely, and is yet to be investigated. Performance may be maximally improved at much lower levels of ketosis, as Cox et al. (2016b) saw a significant effect at more modest blood βHB concentrations (2-3 mM). It did not appear that ketone salt drinks would elevate blood D-βHB to these levels without an undesirable increase in the incidence and severity of lower GI symptoms. In contrast, ketone ester drinks elevated blood D-βHB in a dose-dependant fashion, and whilst some symptoms did occur, fewer were reported when drinks were consumed before exercise, suggesting a role of exercise induced compensation in reducing symptoms. These findings will contribute to the guidelines for the use of exogenous ketones in a healthy population, such as athletes.

8.3 Future directions

The work in this Thesis raised several questions that could be subject to future investigation:

1. The fate of L-βHB could be explored to ascertain its effect on human metabolism.

2. Ketone kinetics could be studied in a more heterogeneous population, including disease, extreme body composition or people of different ethnic backgrounds.

3. The mechanism whereby βHB decreased appetite could be investigated. Human studies could be undertaken to measure gut hormone secretion as well as assessing the effect on food intake. To complement human studies, further experiments using enteroendocrine cells may reveal the mechanism of βHB's effect on hormone secretion.

8.4 Conclusions and implications

Consumption of exogenous ketones has broad implications for metabolic physiology. Whilst some metabolic effects were conserved between ketone salt and esters, such as their ability to lower blood glucose and lipids, there were several fundamental differences, including the isoforms of βHB delivered to the blood and their effects on acid-base balance. Both ketone drinks were tolerable for ketosis, and were associated with low levels of symptoms at the doses studied. As ketone ester drinks rapidly and repeatably induced ketosis in different states, they offer a convenient tool that could facilitate future investigations into the isolated effects of ketosis for a range of applications. One such application is in endurance sport, where supplement use is highly prevalent. As exogenous ketones can be readily oxidised by muscle with high glycogen levels, they may offer a meaningful improvement to elite endurance athletes, who may still follow a normal mixed diet. In conclusion, exogenous ketone drinks represent a convenient alternative to dietary modification to acutely generate 'physiological' levels of ketosis and to modulate human metabolism in health and disease.

Appendix A

Ketone ester and salt drinks for nutritional ketosis

A.1 Nutritional information for sweetener

Sweetener was manufactured by Symrise (UK). Each drink had 6g of sweetener added to the final volume of 300 ml.

Product number: 651757
Product name: PINEAPPLE (FRESH) FLAVOR SD

Nutritional Information

Nutritional data per 100 g product:

Energy value	1.343 kJ (316 kcal)
Fat	0 g
Saturates	0 g
Carbohydrate	73,4 g
Sugars	5 g
Fibre	4 g
Protein	0,1 g
Salt	0,007 g

Figure A.1: *Nutritional information for drink sweetener, manufactured by Symrise (UK). Participants received 6 g of sweetener in each drink*

A.2 Supplemental Methods

A.2.1 Chiral analysis of D/L-βHB

Plasma samples collected from 5 participants who had consumed 3.2 mmoles.kg^{-1} of βHB in a ketone salt drink, were analysed for the presence of L-βHB. Seven samples per subject were analysed, collected at: 0, 30, 60, 90, 120, 180 and 240 min. Analysis was carried out by IPOS (Huddersfield).

A.2.1.1 Sample preparation

A 1000 ppm stock solution of (+/-)-3-hydroxybutyric acid (Aldrich, 95%) was prepared by accurately weighing 100 mg into a 100 ml volumetric flask, diluting to the mark with ultra-pure water and mixing well. A 1000 ppm stock solution of (R)-3-hydroxybutyric acid was prepared by accurately weighing 100 mg into a 100 ml volumetric flask, diluting to the mark with ultra-pure water and mixing well. Each sample (200 μl) was pipetted into 1.5 ml Eppendorf centrifuge tubes with the blank sample (2016/04/59-01) being sampled in triplicate. Two of the three blank sample tubes were spiked, one with 20 μl of 1000 ppm (+/-)-3-hydroxybutyric acid and one with 20 μl of 1000 ppm (R)-3- hydroxybutyric acid (this results in approximately 1 mM HBA spiked samples). Ethanol (1 ml) (Fisher, ACS grade) was pipetted into each tube which were then sealed and vortex mixed for a few seconds to give a homogeneous, pale brown slurry. The samples were then centrifuged for 3 min and the supernatant decanted into 7 ml vials. The vials were then put in an oven at 80°C for 1-2h to evaporate the samples to dryness. Dichloromethane (2 ml) (Fisher, HPLC grade) was pipetted into each vial followed by 200 μl of N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA, Aldrich,>98. 5%). The vials were sealed and placed in an ultrasound bath for 10 min then left overnight. Ultra-pure water (2 ml) was added to each vial, which were well mixed and allowed to separate. The aqueous layer was removed and the dichloromethane layer dried with sodium sulphate. The dried dichloromethane sample solutions were then transferred to GC vials for analysis.

A.2.1.2 Mass spectrometry

Chiral analysis was performed using a Agilent 7890A + 5975 GCMS (Agilent). The ICS-5000+ HPLC system incorporated an electrolytic anion generator (KOH) which was programmed to produce an OH^- gradient over 37 min to achieve ion-exchange chromatographic elution. An in-line electrolytic suppressor removed the OH- ions and sample derived cations from the post-column eluent prior to MS analysis replacing these with water

molecules (Thermo Scientific Dionex AERS 500). A 10 μm partial loop injection was used for all analyses and the chromatographic separation was performed using a Thermo Scientific Dionex IonPac AS11-HC 2 250 mm, 4 μm particle size column with a Dionex Ionpac AG11-HC 4 μm 2x50 guard column in-line. The IC flow rate was 0.250 mL/min. The total run time was 37 min and the hydroxide ion gradient was comprised as follows: 0 min, 0 mM; 1 min, 0 mM; 15 min, 60 mM; 25 min, 100 mM; 30 min, 100 mM; 30.1 min, 0 mM; 37 min, 0 mM. Analysis was performed in negative ion mode using scan range from 80 - 900 and resolution set to 70,000. The tune file source parameters were as follows: Sheath gas flow 60; Aux gas flow 20; Spray voltage 3.6; Capillary temperature 320; S-lens RF value 70; Heater temperature 450. AGC target was set to 1e6 and the Max IT value was 250 ms. The column temperature was kept at 30°C throughout the experiment. Full scan data were acquired in continuum mode. The full scan data was combined with data directed MS2 scanning to provide fragmentation spectra for selected peaks at 17,500 resolution. The MS/MS settings were as follows: AGC 1e5, Max IT 50 ms, loop count 5, isolation window 1.0 m/z, NCE 30, intensity threshold 2e4.

A.3 Statistical effect size

Full results of statistical comparisons are shown below:

Table A.1: *Significance of the differences in kinetic parameters of D-β-hydroxybutyrate (D-βHB) following equimolar ketone ester and salt drinks at two concentrations in subjects at rest (n = 15) calculated using repeated measures one way ANOVA. Abbreviations: AUC, area under the curve; R_a, Rate of appearance in 30 min following drink; T_{max}, time at maximal D-βHB concentration. † = p <0.05 between ketone ester and ketone salt equivalent concentration. Values are mean differences between drinks ± SEM and p values. * = p <0.05 between high and low concentration of same drink.*

		D-βHB R_a (mmoles.min^{-1})	D-βHB T_{max} (min)	D-βHB AUC (mmoles.min)
	Difference			
Δ Ester vs. Salt	Low	0.03 ± 0.01 p = <0.001 †	-26 ± 7 p = 0.011 †	52 ± 16 p = 0.043 †
	High	0.07 ± 0.01 p <0.001	-62 ± 7 p <0.001	249 ± 16 p <0.001
Δ High vs. Low	Ester	0.04 ± 0.01 p <0.001	21 ± 5 p = 0.005	243 ± 16 p = 0.004
	Salt	0 p = NS	36 ± 6 p = <0.001*	45 ± 16 p = 0.0281*

Table A.2: *Significance of the change in urine βHB excretion and urine pH in samples collected following ketone ester and salt drinks (n = 11, 4 subjects excluded). A one way repeated measures ANOVA was performed. Values are mean differences between drinks ± SEM and p values. † = p <0.05 between ester and salt equivalent dose. * = p <0.05 between low and high dose of same drink.*

		Urinary D-βHB excretion (g)	Urine pH
Δ Ester vs. Salt	Low	-0.04 ± 0.01, p = 0.05 †	-2.04 ± 0.24, p = 0.04 †
	High	0.09 ± 0.1, p = 0.81	-2.68 ± 0.16, p = <0.001 †
Δ High vs. Low	Ester	0.29 ± 0.08, p = 0.022*	0.57 ± 0.18, p = <0.001*
	Salt	0.16 ± 0.03, p = 0.002*	0.07 ± 0.06, p = 0.691

Table A.3: *The significance of the change in plasma metabolites over time resulting from study drink consumption, and the difference between each study drinks. Values are p values calculated using a two way ANOVA with corrections for repeated measures and significance was taken as p <0.05 and indicated with *. The difference between the time course of changes with each drink (interaction: time x state) also shown. Abbreviations: KE = ketone ester; KS = ketone salt.*

	Δ due to ketone drinks	Δ KE vs. KS	Interaction: Time x drink
Free fatty acids	F (6, 84) = 16.31, p <0.001	F (3, 42) = 2.15, p = 0.11	F (18, 252) = 0.64, p = 0.872
Triacylglycerol	F (6, 66) = 11.53, p <0.001	F (3, 33) = 1.23, p = 0.31	F (18, 198) = 0.65, p = 0.852
Glucose	F (6, 84) = 22.62, p <0.001	F (3, 42) = 1.91, p = 0.14	F (18, 252) = 2.53, p <0.001
Insulin	F (2, 26) = 14.59, p <0.001	F (3, 39) = 1.00, p = 0.41	F (6, 78) = 1.80, p 0.11

Table A.4: *The significance of changes in plasma electrolytes caused by ketone ester and salt drinks. Values are p values calculated using a repeated measures one way ANOVA with corrections for repeated measures and significance was taken as p <0.05 and indicated with *. Abbreviations: KE = ketone ester; KS = ketone salt.*

		Sodium	Potassium
BL vs. 60	Ester- Low	0.402	0.061
	Ester - High	0.047*	0.002*
	Salt- Low	<0.001*	0.992
	Salt- High	<0.001*	0.691
High vs. Low (60 min)	Ester	1	0.991
	Salt	0.979	0.308
Ester vs. Salt (60 min)	Low	0.159	<0.001*
	High	<0.001*	<0.001*

Table A.5: *Significance of differences between study drinks, and the change following each ketone compound in blood pH electrolyte levels. Values are p values calculated using a two way ANOVA with corrections for repeated measures and significance was taken as p <0.05 and indicated with *. Abbreviations: KE - ketone ester; KS = ketone salt.*

	Δ between ester and salt	Δ following salt	Δ following ester
pH	<0.001*	0.002*	0.003*
Potassium	0.640	0.104	0.005*
Sodium	0.005*	0.011*	0.030*
Chloride	0.001*	<0.001*	0.014*
Bicarbonate	<0.001*	0.004*	0.002*

Appendix B

Repeatability of ketone and metabolite kinetics following ketone ester consumption

B.1 Nutritional information for sports water diluent

NUTRITION INFORMATION			
Per	100ml	500ml	RI(%*)
Energy	58kJ/14kcal	290kJ/70kcal	4
Fat	0g	0g	0
of which saturates	0g	0g	0
Carbohydrate	3g	15g	6
of which sugars	3g	15g	17
Protein	0g	0g	0
Salt	0g	0g	0
*Reference intake of an average adult (8400kJ/2000Kcal)			

Figure B.1: *Nutritional information for citrus flavoured sports water diluent, manufactured by Glaceau ^{TM} (UK). Participants consumed one full 500 ml serving.*

B.2 Statistical effect size

Full results of statistical comparisons are shown below:

Table B.1: *Statistical effect of feeding state (fed or fasted), and of the ketone ester drink (difference over time) on blood and breath ketone levels. The difference between the time course of changes with each drink in different state (interaction: time x state) also shown. Values are p values calculated using a two way ANOVA with corrections for repeated measures and significance ($p < 0.05$) indicated *.*

	Δ fed vs. fasted		Overall difference over time		Interaction: Time x State	
βHB	F (1,31) = 50.65	p <0.001*	F (7,217) = 228.2	p <0.001*	F (7, 217) = 17.60	p <0.001*
Acetone	F (1, 25) = 0.19	p = 0.659,	F (7,175) = 153.7	p <0.001*	F (7, 175) = 0.3811	p = 0.913
AcAc	F (1, 22) = 0.57	p = 0.458	F (7,154) = 26.83	p <0.001*	F (7, 154) = 2.05	p = 0.053

Table B.2: *The significance of the change in plasma metabolites over time resulting from study drink consumption in different states, and the difference between each state. The difference between the time course of changes with each drink in different state (interaction: time x state) also shown. Values are p values calculated using a two way ANOVA with corrections for repeated measures and significance ($p < 0.05$) indicated *.*

	Δ fed vs. fasted vs. control		Overall difference over time		Interaction: Time x State	
Free fatty acids	F (2,28) = 9.46	p <0.001*	F (5,70) = 17.94	p <0.001*	F (10,140) = 9.74	p <0.001*
Triacylglycerol	F (2,26) = 2.24	p = 0.127	F (5,65) = 10.73	p <0.001*	F (10,130) = 1.63	p = 0.104
Glucose	F (2,28) = 0.471	p = 0.629	F (5,70) = 24.13	p <0.001*	F (10,140) = 23.88	p <0.001*
Insulin	F (2, 14) = 0.29	p = 0.750	F (5, 35) = 10.71	p <0.001*	F (10, 70) = 4.32	p <0.001*

Table B.3: *The significance of the changes in ketone kinetic parameters for 3 identical ketone drinks consumed in series. Abbreviations: C_{max} = peak βHB concentration; Elim. = Estimated elimination; Min.= Minimum values taken 180' after each drink; R_a = βHB rate of appearance. Values are p values calculated using a repeated measures one way ANOVA, significance ($p < 0.05$) indicated *.*

	C_{max} (mM)	Rise in βHB (mM)	βHB R_a (mM.min^{-1})	Elim. (mmoles.min)	Min. βHB (mM)
Drink 1 vs. 2	0.269	0.238	0.013*	0.645	<0.001*
Drink 1 vs. 3	0.017 *	0.082	0.032*	0.645	0.001*
Drink 2 vs. 3	0.269	0.054	0.681	0.784	0.082

Appendix C

Systemic effects of exogenous ketones

C.1 Methods for cell culture experiment

C.1.0.1 Cell culture

GLUTag cells were seeded onto 75 cm^2 culture flasks at a density of 26,000-cells/cm^2 in low glucose (1 g/L) Dulbeccos modified Eagles medium (DMEM; Sigma-Aldrich) with 10% heat inactivated fetal bovine serum (FBS; Gibco), Glutamine (Gibco) final conc. 2mM and 1% penicillin/streptomycin (PenStrip; Gibco) solution (growth medium), and cultured at 37 °C. These cells were harvested once 60-80% confluent by removing the growth medium, briefly washing with Dulbeccos modified phosphate buffered saline (DPBS without $CaCl_2$ & $MgCl_2$; Life Technologies) followed by enzymatic treatment with 0.25% trypsin-EDTA (Life Technologies) for 3 min at 37 °C. After proper detachment, GLUTag cells were re-suspended in growth medium and centrifuged at 300 xg for 5 min at 25 °C. The resulting cell pellet was re-suspended in fresh growth medium and the cells were counted using a haemocytometer. Based on the resulting count and viability, the cells were reseeded at a ratio of 1:10 in 75 cm^2 culture flasks for expansion.

C.2 Symptoms questionnaire

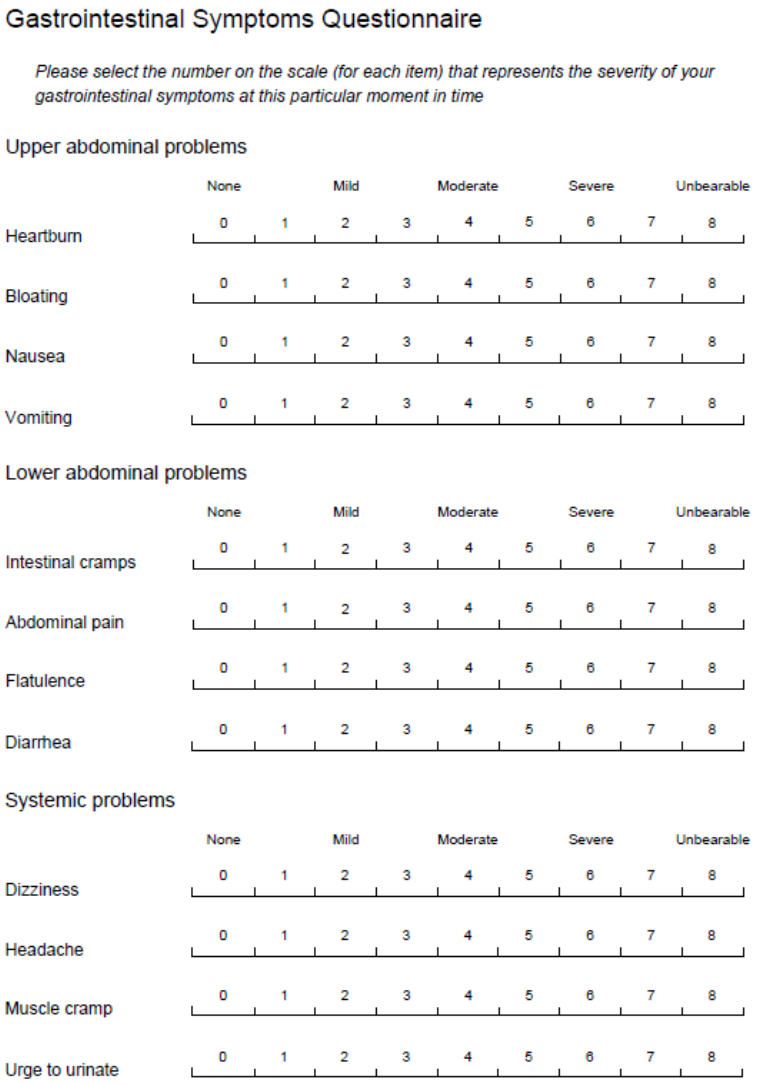

Figure C.1: *Questionnaire used to measure symptoms in all ketone drink studies.*

C.3 Statistical effect size

Full results of statistical comparisons are shown below:

Table C.1: *Level of significance calculated using a repeated measures 2-way ANOVA for the difference between satiety following different study drinks. Values given as p values and significance (p <0.05) is indicated with *.*

Δ Satiety	
KE vs. dextrose	0.005*
Salt: Low vs. High	0.496
Ester: Low vs. High	0.838
Salt vs. Ester (Low)	0.303
Salt vs. Ester (High)	0.595

Table C.2: *Level of significance calculated using a repeated measures 2-way ANOVA for the overall difference between metabolites and hormones following ketone or dextrose drinks. Values given as p values and significance (p <0.05) is indicated with *.*

Δ Metabolites and hormones	KE vs. dextrose
Ghrelin	0.016*
Insulin	0.005*
Glucose	0.060*

Table C.3: *Level of significance calculated using a repeated measures 2-way ANOVA for the difference between GLP-1 secretion by GLUTag cells in different incubation media. Values given as multiplicity adjusted p values and significance (p <0.05) is indicated with *.*

Δ from glucose concentration	0 mM vs. 5.6 mM	0 mM vs. 25 mM	5.6 vs. 25
1h (-) KB, 2h (-) KB	0.09	0.16	0.99
1 h (-) KB, 2h (+) KB	<0.001*	0.003*	0.04*
1h (+) KB, 2h (+) KB	0.02*	0.03*	0.99

Table C.4: *Level of significance calculated using McNemar's or Fisher's exact test for the difference in the incidence of different symptom groups following different study drinks. Values given as p values and significance (p <0.05) is indicated with *.*

Δ Incidence by symptom type	Upper GI	Lower GI	Systemic
Fed vs. dextrose	<0.001*	1.00	<0.001*
Fasted vs. dextrose	0.007*	1.00	<0.001*
Fed vs. Fasted	0.79	0.48	0.73
Rest vs. Exercise	0.02*	0.63	0.03*
Ester: Low vs. High	0.37	0.48	0.62
Salt: Low vs. High	0.22	0.13	0.62
Salt vs. Ester (Low)	1.00	1.00	0.62
Salt vs. Ester (High)	0.37	0.24	0.68

Table C.5: *Level of significance calculated using Wilcoxn's or Mann Whitney test for the difference between overall 'symptom load' (accounting for both incidence AND severity of all symptom groups) per experiment following different study drinks in different conditions. Values given as multiplicity adjusted p values and significance (p <0.05) is indicated with *.*

Δ Overall symptom load	
Fed vs. dextrose	0.005*
Fasted vs. dextrose	0.016*
Fed vs. Fasted	1.000
Rest vs. Exercise	0.006*
Ester: Low vs. High	0.031*
Salt: Low vs. High	0.008*
Salt vs. Ester (Low)	1.000
Salt vs. Ester (High)	0.027*

C.4 Development of a statistical model for symptoms

A mixed effects model was devised in collaboration with the Oxford University Department of Statistics, in order to determine the likelihood of co-variates of interest (e.g. ketone dose, ketone type) being associated with a given symptom group. Mixed effects models are a generalised class of statistical models whereby each individual is allowed to have both person-wise random effects, and population-wise random effects. They can be thought of as a more generalised form of the family of models known as ANOVA, and are particularly able to discriminate between sources of variation within a data set.

There were several potentially confounding co-variates occurring in the study population that may have affected the chance of experiencing symptoms (e.g. body mass, age, sex, ethnicity, metabolic phenotype etc). By initially considering whether or not a particular participant had any symptom as a response to the variety of conditions investigated, it was hoped to determine the importance of co-variates of interest on symptoms. This led to the consideration of a binary/Poisson (success/failure) response model. However, as kinetic properties of ketones and any downstream metabolites that may have caused symptoms (e.g butanediol, butanal) may vary between participants, a nested set of models was required, where observations were considered in the context of each individual. The method of Venables and Ripley (2002) was used (via the `glmmPQL` command of the `MASS` package in R) to fit such multi-level models and ascribe variation in symptoms to different co-variates (e.g: state, ketone dose, time, blood βHB, sex and BMI). Appropriate individually-varying random effects were set *a priori* as the participant identifier and visit number.

It was necessary to perform a log-square-root normality transformation on the time axis of the obtained data. All models initially included sex and BMI as factors, however following initial compilation of the model using R, neither had a significant effect on any of the symptom sub-groups. Therefore, sex and BMI were removed from the model in order to make it parsimonious. The predictors of the model were considered to be dose and blood

Table C.6: *Multi-level mixed effects models were constructed for each symptom group using data from every time point of all studies (1426 time points). The model generated estimates for the importance of βHB dose, ketone compound, blood βHB concentration, feeding state and exercise state in determining if a symptom would occur. Values shown represent p values obtained from the model. Abbreviations: KE = ketone ester; Ex = exercise. Significance taken as p <0.05 and indicated as *.*

	Upper GI	Lower GI	Systemic
Dose	*0.000	*0.000	0.756
D- βHB	0.093	*0.000	0.085
KE Fasted v KE Fed	*0.000	0.575	*0.019
KE Fasted vs. KE Ex	*0.014	0.264	*0.048
KE Fasted vs. Salt	0.412	0.606	0.582
Time x Fast	*0.002	*0.000	*0.001
Time x Fed	*0.000	0.791	0.131
Time x Salt	*0.009	*0.000	*0.003
Time x Ex	0.217	*0.002	*0.002

βHB, and time and state were expected to be mutually co-dependent on each other. The exact library call was:

```
glmmPQL(Lower.GI.TP ~ sqrt(Time)*State + Dose + BHB +
offset(-log(TD)), random = ~ 1|Subject/Run,
family = poisson, data = data).
```

C.4.0.1 Results

The GLME model that was devised evaluated the importance of several co-variates in determining if symptoms were experienced (Table C.6). Dose of ketone administered was a significant determinant of upper and lower GI symptoms but not systemic symptoms. Surprisingly, blood D-βHB was only a significant determinant in the occurrence of lower GI symptoms. Conditions (i.e fed, fasted, rest, exercise) played a significant role in determining the occurrence of upper GI and systemic symptoms, but not lower GI symptoms. In the fasted state, there was no significant effect of ketone compound (ketone ester fasted vs. ketone salt fasted) on any of the three symptom groups.

Appendix D

Nutritional supplement use in athletes

D.1 Full study questionnaire

Use and Perceptions of Sports Nutrition Supplements

1. Please indicate which age group you fall into:

   (a) <18

   (b) 18-25

   (c) 26-32

   (d) 33-40

   (e) 41-50

   (f) 50+

2. Please indicate your sex:

   (a) Male

   (b) Female

3. On average, how many hours of training do you undertake each week? *To help you work this out, in brackets is shown the number of hours per day with a 6 day training week.*

   (a) +36 h/week (+6 h/day)

   (b) 24-35 h/week (4-6 h/day)

   (c) 18-23 h/week (3-4 h/day)

   (d) 12-17h/week (2-3 h/day)

   (e) 6-12 h/week (1-2 h/day)

   (f) >6 h/week

4. In which country do you currently live?

5. Which sports do you participate in?

   (a) Rowing

   (b) Cycling

   (c) Triathlon

6. What level do you participate at?

   (a) Elite

   (b) Serious Amateur

   (c) Casual Competitor

   (d) Recreational

7. What is the highest level event you have competed at (if applicable)?

8. Do you use sports nutrition supplements?

   (a) Yes

   (b) No

9. How often in TRAINING do you use each of the following supplements?

   Options: Hardly ever/ Occasionally/ Sometimes/ Frequently/ Always

   (a) Energy Drink (powdered)

   (b) Energy Drink (pre-mixed)

   (c) Energy Gel

   (d) Energy Bar

   (e) Recovery Drink (pre-mixed)

(f) Recovery Drink (powdered)

(g) Electrolyte Drink

(h) Vitamins or minerals

(i) Protein or amino acids

(j) Bicarbonate

(k) Beta-alanine

(l) Nitrates

(m) Caffeine

10. What other supplements do you use in TRAINING? (if any)

11. How often during COMPETITION do you use each of the following supplements?

    Options: Hardly ever/ Occasionally/ Sometimes/ Frequently/ Always

    (a) Energy Drink (powdered)

    (b) Energy Drink (pre-mixed)

    (c) Energy Gel

    (d) Energy Bar

    (e) Recovery Drink (pre-mixed)

    (f) Recovery Drink (powdered)

    (g) Electrolyte Drink

    (h) Vitamins or minerals

    (i) Protein or amino acids

    (j) Bicarbonate

    (k) Beta-alanine

    (l) Nitrates

(m) Caffeine

12. What other supplements do you use during COMPETITION? (if any)

13. What event/test do you normally use to measure your individual performance? *eg: 2km in a single scull, 50k cycling time trial, 10km run time, 1500m freestyle swimming. TEAM SPORTS- do you complete an individual fitness tests that allows you to asses your progress?*

14. For the event/test you named above, what time or score would you expect to be able to confidently produce with average physical and mental preparation? *(typical course, neutral conditions).*

15. For the event/test you named above, what time or score would you realistically hope to be able to achieve on your best day, with ideal physical and mental preparation? *(identical course and conditions).*

16. Do you have any further comments about your experiences of sports nutrition supplements?

Appendix E

Effect of glycogen on β-hydroxybutyrate oxidation in heart

E.1 Supplemental Methods

E.1.1 Metabolomic analysis of cardiac muscle

Metabolomic analysis was carried out on all hearts that had been perfused with βHB (n = 18). This analysis was performed in collaboration with Oxford University Mass Spectrometry Services.

E.1.1.1 Mass Spectrometry

Metabolic profiling was performed using a Thermo Scientific ICS-5000+ ion chromatography system coupled directly to a Q-Exactive HF Hybrid Quadrupole-Orbitrap mass spectrometer with a HESI II electrospray ionisation source (Thermo Scientific, San Jose, CA). The ICS-5000+ HPLC system incorporated an electrolytic anion generator (KOH) which was programmed to produce an OH- gradient over 37 min to achieve ion-exchange chromatographic elution. An inline electrolytic suppressor removed the OH- ions and sample derived cations from the post-column eluent prior to MS analysis replacing these with water molecules (Thermo Scientific Dionex AERS 500). A 10 L partial loop injection was used for all analyses and the chromatographic separation was performed using a Thermo Scientific Dionex IonPac AS11-HC 2 250 mm, 4 μm particle size column with a Dionex Ionpac AG11-HC 4 μm 2x50 guard column inline. The IC flow rate was 0.250 mL/min. The total run time was 37 min and the hydroxide ion gradient was comprised as follows: 0 min, 0 mM; 1 min, 0 mM; 15 min, 60 mM; 25 min, 100 mM; 30 min, 100 mM; 30.1 min, 0 mM; 37 min, 0mM. Analysis was performed in negative ion mode using scan range from 80 - 900 and resolution set to 70,000. AGC target was set to 1e6 and the Max IT value was 250 ms. The column temperature was kept at 30°C throughout the experiment. Full scan data were acquired in continuum mode. The full scan data was combined with data directed MS2 scanning to provide fragmentation spectra for selected peaks at 17,500

resolution. The MS/MS settings were as follows: AGC 1e5, Max IT 50 ms, loop count 5, isolation window 1.0m/z, NCE 30, intensity threshold 2e4.

E.1.1.2 Data processing

Raw data was processed using Progenesis QI for small molecules (Waters, Elstree, UK). Briefly this encompassed chromatographic peak alignment, isotope cluster recognition (peak picking), compound identification and statistical analysis including principal components analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA). PCA identifies trends and outliers with respect to metabolites and amino acids and helps to visualise how samples group according to their chemical composition across all samples. This also identifies any outliers. OPLS-DA models the data to find more subtle, statistically significant, differences between experimental groups. This works best by comparing binary data sets (ie.'Low' vs. 'High' glycogen). This type of analysis enables elucidation of statistically different variables (metabolites) between sample groups. Identification of compounds in experimental samples was based on matching to an in-house library containing four orthogonal parameters for each compound.

E.2 Exploratory statistics: metabolomics data

E.2.1 PCA plots from metabolomic analysis

PCA plots (Figure E.1) showed significantly greater discrimination between the 'High' and 'Low' groups when 38 identified compounds were selected (41% vs. 25% for the main principal component). The analytical duplicates clustered fairly tightly together, suggesting that analysis of the same sample were strongly reproducible and therefore differences in compounds abundance between experimental samples were unlikely to result significantly from analytical variability.

The PCA analysis for the identified components naturally split the data into two groups

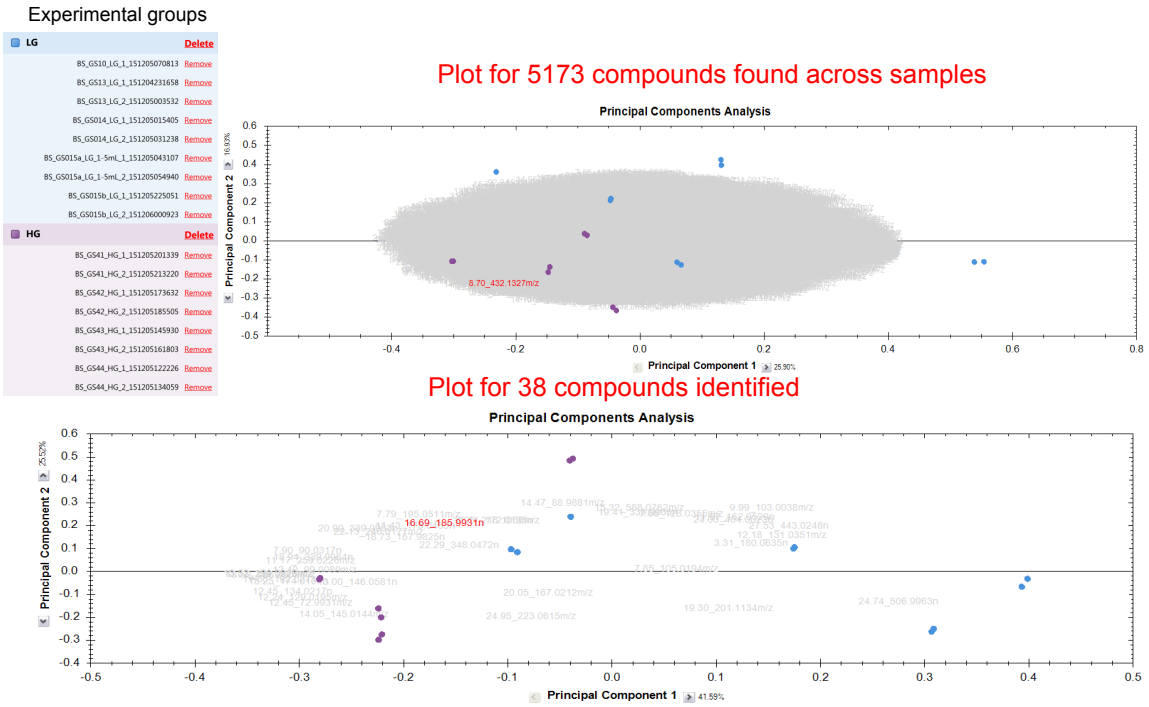

Figure E.1: *Principal components analysis plots, showing the grouping of metabolites in the 'Low' (LG- blue) and 'High' (HG- purple) glycogen groups*

'Low' (LG- blue) and 'High' (HG- purple) glycogen, other than for one sample (duplicate analyses) where the HG sample arguably behaved as if it was in the LG group. The HG group was generally closer together which suggested that their chemical composition (abundance of the 38 compounds) was more homogeneous within this group (i.e. in the LG group metabolite concentrations were more variable)

The PCA plot for all data (5137 compounds) showed a small distinction between the two experimental groups (but this was minimal) suggesting most of the variability between compounds was not significantly different between the groups. This was expected, given that the intervention (high/low levels of a single metabolite: glycogen) would not be expected to change the levels of all cellular metabolites.

E.2.2 OPLS-DA: variable importance plot from amino acids analysis

The VIP values reflect the overall importance of the variables in the model developed to distinguish between the two experimental groups. The amino acids shown in Figure E.2 were the most important 'predictors' of whether a heart had 'Low' or 'High' glycogen.

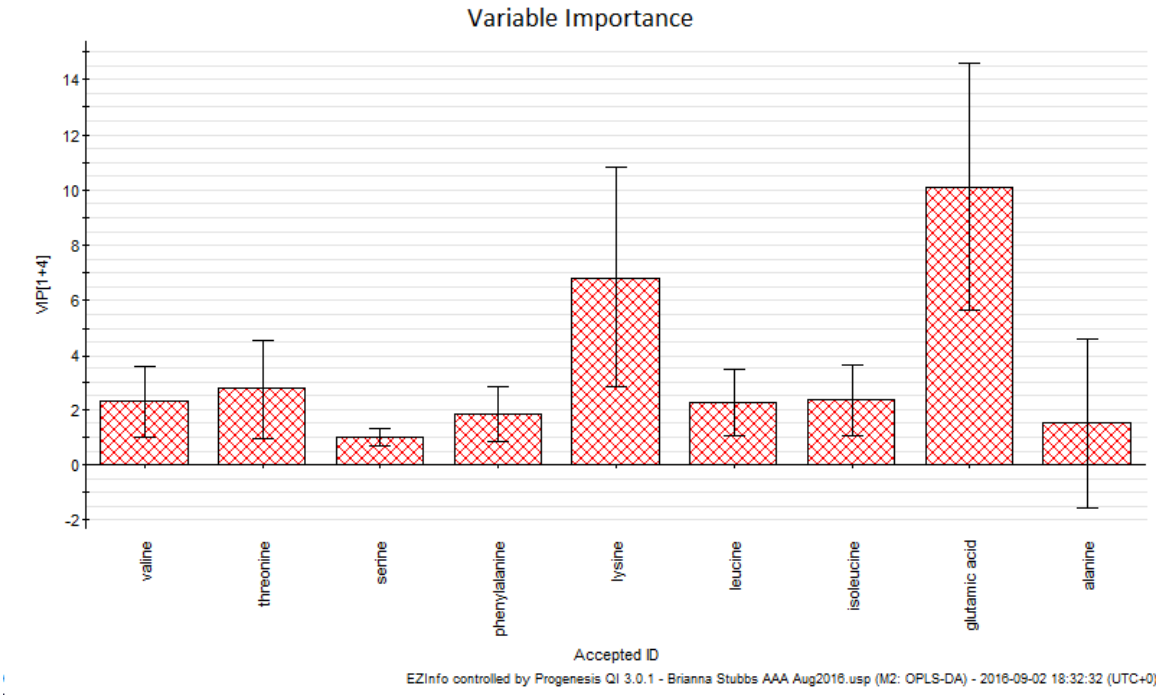

Figure E.2: *Variable importance plot showing which amino acids were most important in predicting if a heart had 'Low' or 'High' glycogen.*

E.3 Statistical effect size

Full results of statistical comparisons are shown below:

Table E.1: *Level of significance calculated using an ordinary 1 way ANOVA for the difference between experimental data either between glycogen levels (L = low, I = intermediate, H = high) or between post-manipulation and post-experimental levels. Values given as multiplicity adjusted p values and significance (p <0.05) is indicated with *.*

		L vs.I	L vs. H	I vs. H
βHB oxidation	+K	0.71	0.03*	0.23
Glycogen	-K	0.05	<0.01*	<0.01*
	+K	0.69	0.01*	0.09
TAG	-K	0.42	0.47	0.97
	+K	0.02*	0.05*	>0.99
		Low	Intermediate	High
-K vs. +K	Glycogen	<0.01*	0.01*	0.15
	TAG	<0.01*	0.73	0.47

Table E.2: *Level of significance calculated using an ordinary 1 way ANOVA for the difference between metabolite levels in hearts with different glycogen levels (L = low, I = intermediate, H = high). Values given as multiplicity adjusted p values and significance (p <0.05) is indicated with *.*

	H vs. L	H vs. I	I vs. L
Glucose-1-phosphate	<0.001*	<0.001*	0.872
Fructose-6-phosphate	<0.001*	<0.001*	0.993
Fructose-1,6-bisphosphate	0.424	0.994	0.464
Dihydroxyacetone-phosphate	<0.001*	<0.001*	0.313
3-phosphoglycerate	0.991	0.564	0.512
Phosphoenolpyruvate	0.015*	0.048*	0.971
Pyruvate	<0.001*	<0.001*	0.686
Citrate	<0.001*	<0.001*	0.111
Aconitate	<0.001*	<0.001*	0.034
α-ketoglutarate	<0.001*	0.002*	0.412
Succinate	<0.001*	<0.001*	0.099
Malate	<0.001*	<0.001*	0.152

Table E.3: *Level of significance calculated using an unpaired t-test for the difference between metabolite levels in hearts with different glycogen levels (L = low, H = high). Significance (p <0.05) is indicated with *.*

	H vs. L
Leucine	0.029*
Alanine	0.479
Lysine	0.074
Glutamic acid	0.031*
Valine	0.033*
Isoleucine	0.025*

E.4 The effect of exogenous ketones on pyruvate dehydrogenase flux in twitching skeletal muscle

E.4.1 Abstract

Pyruvate dehydrogenase (PDH) acts a metabolic control point to determine entry of pyruvate from carbohydrate metabolism into the Krebs cycle. It is widely believed that ketone bodies inhibit PDH activity through increasing levels of acetyl-CoA, and thereby decrease carbohydrate oxidation. This experiment examined the activity of PDH in skeletal muscle during exogenous ketosis. Mice were given an oral gavage of ketone ester and underwent sciatic nerve stimulation of hind-limb muscle with ^{13}C magnetic resonance spectroscopy following an injection of hyperpolarized [1-^{13}C]-pyruvate. Real-time conversion of pyruvate to lactate and bicarbonate was measured at rest and during muscle contraction, with and without ketosis. Whilst a 3- and 3.3-fold increase in pyruvate conversion to lactate and bicarbonate respectively was detected during exercise vs. rest, no effect of ketosis was observed. Therefore, KB-mediated inhibition of carbohydrate metabolism during exercise may have occurred upstream of PDH, or the technique was not sensitive enough to detect any changes.

E.4.2 Introduction

To extend the investigation of the relationship between elevated βHB and carbohydrate metabolism seen in Chapter 7, a novel murine exercising skeletal muscle model was developed, which combined *in vivo* gastrocnemius muscle stimulation (Cole et al., 2002) with MR spectroscopy of hyperpolarized [1-^{13}C]-pyruvate (Schroeder et al., 2008) to measure flux through PDH. PDH was considered to be an important target because it is at a key junction that determines the balance between oxidation of carbohydrate in the Krebs cycle or its use in anaplerosis (Gibala et al., 2000). Ketone bodies may exert an inhibitory effect on PDH as part of the 'Randle cycle,' which describes how the metabolism of fatty acids inhibit carbohydrate oxidation (Randle et al., 1963), mediated by an increased ratio of acetyl-CoA:CoASH and NADH:NAD$^+$.

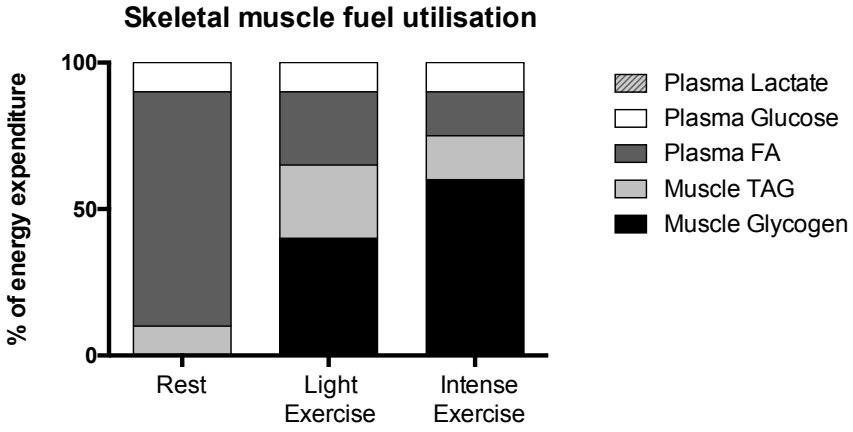

Figure E.3: *Typical fuel use in skeletal muscle. Based on figures from: Lopaschuk et al. (2010); van Loon et al. (2001b)*

At rest, skeletal muscle energy requirements are almost exclusively met through the uptake and oxidation of plasma free non-esterified fatty acids (Jeukendrup, 2002), but during intense exercise, carbohydrate from muscle glycogen becomes the dominant fuel source for the contracting muscle (van Loon et al., 2001a) (Figure E.3) and flux through glycolysis and PDH is increased (Dyck et al., 1993). It has been suggested that the conventional

'Randle Cycle' may not operate in skeletal muscle during intense exercise (Hue and Taegtmeyer, 2009), as the increased energy demand over-rides fatty acid-mediated inhibition of glycolysis, so carbohydrate metabolism becomes dominant. As exogenous ketones decrease muscle lactate production and glycogen use during exercise (Cox et al., 2016b), it is possible that high levels of KB may inhibit PDH, decreasing carbohydrate oxidation whilst oxidising ketone bodies. Therefore, the aim of this work was to measure the effects of exogenous ketosis on PDH flux in exercising skeletal muscle *in-vivo*.

E.4.3 Methods

Skeletal muscle metabolism has been measured in electrically stimulated myocytes (Lynge et al., 2001), isolated muscle bath preparations (Aslesen et al., 2001) or isolated hindquarter perfusion (Ruderman et al., 1971), none of which provides real-time information on metabolic flux during exercise. In order to study exogenous ketone metabolism in working skeletal muscle, an animal model was developed to obtain real-time measurements of metabolic flux during exercise.

Initially, mice (n = 6) underwent *in-vivo* gastrocnemius muscle stimulation protocol with two hyperpolarized [1-^{13}C]-pyruvate scans, one at rest and the other during muscle stimulation to validate the technique (Figure E.4). A further group of mice (n = 17) underwent identical *in-vivo* gastrocnemius muscle stimulation with one hyperpolarized scan, following either ketone ester (KE) gavage or saline. Infusion of hyperpolarized [1-^{13}C]-pyruvate allowed real time measurement of [1^{13}C]-pyruvate conversion into [^{13}C]-lactate and [^{13}C]-bicarbonate using an MR scanner (Figure E.4).

E.4.3.1 Animals, surgical preparation and exercise protocol

Three month old C57BL/6 male mice were purchased from Harlan (UK) and kept in controlled conditions (19-23 °C, humidity 45-65%, 12:12-h light-dark cycle) in the animal facilities at the University of Oxford. Animals were provided with standard rodent chow

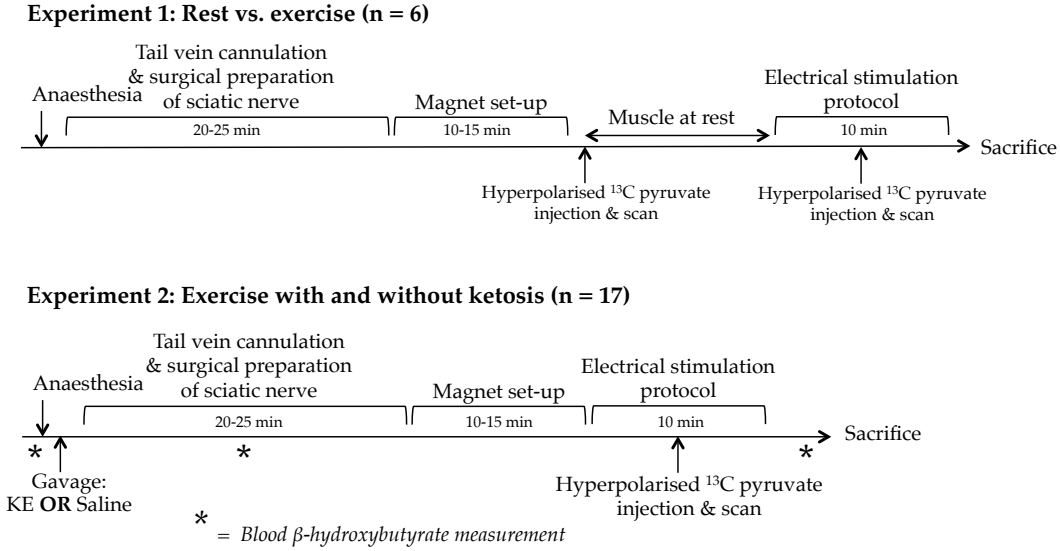

Figure E.4: *Schematic illustrating the overall study design for the effect of ketosis on PDH activity. One group of animals underwent two hyperpolarised scans: one at rest and one during electrical stimulation of the gastrocnemius. The second group were administered either ketone ester or saline through an oral gavage and underwent one hyperpolarised scan during electrical stimulation of the gastrocnemius.*

(Teklad Global Diets 2186) *ad-libitum* and were not fasted prior to experiments. Anaesthesia was induced using 2.5-3% (v:v) isoflurane in oxygen and nitrous oxide (4:1, total of 2 l/min). Anaesthesia was maintained by means of 2% (v:v) isoflurane delivered to, and scavenged from, a nose cone during the experiment. Once sedated, mice were gavaged with 2 g/kg of KE or an equivalent volume of saline. Blood βHB levels were assayed on capillary samples before the gavage and at 15 and 75 min after the gavage using a handheld monitor and reagent strips (Abbott -UK). A catheter (32 gauge needle) was introduced into the tail vein for intravenous infusion of hyperpolarized solutions. The sciatic nerve was isolated surgically and electrodes were placed distal to the tibial nerve branch. Knee and ankle joints were immobilised, the calcaneal tendon was attached to a force transducer, via a suture thread. Mice were placed in a bespoke perspex cradle (Figure E.5). Isometric force production of the hindlimb muscles was measured using a MacLab computer system connected to a force transducer. Resting tension was set at 50 g, and stimulation intensity

was determined by delivering a series of single stimuli of increasing current. The current was increased from 1 mA, at 10 mA increments, until no further increase in force was seen (supra-maximal stimulus), typically 60 mA. To mimic exercise, a stimulation protocol (Cole et al., 2002) consisting of a train of eight pulses of 100 μs at 30 Hz, followed by a rest period of 1.25 sec, was repeated over 10 min and force recorded over this time using a PowerLab system. A home-built ^{13}C saddle-shaped RF surface coil was placed over the muscle (Figure E.5) and the cradle was positioned inside a 7 T Agilent MR system. Images of cross-sectional area (CSA) of the gastrocnemius muscle were obtained using a ^{1}H volume coil. ECG and respiration were monitored throughout the experiment by electrodes placed under the skin. Air heating maintained body temperature.

E.4.3.2 Hyperpolarized [1-^{13}C] CMRS protocol

Hyperpolarized [1-^{13}C] pyruvate was infused into the tail vein and spectra acquired either at rest or when the gastrocnemius muscle was exercising at a steady state, at around 4 min into the exercise period. [1-^{13}C]-pyruvate was hyperpolarized and dissolved as previously described (Dodd et al., 2013). An aliquot of 0.2 mL of 80 mM hyperpolarized [1-^{13}C]-pyruvate solution was infused over 10 sec, via a tail vein cannula, into the anaesthetised mouse positioned in the 7 T MR scanner. Following infusion, spectra were acquired for one minute with 1 sec temporal resolution, using a 15° RF excitation pulse. Signal was localised to the gastrocnemius muscle using a home-built ^{13}C RF surface coil.

E.4.3.3 Statistical analysis

The first 30 spectra after the appearance of the pyruvate peak were summed and analysed using the AMARES algorithm in the jMRUI software package (Naressi et al., 2001) and results shown as a ratio of the peak of the metabolite of interest to the pyruvate peak. The appearance of the label in lactate, bicarbonate and alanine was measured. Difference in the ratios between rest and exercise in one animal were tested using a paired Student's t-

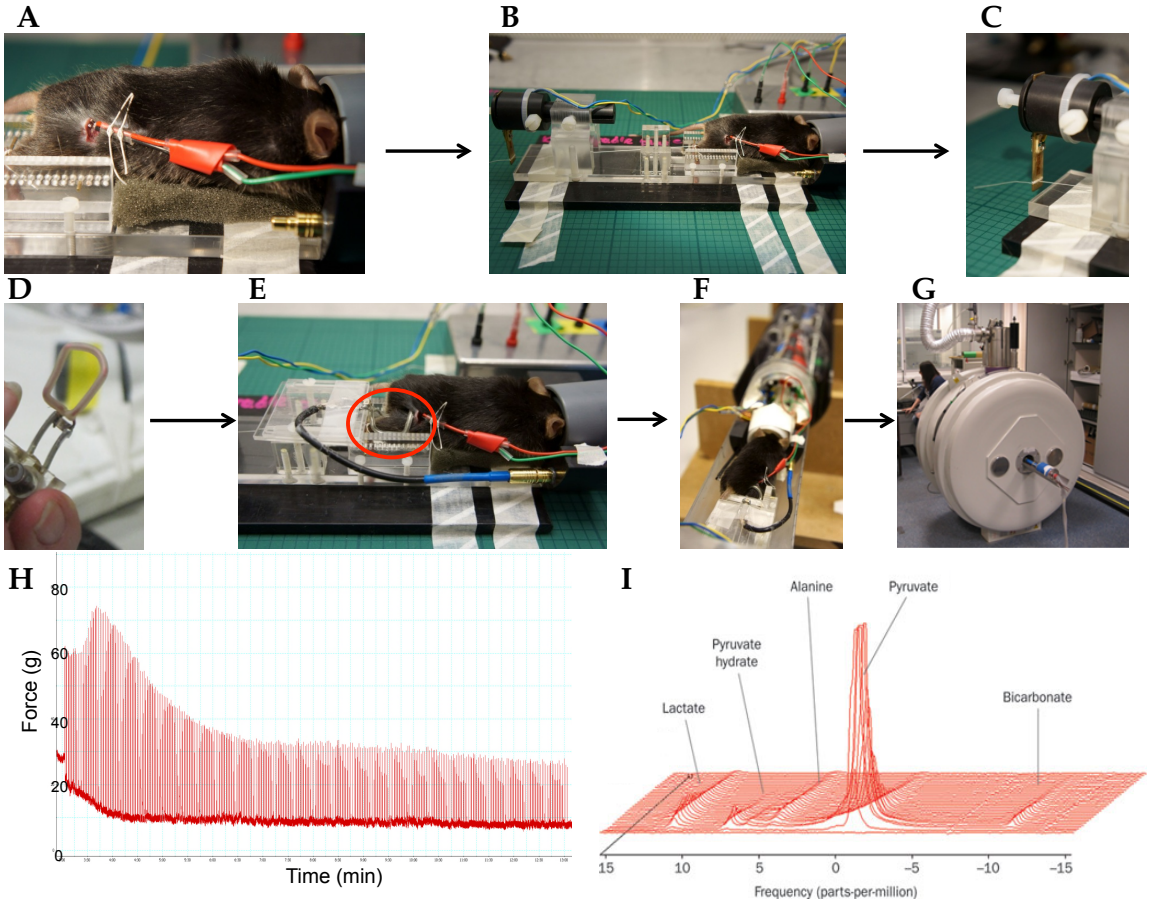

Figure E.5: *Experimental set up for investigating skeletal muscle metabolism using hyperpolarized [1-^{13}C] pyruvate. A: Electrodes placed around the sciatic nerve. B and C: Knee and ankle joints immobilised. Suture thread tied around the calcaneal tendon and attached to a force transducer. D and E: ^{13}C RF surface coil placed over the gastrocnemius muscle. F and G: Mouse placed in 7 Tesla magnet. H: Example trace showing muscle force during stimulation. I: Example spectra showing ^{13}C label incorporation over time following hyperpolarized [1-^{13}C] pyruvate injection.*

test, and differences between exercise with and without ketosis were compared by unpaired Student's t-test.

E.4.4 Results

An oral gavage of ketone ester (KE) administered to a pilot group of mice (n = 4) raised blood βHB levels to ≈ 5 mM for 90 min (Figure E.6 A). The oral gavage of KE given prior to the muscle stimulation experiments elevated blood βHB to 4.5 ± 0.5 mM, and remained elevated, at 4.7 ± 0.4 mM, to the completion of the experiment. There was no increase in blood βHB levels when the oral gavage contained saline (Figure E.6).

Table E.4: *Level of significance calculated for the difference between experimental data between rest vs. exercise and between exercise following an oral gavage of ketone vs. saline. Values given as multiplicity adjusted p values and significance of p <0.05 is indicated with *)*

	Lactate	Bicarbonate
Rest vs. exercise	0.03*	0.04*
Ketone vs. saline	0.47	0.61

Conversion of [1^{13}C]-pyruvate to [^{13}C]-lactate was increased 3-fold by exercise (p = 0.03) (Figure E.7 A). During exercise the conversion of [1^{13}C]-pyruvate to [^{13}C]-bicarbonate was 3.3-fold greater than at rest (p = 0.04) (Figure E.7 B). However, there were no further differences in either lactate:pyruvate or bicarbonate:pyruvate during exercise when blood ketones were elevated using the oral ketone gavage vs. exercise (Table E.4).

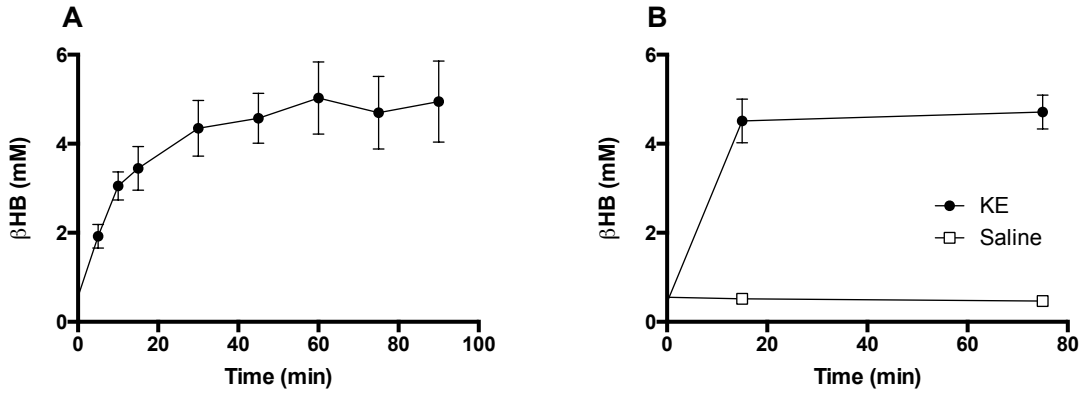

Figure E.6: *Blood βHB levels measured in mice administered 2 ml/kg KE by oral gavage. A: βHB was measured from capillary samples at regular intervals in anaesthetised animals without muscle stimulation (n = 4). B: βHB was measured from capillary samples in animals undergoing muscle stimulation before oral gavage of 2 ml/kg KE or an equivalent volume of saline, and 15 and 75 min after the gavage (n = 17). Values are shown as the mean ± SEM.*

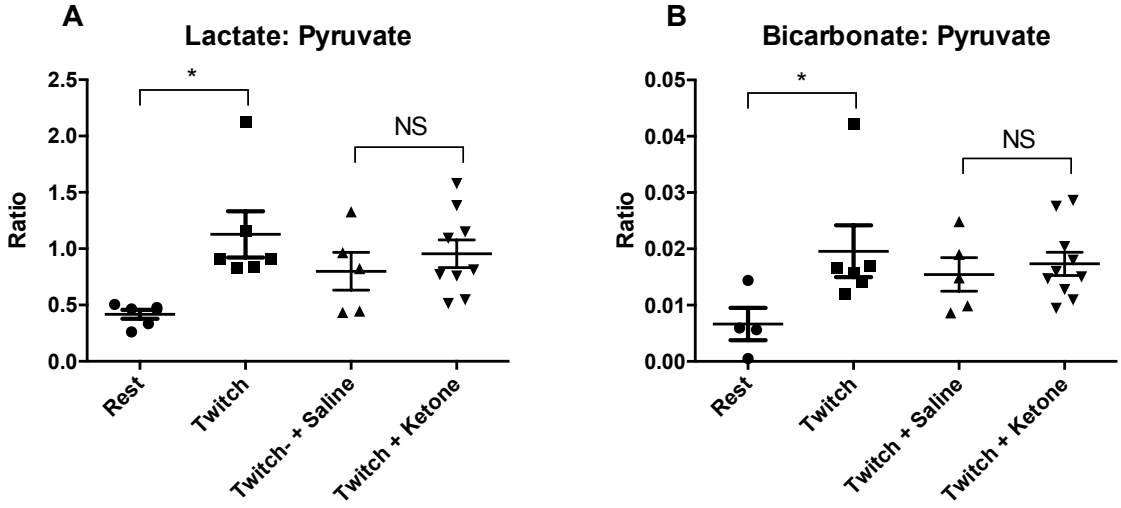

Figure E.7: *The conversion of [1^{13}C]-pyruvate to [^{13}C]-lactate and [^{13}C]-bicarbonate was measured in mouse gastrocnemius using an injection of hyperpolarised [1^{13}C]-pyruvate with an MR scan. A group of mice underwent two scans, one at rest and one during exercise (n = 6). A separate group were given an oral gavage containing ketone ester or saline, and underwent one scan during exercise. The ratio of lactate:pyruvate (A) and bicarbonate:pyruvate (B) are shown for all groups. Bars indicate the mean ± SEM. Significance is shown as ⋆ and was taken at p <0.05.*

E.4.5 Discussion and conclusions

Use of hyperpolarized [1-^{13}C]-pyruvate spectroscopy circumvented some limitations of tissue analysis in describing dynamic metabolic changes in working skeletal muscle. PDH plays a key role in directing pyruvate to acetyl CoA vs. anaplerosis, and is strongly activated during exercise. The twitching skeletal muscle technique developed here successfully detected the increase in production of lactate and bicarbonate occurring as a result of exercise. The increase in labelled lactate production reflected a mismatch between the flux through glycolysis and the oxidation of pyruvate through PDH (Dodd et al., 2013; Spriet and Heigenhauser, 2002), whereas the increase in bicarbonate reflects an overall increase in the terminal oxidation of pyruvate in the Krebs cycle (Dodd et al., 2013).

It is widely believed that ketone bodies inhibit carbohydrate metabolism through inhibition of PDH (Williamson and Krebs, 1961; Sultan, 1992). The work of Cox et al. (2016b) demonstrated that ketone bodies decreased carbohydrate metabolism at exercise intensities that would usually be heavily reliant on carbohydrate; one explanation for this may have been ketone-mediated inhibition of PDH, strongly reinstating the 'Randle cycle'. Despite the 5 mM ketosis following a ketone ester gavage, here there were no differences in the lactate:pyruvate or bicarbonate:pyruvate ratios seen during exercise. The data were highly variable and therefore it was not possible to determine the true effect of exogenous ketones on PDH flux. However, with further development this technique may offer a useful tool to characterise muscle metabolism during exogenous ketosis.

Appendix F

Publications and conference abstracts

F.1 Planned publications

1. **Metabolism of oral exogenous ketone compounds in healthy humans**: Stubbs, Cox, Evans, Miller, Hiyama, King, Veech, Clarke. In preparation for submission to The American Journal of Clinical Nutrition.

2. **The effect of D-βHB on appetite and enteroendocrine cell hormone release**: Stubbs, Cyranka, Clarke, de Wet. Further experiments planned pre-submission.

3. **The relationship between D-βhydroxybutyrate oxidation & cardiac glycogen**: Stubbs, Evans, Mccullagh, Cox, Clarke. Further experiments planned pre-submission.

F.2 Conference abstracts

1. **Glycogen availability profoundly alters oxidation of β-hydroxybutyrate in the perfused rat heart**: Stubbs, Evans, Mccullagh, Pires, Clarke, Cox. *The Physiological Society Annual Conference, Dublin 2016.*

2. **Ketone ester drinks increase blood ketone levels more effectively than ketone salt drinks**: Stubbs, Evans, Clarke, Cox. *The Physiological Society Biomedical Basis of Elite Performance, Nottingham 2016.*

3. **Using hyperpolarised 13C-MRS to explore murine skeletal muscle metabolism during exercise**: Curtis, Stubbs, Ball, Cochlin, Cole, Miller, ONeill, Clarke, Robbins, Tyler. *ISMRM Annual Conference, Signapore 2016.*

4. **A ketone ester drink results in nutritional ketosis with low within and between subject variability when fed or fasted**: Stubbs, Clarke, Cox. *British Pharmacological Society Annual Conference, London 2015.*

5. **A ketone ester drink reduces appetite compared to an isocaloric carbohydrate drink**: Stubbs, Willerton, Clarke, Cox. *The Physiological Society Annual Conference, Cardiff 2015.*

6. **A ketone ester drink alters levels of circulating lipids and glucose**: Stubbs, Willerton, Clarke, Cox. *The Physiological Society Annual Conference, Cardiff 2015.*

7. **Concomitant meal ingestion alters levels of circulating ketone bodies following a ketone ester drink**: Stubbs, Willerton, Clarke, Cox. *The Physiological Society Annual Conference, Cardiff 2015*

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